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Differential impact of nutrition on developmental and metabolic gene expression during fruiting body development in *Neurospora crassa*

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Abstract

Fungal fruiting body size and form are influenced by the ecology of the species, including diverse environmental stimuli. Accordingly, nutritional resources available to the fungus during development can be vital to successful production of fruiting bodies. To investigate the effect of nutrition, perithecial development of *N. crassa* was induced on two different media, a chemically sparsely nutritive Synthetic Crossing Medium (SCM) and a natural Carrot Agar (CA). Protoperithecia were collected before crossing, and perithecia were collected at 2, 24, 48, 72, 96, 120, and at full maturity 144 hours after crossing. No differences in fruiting body morphology were observed between the two media at any time point. A circuit of microarray hybridizations comparing cDNA from all neighboring stages was performed. For a majority of differentially expressed genes, expression was higher in SCM than in CA, and expression of core metabolic genes was particularly affected. Effects of nutrition were highest in magnitude before crossing, lowering in magnitude during early perithecial development. Interestingly, metabolic effects of the media were also large in magnitude during late perithecial development, at which stage the lower expression in CA presumably reflected the continued intake of diverse complex initial compounds, diminishing the need for expression of anabolic pathways. However, for genes with key regulatory roles in sexual development, including pheromone precursor *cgc-4* and *poi2*, expression patterns were similar between treatments. When possible, a common nutritional environment is ideal for comparing transcriptional profiles between different fungi. Nevertheless, the observed consistency of the developmental program across media, despite considerable metabolic differentiation is reassuring. This result facilitates comparative studies that will require different nutritional resources for sexual development in different fungi.

Keywords

perithecial development; medium impact; transcription; microarray

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1. Introduction

Fungi form a significant proportion of the microbial community in diverse ecosystems as decomposers, pathogens, and symbionts, and they play fundamental roles in biogeochemical cycles of organic/inorganic elements (Stajich et al., 2009). In natural environments, limitations to the distribution, growth, and morphological development of fungal saprobes are mainly imposed by nutritional and other abiotic constraints (Fomina et al., 2003; Wainwright 1993; Wang et al., 2009). However, most of our knowledge about the impact of nutrition on fungi has been obtained by studies culturing lab strains on controlled artificial media. Ever since it provided strong support for the one-gene-one-enzyme hypothesis (Beadle and Tatum, 1941), studies of fungal genetics and biology of the model *Neurospora crassa* have typically employed synthetic media that have facilitated remarkable improvements of our knowledge about metabolism and development in eukaryotic cells in the lab.

In order to manipulate and recapitulate the life-histories of some fungal models in the lab, most molecular work has required highly controlled biotic and abiotic conditions that are not present in any natural environment (Stajich et al., 2009). As in human industrial food products, carbon and nitrogen resources for lab organisms tend to be simplified and standardized, and are composed sparsely of just a few pure chemicals. It is unclear whether such sparse nutritional environments affect the expression of developmentally important genes, or if the developmental process cannot be altered much by nutritional environment. The expression of other genes, especially those involved in nutrient storage, processing, and metabolism, is dynamic, presumably maintaining a homeostatic internal environment for the process of development, and would presumably be highly regulated in response to changes of a cell's environment. The nutrigenomics adage "you are what you eat" begs the question as to whether the expression of many genes are dynamically regulated by the nutrient conditions an organism encounters.

Abiotic and biotic environmental effects on gene expression have been investigated in diverse studies utilizing prokaryotes, yeasts, animals and plants (Hodgins-Davis and Townsend, 2009). Unicellular organisms like yeasts adjust their metabolic rate directly in response to nutrient supply or availability (Rutherford, 2011). In yeasts, transcriptional regulation appears to be the fundamental regulatory process, and in some fungal models DNA-binding transcription factors and genes under their control are well characterized for most metabolic pathways (Nishizawa et al., 2008, Adams et al., 2010). In multi-cellular organisms with extensive tissue differentiation, like mammals, regulation of gene expression involves complex interactions of hormones, neural activity, and exposure to nutrients (Vaulont et al., 2000). In filamentous fungi such as *Neurospora*, *Trichoderma*, and *Rhizopus* species, nutritional influence and nutrient-dependent dynamics of transcription have been investigated during asexual development (Gadd et al., 2001; Aign and Hoheisel, 2003; Fomina et al., 2003). Although the induction of sexual development for some fungi under laboratory condition requires special nutritional and abiotic conditions, the influence of nutrition on gene expression during sexual development has not yet been examined in fungi using genome-wide approaches.

For most fungal strains in molecular laboratories, colony growth and asexual development (conidiation) can be easily induced and maintained on defined, sparsely nutritive media composed primarily of two simple carbon and nitrogen sources. In contrast, media used for sexual development typically vary among different fungal species. During sexual reproduction, filamentous fungi such as *Neurospora* and *Fusarium* species produce multicellular structures (perithecia) and sexual spores (ascospores) (Poeggeler et al. 2006). For these heterothallic species, this developmental process usually starts with a cross

between two strains of opposite mating types (Glass and Keneko, 2003). In *Neurospora*, sexual development can be easily induced in the lab using Synthetic Crossing Medium (SCM), a nitrogen-poor defined medium with up to 1.5% sucrose as sole carbon resource (Westergaard and Mitchell, 1947). In contrast to the simple, sparsely nutritive media used for *Neurospora* sexual development, sexual structures for *Fusarium* species are typically induced in lab conditions on the natural Carrot Agar medium (CA, Klittich and Leslie, 1988). The close phylogenetic relationship, parallel morphological development of the perithecium, and complete sequenced genomes make *Neurospora* and *Fusarium* species ideal models to study the genetic basis of mycelial growth and development of multi-cellular sexual reproduction. Previous studies of gene expression shifts during sexual development were undertaken in *Neurospora* on SCM (Clark et al., 2008) and in *Fusarium* on CA (Hallen et al., 2007). Therefore, to reveal the impact of nutritional composition on sexual development and to serve as a basis for comparative development, a comparison of expression differences attributable to the nutritive environment is required.

To address the impact of media composition on gene expression, especially gene expression of core metabolic genes and developmental genes, we performed transcriptomic analysis using genome wide microarrays on the sexual development of *Neurospora crassa*. Genome-wide transcriptional profiling in *N. crassa* has been applied to identify genes expressed in diverse stages of asexual development (Kasuga and Glass, 2008; Greenwald et al., 2010), it has not been applied to investigate perithecial development in *N. crassa*. In this experiment we observed that, for most differentially expressed genes, the expression level was typically higher in SCM than in CA. We identified the expression of many core metabolic genes was affected by the nutritional environment. Nevertheless, fruiting body development and ascospore production on CA was not debilitated. Our results validate the potential to gain insight from further experimental work on the comparative sexual development of fungi, facilitating informed comparative analyses of fungi that require distinct nutritional resources.

2. Materials and methods

2.1. Strains and culture conditions

Two strains of *N. crassa* of complementary mating types *mat a* (FGSC4200) and *mat A* (FGSC2489) were kindly provided by the Fungal Genetics Stock Center (McCluskey et al., 2010). The strains were grown on either nitrogen-poor Synthetic Crossing Medium (SCM) agar with 1.5% sucrose as a carbon source (Clark et al., 2008), or Carrot Agar (CA; Hallen et al., 2007). In either case, growth medium was covered by a cellophane membrane (Fisher Scientific Company) and growth occurred at 26°C under constant artificial light provided by several Ecolux bulbs (F17T8.SP41-ECO, General Electric Company). For CA, 350g carrots were washed, cut into 2 cm pieces and 400 ml distilled water was added prior to autoclaving for 30 min. Carrots were decanted into a blender and blended, and agar (2%) was added followed by sterilization of 35 minutes. Conidia from the *mat a* strain were collected and suspended in 2.5% Tween 60 ($10^5 - 10^6$ conidia/ml). Cultures of the *mat A* strain on both SCM and CA were examined using a stereomicroscope for the formation of protoperithecia in 5 to 7 days, and areas with evenly distributed protoperithecia of a common size were delineated with a marker on the bottom of the plate.

Crosses were performed by applying 2 ml *mat a* conidia suspension in 1.5% Tween 60 to the surface of the *mat A* protoperithecia plates, at which point considerable disturbance to surface hyphae and other fungal tissues was unavoidable. Sexual development then proceeded from the cross (0 h) to fully developed perithecia (144 h; Figure 1) under the same conditions for both SCM and CA cultures as before the crossing, and was monitored with a stereomicroscope. Tissues were scraped from plate surface with a razor blade

separately for the SCM and CA cultures of the *mat A* strain in the marked areas right before the crossing and at 2, 24, 48, and 72 hours after crossing.

Macro-morphology (size and color) of perithecia experienced dramatic changes at later stages (Fig. 1B–J). Many perithecia could be clearly spotted with the naked eye by 72 hours after crossing. For these time points, perithecia at matched development stages (based on size and color under stereomicroscope) were collected from the plate surface with a razor blade separately for the SCM and CA cultures of the *mat A* mated perithecial plates within the marked areas at 96, 120, and 144 h after crossing. Similar morphological development, indicated by macro-morphology of perithecia as well as microscopic development of parenchyma, asci and ascospores, and paraphyses was validated in CA and SCM at all sampled time points. Developing and matured asci and ascospores were observed in perithecia from 120 h to 144 h samples (Fig. 1E,I and F,J). For transcriptomic analysis, all tissues and perithecia were immediately and rapidly frozen in liquid nitrogen as they were sampled, then stored at -80°C .

2.2. Fixation and microscopy

Fungal material from selected time points was subjected to light microscopy, revealing morphological stages of perithecial development (Fig. 1B–J). Rectangles (5×2 mm) of *N. crassa* cultures growing on cellophane were cut out and fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde over night. The samples were then washed with 0.1 M sodium cacodylate buffer, postfixed with 1% osmiumtetroxide for 1 h, washed with distilled water, and contrasted with 1% uranylacetate for 1 h. The samples were then washed again with water, dehydrated in an increasing series of acetone and infiltrated in an increasing series of Spurr's plastic (ERL) over night. Infiltration of ERL into the sample was facilitated by vacuum treatment for 1 h prior to hardening for 24 h at 65°C . Semi-thin sections ($0.75\ \mu\text{m}$) were made with a glass knife using an ultramicrotome. Additionally, fresh perithecia were squashed in a drop of water between a glass slide and a cover slip, then imaged under a light microscope (Nikon Diaphot 300).

2.3. Sample preparation and hybridization to microarrays

Total RNA was extracted from 300 to 400 mg of tissue from at least three biological replicates (each plate representing one biological replicate), and homogenized with 3 to 4 ml TRI REAGENT (Molecular Research Center). Messenger RNA was purified using Oligo(dT) Cellulose Columns (Molecular Research Center) as in Clark et al. (2008). Two μg of purified mRNA were used for reverse transcription, including 0.25 μg oligo(dT) mixed with 0.25 μg *Neurospora*-specific multi-targeting primer (Adomas et al., 2010) in order to achieve the greatest transcript coverage possible. The resulting cDNA was labeled reciprocally with cyanine dyes (Townsend, 2004) and used for hybridization. Production of oligonucleotide microarrays and hybridization was performed as described in Clark et al. (2008) and Kasuga et al. (2005). The microarrays were composed of 70mer oligonucleotides synthesized by Illumina (San Diego) for 9826 ORFs identified by the Broad Institute (<http://www.broad.mit.edu/annotation/genome/neurospora>), and robotically printed on CMT-GAPS-aminopolysilane-coated glass slides (Corning, Corning, NY) at the Yale University Center for Genomics and Proteomics. A total of 48 hybridizations were performed, including dye-swaps originating from independent reverse transcription reactions (Fig. 1A).

2.4. Data acquisition and analysis

Hybridized microarray slides were scanned with a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA), using the GenePix 4000 software package to locate spots on the microarray. All spots were verified by eye, and those with unusual morphology

or with erratic signal intensity distribution were excluded from later analyses. Acquired raw data were normalized by background-subtracted mean-by-mean normalization as described in Townsend, Cavalieri, and Hartl (2003), and normalized data were then statistically analyzed using Bayesian Analysis of Gene Expression Levels (BAGEL, Townsend and Hartl, 2002; Townsend, 2004). Fluorescence intensity values were adjusted by subtracting background from foreground, and a gene was considered well measured only if the foreground fluorescence signals were higher than three standard deviations of the distribution of intensities of the background pixels for that gene. After normalization and BAGEL analyses, we obtained well measured gene expression data for 902 genes out of 9826 genes printed on the array (Supplemental Table S1, S2, S3). Genes whose expression differed statistically significantly based on developmental stage or based on growth medium in the same developmental stage were assessed by BAGEL *P*-values. BAGEL results were also subjected for clustering analysis using web-based MetATT, a tool for analyzing time-series and two-factor data sets (Xia et al. 2011). To identify gene expression levels for genes only expressed at early or only at late stages of the development, or only on one of the two media, we also analyzed subsets of the 48 hybridizations, including SCM and CA only comparisons, early development during 0 h to 48 h, and late development during 48 h to 144 h.

The Functional Catalogue (FunCat: <http://mips.helmholtz-muenchen.de/proj/funcatDB/>) annotation scheme (Ruepp *et al.*, 2004) was applied to group genes according to their cellular or molecular functions (Supplemental Table S4, S5). The statistically significant overrepresentation of gene groups in functional categories relative to the whole genome was determined by the hyper geometric distribution for *P* value calculation facilitated by the MIPS FunCat online web application. Further functional annotation of genes with significantly differential expression pattern in metabolic pathways was characterized using biochemical pathways and annotation data from the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa and Goto, 2000).

2.5. Verification of transcription profiles of CA samples using transcriptomic sequencing

Transcriptomic sequencing was used to verify the present microarray data. Briefly, a multi-targeted primer (Adomas et al. 2010) was used for reverse transcription of the first strand cDNA, and random hexamers (N₆, Invitrogen) were used to recover the second strand cDNA. After ligation of standard adaptors for Illumina sequencing, gel purification was applied to select 200 – 400bp cDNA fragments. cDNA samples were enriched with PCR using Pfx DNA polymerase (Invitrogen). Purified PCR products were sequenced for one end with 35bp in length on eight lanes of an Illumina Genome Analyzer at Yale Center for Genomic Analysis (YCGA). We used Tophat v1.2.0 (Trapnell et al. 2009) to perform spliced alignments of the reads against the *N. crassa* genome (<http://www.broadinstitute.org/annotation/genome/neurospora>). Only reads that mapped to a single unique location with in the genome with a maximum of two mismatches in the anchor region of the spliced alignment were reported in these results. To obtain a tally of the number of the reads that overlapped the exons of a gene, we analyzed the aligned reads with HTSeq v0.4.5p6 (unpublished; <http://www-huber.embl.de/users/anders/HTSeq/doc/>) and the gene structure annotation file for the reference genome. The tally for each sample was then processed with LOX v1.6 (Zhang et al. 2010) to estimate gene expression levels and determine statistical significance. Reads Per Kilobase of exon model per Million mapped reads (RPKM; Mortazavi et al. 2008) were also estimated for well measured genes.

2.6. Supplemental materials and database

Refer to Web version on PubMed Central for supplementary material.

3. Results

3.1. Similar morphological development of *N. crassa* on SCM and CA media

No differences were observed in number, color, and size of perithecia between SCM and CA media. Crossing between two mating types was accomplished by distributing *mat a* conidia onto fully developed *mat A* protoperithecia (Fig. 1B). With a careful selection of representative portions of plates, nearly all perithecia in each sample were at approximately the same development stage. Except for a slight increase in size and a slight darkening in color, no obvious tissue differentiation was observed for protoperithecia and perithecia within the 24 h immediately following crossing (Fig. 1B). Differentiated centrum parachyma was present in samples from 48 h (not shown) to 72 h (Fig. 1C, G). Asci and paraphyses became apparent in samples after 96 h (Fig. 1D, H). From 120 h (Fig. 1E, I) to 144 h (Fig. 1F, J), we observed the development of mature asci and ascospores.

3.2. Transcripts detected during sexual development for *N. crassa*

Among 9806 ORFs probed on the 70mer oligonucleotides microarray (Kasuga et al., 2005), 902 were expressed at a level permitting accurate measurement by microarray at all eight sampled time points during sexual development of *N. crassa* on both SCM and CA media (Fig. 2, Table S1, S2, S3). Most of these genes (>75%), while expressed, exhibited no significant difference in expression level between SCM and CA samples extending across development from 0 h to 96 h. Of genes that were differentially expressed between the two treatments, from 7% to 44% of measured genes were expressed significantly higher ($P < 0.05$) in the SCM samples than in the CA samples in sampled time-points (Fig. 3). Nearly 60 genes exhibited a consistent up-regulation of expression during sexual development for both SCM and CA samples (Fig. 2 cluster B), and they were enriched for functional categories of cell motility, detoxification, and fatty acid metabolism. Much greater diversification in expression between the SCM and CA samples was observed at 120 h and 144 h, during late sexual development, at which stages more than 80% of well measured genes showed a significant difference in expression level between cultures on different media (Fig. 2 cluster C and E).

Genes differently expressed between SCM and CA samples at every sampled time-point were significantly enriched in metabolic functions, transcriptional functions, and protein functions that involved a binding function or cofactor requirement (Table S4). Although genes differentially expressed between the CA and SCM samples exhibited a similar enrichment in functional categories across all eight time-points in *N. crassa* sexual development, CA and SCM samples at the same time point presented different enrichment patterns for genes with significantly higher expression levels ($P < 0.05$). Namely, during early sexual development stages before 96 h, genes highly expressed in the CA samples were narrowly enriched in only four functional categories, including proteins with binding function, biogenesis of cellular components, metabolism, and transcription. However, during late sexual development, genes highly expressed in the CA samples were enriched in 15 diverse functional categories. For genes highly expressed in the SCM samples, there was little difference in enrichment patterns between early and late sexual development stages (Table S4). Also worthy of mention, some metabolic genes showed a consistently higher expression during sexual development for the CA samples, including genes similar to transporters of sugar (NCU09550), amino acids (NCU08066), and iron (NCU03497), genes involved in ribosome biogenesis, and genes involved in phosphatidylinositol metabolism and signaling (NCU05644, NCU06348).

Microarray results based on SCM samples showed fewer genes being well measured cross all eight time points than those based on CA samples. Inclusion of hybridizations between

SCM and CA samples significantly improved the numbers of genes that well measured for SCM samples, and exclusion of SCM samples did not increase the number of well measured genes for CA only subset. Fewer than 500 genes were well measured for early development of perithecia from 0 to 48 h. However, more genes were well measured for subsets of hybridizations including only samples after 72 hours, and the most additional 344 genes were detected only as expressed from 96 h to 144 h using both SCM and CA samples (Table S5). Genes involved in apoptosis, secondary products, retrograde transport, disease, virulence and defense, were found significantly enriched in these 344 genes, of which most showed low-magnitude changes in expression (less than 2 fold) during the three time points in both CA and SCM samples. Comparatively, SCM samples showed a higher expression level than CA samples in genes detected only in the 96 h to 144 h subset. Genes including an exoglucanase I precursor (NCU07340), two 60S ribosomal proteins (NCU05804, 00706) and an aldehyde dehydrogenase (NAD⁺, NCU03415) were significantly down-regulated during late development in SCM samples (Table S5).

3.3. Transcriptional profiling of *N. crassa* on SCM

Growing on SCM, expression patterns for well measured genes typically exhibited dramatic changes along the complete time course of sexual development. More than 300 genes were up-regulated (Fig. 2 cluster C), and about 250 genes were down-regulated across sexual development (Fig. 2 cluster E). Among these dynamically regulated genes, there was no significant enrichment of genes annotated as related to sexual development or cell-type differentiation. Most of the corresponding expression changes were observed just after crossing (2 h) or during late development (96 h to 120 h). Fifty-six genes, enriched in cell type differentiation and energy function, were up-regulated at 2 h after crossing, while 63 genes, enriched in translation and ribosome biogenesis function, were down-regulated (Table S6). One hundred and eighty eight genes, enriched in transmembrane signal transduction and glycolysis and gluconeogenesis functions, were up-regulated from 96 h to 120 h. The gene encoding the key enzyme in melanin syntheses, tetrahydroxynaphthalene reductase (4HNR), showed a maximum expression level at 120 h. More than 224 genes, enriched for proteins with a binding function or a cofactor requirement, and for function in the biogenesis of cellular components, were down-regulated from 96 h to 120 h. From 24 h to 96h, we observed significant changes in expression for genes related to cell-type differentiation, when significant morphological changes were observed in the perithecia.

3.4. Transcription profiling for *N. crassa* on CA

Growing on CA, expression patterns for well measured genes typically did not exhibit dramatic changes along the complete time course of sexual development. Most genes showed a fairly consistent expression level across the sexual development (Fig. 2). Genes typically exhibited their maximum or minimum expression at 72 h or 96 h; however, most of the corresponding changes in gene expression were much smaller in magnitude than those observed on SCM, and were not statistically significant.

The number of genes significantly up-regulated and down-regulated in samples grown on CA varied from stage to stage across sexual development. Many genes exhibited significant changes in expression immediately following crossing (0 h to 2 h), from 2 h to 24 h, from 48 h to 72 h, and from 96 h to 120 h (Table S6). From 0 h to 2 h, more than 140 genes, which were enriched in functions of energy and cellular communication/signal transduction, were up-regulated, and about 100 genes, which were enriched in metabolism of cobalamins, serine and methionine, and involved in non-vesicular cellular import, were down-regulated after crossing. Genes functioning in translation and ribosome biogenesis were at the lowest expression level from 0 h to 2 h, consistent with expression in samples grown on SCM. From 2 h to 24 h, only 24 genes, enriched for secondary metabolism, translation, and

ribosome biogenesis, showed a significant up-regulation in expression, but during the same time period more than 120 genes, enriched in fungal cell type differentiation and transmembrane signal transduction, were significantly down-regulated. From 48 h to 72 h, 103 genes, enriched in cell type differentiation and cell adhesion, were up-regulated, while 151 genes, enriched in peptide-derived compounds metabolism, were down-regulated. During the late perithecial development (96 h to 120 h), 38 and 72 genes were up- and down-regulated, respectively. Up-regulated genes were significantly enriched in transport functions, while down-regulated genes were enriched in protein synthesis and cell type differentiation. Expression of *con-8*, a gene highly expressed in conidia, was detected across sexual development in both the SCM and CA samples. Expression of *con-8* during sexual development is consistent with a previous study demonstrating the expression of *con-8* in ascospore production (Springer and Yanofsky, 1992).

3.5. Impacts of nutrition on expression of selected genes in sexual developmental and metabolic pathways

A total of 16 genes that were annotated with a function in sexual development were well measured across all samples. Three of the genes, encoding the 14-3-3 protein, hydrophobin, and proteasome component PRE6, are involved in basic cellular biology in *Neurospora crassa* (Borkovich et al., 2004), as well as being classified as sexual development related genes. However, their function in sexual development in *N. crassa* has not been clearly demonstrated. Furthermore, these three genes exhibited different expression patterns when grown on SCM and CA. Eleven genes did not show significant changes in expression patterns between the different media after crossing in perithecial development from 24 h to 144 h, including *ccg-6*, *isp-4*, *poi-2*, *mkr5*, and *eif-5a* like, whose primary function may be in sexual development and cell type differentiation (Fig. 4). The gene *poi-2* is predicted to function in sexual development and is highly expressed in sexual and perithecial tissues of *N. crassa* (Kim and Nelson, 2005). MAP kinase pathway gene *mkr5*, encoding an aerial hyphae development related protein, plays an important role in cell-type differentiation in *Neurospora crassa* (Li et al., 2005). The gene similar to *eif-5a* (NCU08332) encodes a protein similar to a Magnaporthe grisea Hex1, a Woronin body major protein required specially during cell damage, and expression of this gene showed a peak right after crossing in 2 h samples for both treatments. We observed highly similar differential expression patterns across the time course of sexual development between the SCM and CA samples for these genes (Fig. 4). Expression of the clock-controlled gene *ccg-4* exhibited an increase on SCM at 24 h and 48 h, but no significant changes during later sexual development for both treatments (Fig. 4). Encoding a pheromone precursor, *ccg-4* is essential for the mating process in *N. crassa*, but its function in late stages of perithecial development requires further investigation (Kim and Borkovich, 2006; Wang et al., unpublished).

The *4hnr* and *al-1* genes are involved in pigment production during asexual and sexual development in *N. crassa*, and exhibited differential expression patterns during sexual development between growth on SCM and CA (Fig. 5). Expression of gene *4hnr* was modulated significantly during the sexual development, dropping in abundance at early stages and increasing in abundance at late stages. The *al-1* is a component of carotene metabolism, encoding a carotenoid dehydrogenase (Schmidhauser et al., 1990). The gene *4hnr* is involved in melanin synthesis, encoding a tetrahydroxynaphthalene reductase (Thompson et al., 2000). The gene *al-1* exhibited diminishing abundance during sexual development on sparsely nutritive SCM (Fig. 5A). In carotene-rich carrot agar, in contrast, it was maintained at a consistent expression level. Expression of *4hnr* was higher during sexual development on SCM than on CA for all sampled time-points, consistent with the typical pattern for differentially expressed genes measured in this study (Fig. 5B).

Because of the different carbon and nitrogen resources supplied, we compared expression of genes involved in cellulose metabolism, glycolysis/gluconeogenesis, and nitrate transport. We found significant differences in expression level and pattern (Fig. 6) specifically at earlier time-points between the SCM and CA samples for genes encoding endoglucanase II (*egl2*, NCU01050) and cellobiohydrolase 2 (*cbh2*, NCU09680) and specifically at late perithecial development for genes encoding for triosephosphate isomerase (*tim*, NCU10106), and glucose-6-phosphate (*pgi*, NCU02786). Expression of a nitrate transporter, NCU07205, was unchanged during the experiment for all samples. Consistent with findings for many other genes, the expression of these genes measured in the CA samples showed less dynamic differential expression across the time course of sexual development.

3.6. Verification of transcription profiles of CA samples using transcriptomic sequencing

Significant changes in gene expression from all eight time points across sexual development in CA were validated by comparison to an Illumina transcriptomic sequencing data set (Wang and Townsend, unpublished). Average RPKM reads of sequenced tags across all time points ranged from 0 to more than 2,000, providing measurements of expression level for 9717 genes. Among these 9717 genes, a lower coverage (below 7%) for weakly expressed gene (average RPKM reads lower than 100) was observed for the genes detected with microarrays (Fig. S1). For moderately expressed genes (an average RPKM read between 100 to 800), microarrays provided a consistent lower coverage (about 10%) compared with transcriptomic sequencing. A higher sensitivity of up to 44% was reached for highly expressed genes (Fig. S1). For genes of interest mentioned in the text above, except for the *elf-5a* like gene, expression profiles for CA samples were detected and validated by comparison with transcriptomic sequencing data (Fig. S2). For genes exhibiting large changes expression level (over 100 fold in LOX measurement), such as *mkr5*, *poi-2*, *egl2*, and *cbh2*, our transcriptomic sequencing data confirm the expression patterns observed with the microarrays across the sexual development. For genes exhibiting smaller changes in expression level, such as *cgg-4*, *cgg-6*, and *4hnr*, transcriptomic sequencing provided highly similar expression profiles with a much finer resolution. Two genes, *tim* and *pgi*, exhibited less than a 3 fold change in our experiment with transcriptomic sequencing, but no significant difference in the microarray measurement. The genes *al-1* and *isp-4* exhibited no significant change in expression level across all time points using microarrays due to the large confidence intervals, and a maximum of 10 to 18 fold change with transcriptomic sequencing with similar up- or down-regulation patterns between time points. The expression pattern of these genes was similar in both microarray and transcriptomic sequencing data.

4. Discussion

We have demonstrated that genomic gene expression is markedly different during sexual development on a chemically sparse synthetic source of nutrition and on a chemically diverse carrot agar medium. In particular, metabolic genes exhibited striking differences in expression both across stages of sexual development and comparing one nutritive medium to the other. Intriguingly, many more differentially expressed genes exhibited a significantly higher expression level in the SCM samples than in the CA samples, especially during early sexual development (Fig. 3). Nevertheless, expression of genes known to be highly important for sexual development exhibited relatively consistent expression across nutritive environments.

Genes significantly differentially expressed between cultures from the two media were found to be enriched in specific functional categories, including metabolism, regulation of metabolism and protein function, energy, and biogenesis of cellular components. We also detected expression differences in genes involved in cell type differentiation, cell cycle and

DNA processing, and cellular communication/signal transduction mechanism. In samples growing in the defined medium, increased expression of genes is presumably due to heavy traffic along metabolic pathways from a few abundant, undifferentiated molecular sources of carbon and nitrogen. Accordingly, diverse compounds of carbon and nitrogen in CA provide widely distributed starting points for material/energy flows through metabolic networks, and thus lower differential expression, a pattern expected to be especially strong early in growth when the organism has processed fewer nutrients. Interestingly, more than two thirds of measured genes also were differently expressed between the SCM and CA samples during the late developmental stages. This differential expression may arise due to the provision by rich media of more complex compounds for anabolic pathways after a long culture period, when easily metabolized undifferentiated carbon and nitrogen compounds in SCM had been exhausted.

Expression impacts were greatest on genes functioning in metabolic pathways, as may be expected due to the differences in nutrients between the two media. For example, expression of genes involved in carotene synthesis and glycolysis metabolism exhibited different patterns between the SCM and CA samples (Fig. 5, 6). The dynamic expression patterns observed in SCM during early phases may indicate a quick response from cells to transform abundant supplies of limited range of nutrients to diverse metabolites. In contrast, the less dynamic expression of these genes across the sexual development in the CA samples may be attributed to a simpler steadily diminishing supply of diverse metabolites. Interestingly, we observed two peaks of expression (at 0 h and at 72 h) of both endoglucanase II and cellobiohydrolase 2 RNAs, involved in cellulose metabolism and likely regulated in a similar pattern (Mernitz et al., 1996; Sun and Glass, 2011), in the CA samples but only one peak (72 h) of expression of this gene was present in the SCM samples. The second expression peak at 72 h might correspond to a shift toward usage of the cellophane membrane covering the media surface as a carbon resource. Being aware of this potential metabolic shift toward decomposition of cellophane membranes is important for studies of fungal metabolism. An alternative would be use of perforated polycarbonate membranes that have been used in study physiology of *Aspergillus niger* (Levin et al., 2007), and have been advocated as a reliable system to access protein secretion and fungal growth (de Vries et al., 2011). Expression of the gene *4hnr*, which catalyzes melanin synthesis and contributes to the dark-colored perithecial walls, dropped significantly after the 2 h time point in CA, and maintained a low level of expression at 24 h and 48 h. In SCM, expression of *4hnr* also decreased after the 2 h time point, then gradually increased until at 120 h and drops again slightly (Fig. 5). As the application of conidia in Tween during crossing constitutes a significant physical disturbance of the surface culture in the protoperithecial plates, these expression differences might be a consequence of a differential reaction of the fungus to the disturbance in SCM and CA. Certainly, the damage to the hyphae would lead to the entry of different ambient compounds in the two media.

Some categories of genes, in contrast, reacted similarly between environments: genes involved in translation and ribosome biogenesis were enriched in genes down-regulated at 2 h, and up-regulated at 24 h in both the SCM and CA samples. Expression of genes related to sexual development, such as *ccg-4*, *poi2*, and *isp4*, exhibited consistent expression patterns between the SCM and CA samples. Morphologically, there were no apparent differences in number, size and color of the developing perithecia between samples from different media across stages of sexual development. A gene related to aerial hyphae development in the MAP kinase pathway, *mkr5*, (Li et al., 2005) also exhibited the same expression pattern in both the SCM and CA samples, except for higher expression before the crossing for the CA sample, which was observed with abundant aerial hyphae not seen in the SCM protoperithecia plate (Fig. 4). With the development of perithecia, plenty of aerial hyphae were present in both the SCM and CA plates, and correspondingly the expression of *mkr5*

was up-regulated during the late sexual development stages. Interestingly, the expression profile of *mkr5* was similar in pattern changes with the expression of sexual development gene *poi2* for both the SCM and CA samples, and this agrees with previous studies suggesting a potential function of the MAP kinase pathway in sexual development of *Neurospora crassa*.

Conclusions

We have demonstrated that different nutritive environments have significant impact on genome wide transcription profiles in fungi with different nutrient preferences, and a common nutrient background would be ideal for direct comparisons between species. Less dramatic changes in most metabolism genes in CA suggested it as a more natural system than a sparse synthetic media for study of the genetic basis of sexual development and for comparison of development in *Neurospora* and other fungal species. Despite the considerable metabolic differences between media, the consistency of the developmental genetic program across media is reassuring, and facilitates informed analysis of comparative studies that require different nutritional resources for sexual development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- We examine sexual development in *N. crassa* in synthetic and natural media.
- We assess genomic gene expression across eight developmental stages.
- Metabolic genes are differentially expressed in different environments.
- Metabolic gene expression is more dynamic in synthetic medium.
- Sexual development genes are consistently expressed across media.

Figure 1.

Stages of perithecial development examined in this study. A. Experimental design of microarray hybridizations of reciprocally labeled cDNA samples from eight developmental stages of *N. crassa* grown on SCM and CA. Cultures of *mat A* were sampled from SCM and CA for the zero time point immediately prior to crossing by fertilizing protoperithecia from a seven day old culture of *mat A* with *mat a* conidia. B–F: Longitudinal section ($\times 200$) and squash mount of perithecium ($\times 100$) illustrating sexual development in *N. crassa*. B. Perithecium just after crossing (2 h). C. Longitudinal section and G. squash mount of immature perithecium, showing centrum parenchyma (CP, 48 h). D. Longitudinal section and H. squash mount of developing perithecium displaying development of asci (AC) and paraphyses (PH, 96 h). E. Longitudinal section and I. Squash of developing perithecium with young asci (AC, 120 h). F. Longitudinal section and J. Squash of mature perithecium with asci and ascospores (AS, 144 h).

Figure 2.

Heat map depicting gene expression levels for well measured genes at all eight sampled time points for *N. crassa* grown on SCM and CA. Genes were hierarchically clustered with MetATT (Xia et al., 2011) by Pearson correlation of BAGEL estimates of gene expression, which had been mean-centered and divided by the standard deviation of each variable. A large portion of measured genes exhibited different expression profiles between the two nutrient environments. These differences in gene expression profiles contributed to a hierarchical clustering that recognized five main clusters (A to E).

Figure 3.

Distribution plots of expression levels across genes (gene expression measures are relative to the lowest expression level observed in the experiment for that gene), for *N. crassa* grown on SCM (blue) and CA (yellow), at each sampled time point. Quantiles are indicated at 5% (lowest point of line), 25% (lower edge of box), 50% (middle line), 75% (upper edge of the box), and 95% (highest point of line).

Figure 4.

Genes annotated for a function in sexual development in *N. crassa* exhibited similar expression patterns grown on CA (yellow) and SCM (blue), and BAGEL estimates of gene expression (gene expression measures are relative to the lowest expression level observed in the experiment for that gene) were compared: A. “clock-controlled gene” *ccg-6*, B. MAP kinase regulatory gene *mkr5*, C. NCU08332, a gene likely encoding an eIF-5a factor, D. *poi-2*, a gene predicted to be function in sexual development, E. NCU08397, a gene encoding an ISP-4-like protein, F. “clock-controlled gene” *ccg-4*.

Figure 5.

Carotene and melanin metabolism genes in *N. crassa* grown on SCM (blue) and CA (yellow) exhibited different expression profiles across sexual development, and BAGEL estimates of gene expression (gene expression measures are relative to the lowest expression level observed in the experiment for that gene) were compared: A. Expression of *al-1* in *N. crassa* grown on SCM and on CA. B. Expression of *4hnr* in *N. crassa* grown on SCM and on CA.

Figure 6.

Metabolic genes involved in cellulose processing and glycolysis exhibited globally similar expression profiles in *N. crassa* grown on SCM (blue) and CA (yellow), with some stage-specific differential expression, and BAGEL estimates of gene expression. Gene expression levels are scaled relative to the lowest expression level observed in the experiment for that gene. A. Endoglucanase II (NCU01050), B. Triosephosphate isomerase (NCU10106), C. Cellobiohydrolase 2 (NCU09680), D. Glucose-6-phosphate (NCU02786).