

Metabolism and development during conidial germination
in response to a carbon-nitrogen-rich synthetic or a natural source of nutrition in
Neurospora crassa

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Abstract

Fungal spores germinate and undergo vegetative growth, leading to either asexual or sexual reproductive dispersal. Previous research has indicated that among developmental regulatory genes, expression is conserved across nutritional environments, whereas pathways for carbon and nitrogen metabolism appear highly responsive—perhaps to accommodate differential nutritive processing. To comprehensively investigate conidial germination and the adaptive life history decision-making underlying these two modes of reproduction, we profiled transcription of *Neurospora crassa* germinating on two media: synthetic Bird medium, designed to promote asexual reproduction; and a natural maple sap medium, on which both asexual and sexual reproduction manifest. There were no observable epigenetic effects on conidial germination due to previous culture on synthetic medium, and a later start to germination but faster development was observed on synthetic medium. Metabolic genes exhibited altered expression in response to nutrients—34% of the genes in the genome were significantly (Bonferroni adjusted $P < 0.01$) down-regulated during the first two stages of conidial germination on synthetic medium. Knockouts of genes exhibiting differential expression across development altered germination and growth rates as well as causing abnormal germination. A consensus Bayesian Network of differentially expressed genes indicated especially tight integration of environmental sensing, asexual and sexual development, and nitrogen metabolism on a natural medium, providing a comprehensive view of the impact of nutrition: in natural environments, a more dynamic and tentative balance of asexual and sexual development may be typical in the growth and development of *N. crassa* colonies.

Importance

One of the most remarkable successes of life is its ability to flourish in response to temporally and spatially varying environments. Fungi occupy diverse ecosystems, and their sensitivity to environmental changes often drives major fungal life-history decisions, including the major switch from vegetative growth to asexual or sexual reproduction. Spore germination comprises the first and simplest stages associated with the switch. We examined the dependence of early life history on nutritional environment using genome-wide transcriptomics. We demonstrated that for developmental regulatory genes in general, expression was conserved across nutritional environments, whereas metabolic gene expression was highly labile. Parents' nutritional conditions had no impact on asexual growth, however the level of activation of developmental genes did depend on current nutrient conditions, as did the modularity of metabolic and developmental response network interactions. This knowledge is critical to future technologies that could manipulate fungal growth for medical, agricultural, or industrial purposes.

Introduction

Fungi exhibit a extensive diversity in morphology and natural history and can be found in nearly every environment inhabited by living organisms. Their dispersal via spores—and in some cases hyphal fragments— spawns new opportunities over long distances, but also creates unexpected environmental challenges for the initial growth of individual fungi (1–4). How fungi physiologically adapt to the stochastic nature of the environment they encounter and synchronously regulate metabolism and development toward a successful colony remains unclear. In response to exposure to a harsher environment, many ascomycetes produce meiotic spores (ascospores) via sexual reproduction instead of producing multiple bouts of large numbers of asexual mitotic spores (conidia; 5). Conidia, in contrast, usually lack thick cell walls or dark pigments that provide resistance against radiation or drought conditions that are characteristic of ascospores. In the typical life cycle of ascomycete fungi, vegetative growth entails an adaptive decision regarding maintenance of asexual reproduction via continued hyphal growth and production of conidia, versus sexual reproduction of resistant meiotic spores that can survive harsh changes in the environment. Illumination of the genetics and biology of conidial germination would provide insights into mechanisms of response to environmental signals in fungi, especially those mechanisms of response that are active during their early vegetative growth leading to asexual and/or sexual reproduction. Such knowledge would also aid in the development of efficient and powerful approaches to control fungal growth, with applications to pathogen control, decay prevention, environmental protection, fermentation optimization, and bioenergy generation (6–10).

Neurospora crassa, a model filamentous fungus that flourishes in post-fire environments, has long been studied to understand fungal biology and ecology (11, 12). Morphological development during asexual growth in *N. crassa* had been characterized mainly via gene-by-gene study of specific developmental stages, ranging from conidial germination to hyphal elongation and branching and the formation of new dispersal units (11, 13-21). Other genes have been identified that are responsive to external environmental as well as internal environmental factors during *N. crassa* growth (13-16). Intensive research has been focusing on genes responsive for light, temperature, ROS, and nutrients (15, 17, 18) as well as G-protein signaling that is essential for conidia germination and normal hyphal growth in *N. crassa* (19). Furthermore, computational annotation of metabolic pathways associated with the *N. crassa* genome have improved (20-24), incorporating the extensive available biochemical genetics of *N. crassa* metabolism. Well-annotated fungal genomes and current transcriptomic tools also make it possible to efficiently characterize groups of genes and gene networks that underlie specific biological states or processes (25-28).

Regulation in response to properties of the environment plays a key role in the fundamental life-cycle fork governed by the classic autoregulatory asexual-sexual switch. One of two competing forms, asexual or sexual reproduction, is selected via a process in which execution of either developmental program inhibits execution of the other (29, 30). As a general rule for fungi, nitrogen starvation inhibits conidiation and induces sexual development, resulting in slow, robust dispersal, and carbon starvation leads to conidiation and sexual development (31, 32). Nitrogen starvation has long been known to induce or up-regulate synthesis of “sexual development genes” (*sdv*), most of which are responsive to mating-type expression, suggesting that fixed

nitrogen is one of the key environmental regulators in *N. crassa* sexual development (Nelson and Metzenberg 1992). The effects of carbon starvation on induction of reproduction are also associated with the specific down-regulation of expression of a large set of genes that is termed carbon catabolite repression (33). Abundant carbon and nitrogen, in contrast, promote asexual growth, resulting in rapid dispersal. As the environment is exhausted of nutritional sources, fungi such as *N. crassa* are thought to switch to sexual development to produce resistant sexual spores.

Although *N. crassa* has been a model for filamentous fungi for both sexual perithecium development and asexual conidia development, there are few genetics-of-development studies during early stages in conidial germination, and none that compare effects of carbon and nitrogen supply (Horowitz et al. 1976; Kasuga 2005; Kawanabe et al. 1985; Rao et al. 1997; Eaton et al. 2012). Conidial germination is rapid and dramatic, constituting a suite of morphological changes that must be a challenge to regulate in the face of sparsity of nutrients within the environment that conidia of *N. crassa* often encounter. Most studies on conidial germination of *N. crassa* use standard artificial media, such as Vogel's medium (34) and Bird medium (35), which contain an abundance of carbon and nitrogen sources that repress sexual development. With results on artificial media available as a basis for comparison, it is becoming increasingly feasible to design experiments that will illuminate fungal ecology (36). Conclusions based on analysis of cultures on artificial media need to be investigated with nutritional resources approximating the natural environment in which the fungus grows, for which metabolic and developmental pathways have been naturally selected. These natural environments likely contain low concentrations of simple carbohydrates and organic acids.

Using artificial Bird medium (BM) that promotes asexual development as well as a more natural maple-sap medium (MSM) that supports both asexual and sexual reproduction, we investigated the synchronous metabolic and developmental processes during the germination of *N. crassa* conidia. Transcriptomics were profiled for this germination process that commences with a newly produced asexual spore, proceeds with polar growth of the germination tube, and leads to hyphal growth and the first branching event. Divergent expression across the process and between the culture condition were used to guide knockout phenotype investigation to get insights of the genetic regulation during the conidial germination.

Results

Our analyses have been focused on groups of regulatory genes, including histone activities and genomic methylation, hyphal development characterized for conidial germination, transcription factors and response to environmental signals during major morphological development in conidial germination (Figs. 1, 2). Between cultures on BM and MSM, these genes exhibited differential regulation across conidial germination (Fig. 2A–E). Expression profiles of these genes enabled reconstruction and comparison of regulatory networks and metabolic pathways relevant to development on BM and MSM. Lastly, this study also collected new data about expression of isoforms during conidial germination, and their relation to conidial germination on hyphal development.

Conidial germination on different media—nearly 50% of typical wild type *N. crassa* conidia germinated within three hours after plating out onto BM and MSM solid media, which are similar in concentration of total carbohydrates but very different in concentration of nitrogen and mineral elements (**Fig. 1, Table S1**). Nitrogen content in MSM is too low to be detectable

(Table S1), and nitrogen starvation is known as a factor promoting sexual development in *N. crassa* (Park et al. 2008). Conidial germination started earlier on MSM (20% conidia germinated during the first 60 mins of inoculation) when compared to BM (almost no conidia germinated during the first 60 mins of inoculation). Extension of the germ tube and hyphal development were slower on MSM than on BM (**Fig. 3**). In addition, although in plate tests more conidia failed to germinate on MSM (6 / 60 = 10%) than on BM (3 / 60 = 5%), the difference was not statistically significant (Fisher Exact Test, $P = 0.4906$). Cultures on MSM started to produce protoperithecia and then perithecia within 10 days after inoculation—yet at a visibly lower density than typically observed on Synthetic Crossing Medium (SCM; Westergaard and Mitchell 1947), which has frequently been used as a medium with low nitrogen content that induces sexual development in *N. crassa*. (**Fig. S1**).

Sequencing mRNA during germination of conidia—We sampled RNAs from four conidial germination stages, including fresh conidia, first polar growth of germ tube, doubling of the germ-tube length, and appearance of the first branch, cultured on both BM and MSM. In total, 40.6–94.5 million 76 bp pair-end reads were obtained from all samples (GEO accession GSE101412). Total reads and mapped reads from RNA extracted from cultures on BM were slightly higher (on average, 6.8% and 7.3% respectively) than those from cultures on MSM (Table S2). The average coverage depths for the RNA-seq data sets were 70–120X, and the rate at which reads mapped to the genome ranged from 93.7%–96.6% (Table S2). Average coverage depths were calculated following Illumina guidelines: $C = LN / G$ (C , coverage; G , haploid genome length; L , read length, and N , number of reads). The number of genes for which

expression was detected for one time-point on BM or MSM ranged from 9167 to 9202 (**Tables S3, S4**).

Transcriptomics profiles during germination of conidia in different nutrient conditions—

Between stages of fresh conidia to polar growth, a larger portion (1818 genes) of the genome was significantly (Bonferroni adjusted $P < 0.01$) down-regulated, and these genes are associated with regulation of transcription, DNA binding RNA polymerase II transcription factor activities and zinc ion binding (**Tables S3, S4**). Functional enrichment analyses identified differently enriched gene activities, especially signaling pathways, cell cycle control, carbon and nitrogen metabolism as well as biosynthesis of amino acids between BM and MSM cultures across different morphological stages (Table S5). Among 734 genes that were up-regulated in both media during this stage, nitrogen and carbon metabolic pathway were significantly ($P < 0.01$, FungiFun, Priebe et al. 2015) enriched. Interestingly, at the last conidial germination stage 2657 genes were up-regulated on MSM but down-regulated on BM; these genes were enriched for MAPK signaling pathways.

*Expression of genes in histone activities and genomic methylation—*Among 70 genes annotated for histone functions, 30 genes showed up-regulated expression, and expression of the other 40 genes was generally down-regulated for all sampled cultures during conidial germination (**Tables S3, S4**). For genes that were up-regulated across most of germination, expression levels on MSM increased steadily, while on BM, up-regulated genes typically slightly decreased at the germ tube stage before increasing afterwards.

Expression of genes in metabolic pathways characterized for conidial germination—In general, two major types of expression patterns, down-regulated through all four stages and up-regulated after the second stage of germ tube appearance, can be recognized during conidial germination on BM, while expression patterns for this process on MSM were more multifarious with many genes up-regulated towards the first branching event. Carbon and nitrogen play critical roles in *N. crassa* growth and development, and G-proteins and coupled receptors (gpr) that are critical regulators of fungal response to carbon and nitrogen nutrition are among the major regulators of *N. crassa* development (40–43). Gene *gna-1* is coupled with *gpr-4* in regulating the response to carbon source (44). Expression of *gna-1* and *gpr-4* were highly coordinately down-regulated in cultures on BM but were opposite in cultures on MSM with *gna-1* up-regulated (**Fig. 4A**). Upregulation of *gna-1* in cultures on MSM is consistent with previous suggestions for additional function of *gna-1* in sexual development (45). Expression of *gna-1* was downregulated in cultures on BM, which is consistent with our observation of repression of the sexual development in cultures on BM. *Gpr-5* and *Gpr-6* are homologs of Stem1, a transmembrane protein in yeast that signals upon nitrogen starvation (11, 40, 46). Their roles as nitrogen sensors in *N. crassa* growth and development have not yet been confirmed (Cabrera et al. 2015). In our experiments, expression of *gpr-5* and *gpr-6* and their potentially coupled gene *gna-3* were highly coordinately down-regulated on nitrogen-rich BM (**Fig. 4B**). Up-regulated expression of *gpr-6* and *gna-3* in extremely low nitrogen MSM invites further investigation of their roles as potential nitrogen sensors in *N. crassa*. For 17 genes involved in nitrogen metabolism, 14 were dramatically up-regulated, including up to 51-, 81-, and 147-fold increases respectively for nitrate nonutilizer-10 (*nit-10*), NCU02361 (formamidase), and *nit-6* in conidial germination on nitrogen-deficient MSM. MAPK regulatory networks are involved in

response to nutrition, including regulation of entry into serial morphological development stages as well as in the activation of the switch from the asexual to the sexual phases of the life cycle (47–50). For 59 genes that can be mapped to major functions of the MAPK signaling pathway of yeast (KEGG pathways) (51), expression showed much greater changes during germination on MSM than during germination on BM (**Tables S3, S4**). Most MAPK pathway genes in response to starvation were significantly up-regulated during conidia germination on MSM (**Fig. S2**). As sucrose is the dominant carbon source in MSM, we observed a stably high expression of invertase (*inv*, NCU04265) during the first two stages of conidia germination on MSM but an abrupt down-regulation in expression of this gene on BM.

Expression of genes in hyphal development characterized for conidial germination—The roles of some genes involved in conidial germination are known, and expression of these genes showed different patterns between BM and MSM cultures (**Fig. 5**). Genes associated with carotenoid synthesis were up-regulated 2–7-fold during conidial germination in cultures on MSM; but with a nearly 2-hour delay in up-regulation in cultures on BM (**Fig. 5A–B**). Several genes, when mutated, have shown to confer colonial temperature sensitive (*cot*) phenotypes (52–56). Expression of these *cot* genes were first down-regulated and then up-regulated during germinating tube extension for cultures on BM. These genes, excluding *cot-3*, were generally up-regulated throughout the process for MSM cultures (**Fig. 5C–D**). Similar expression differences between the two nutrient conditions were also observed for genes related to polarity establishment, and genes involved in chitin synthesis, septation, and budding (**Fig. S3, S4**). In contrast, expression of genes required for sexual development was generally down-regulated during conidial germination on BM while up-regulated for MSM cultures (**Fig. 5E–F**).

Expression of transcription factors in conidial germination—Among the 100 TFs profiled (Table S3, S4), some regulate asexual and sexual development ([Colot et al. 2006](#); [Fu et al. 2011](#); [Boni et al. 2018](#); [Carrillo et al. 2017](#)). Two expression patterns were commonly observed for TFs in culture on BM: 48 TFs were steadily downregulated, exhibiting lowest expression at the last stage, and 36 exhibited their lowest expression at the second stage (polar growth). Expression of these TFs showed various expression patterns for MSM cultures. The TFs regulating both basal hyphal growth and asexual development showed similar down-regulated expression patterns in RNAs obtained from cultures grown in both nutrient conditions. The exceptional transcription factors were *tah-1* (tall aerial hyphae) and *tah-4*, which were both significantly up-regulated in both BM and MSM cultures. Interestingly, expression of *sub-1*, a key regulator in the asexual-sexual switch, showed opposite regulation patterns between the two nutrient conditions with significant up-regulation for conidia germination on MSM, which is preferred for sexual development.

Expression of genes in response to environmental signals during conidial germination—Chen et al. (15) identified genes that exhibited early responses (ELRGs) and late responses (LLRGs) to light stimulation during *N. crassa* asexual growth. Our analysis demonstrates that nutrients affect expression of light-responsive genes during conidia germination. Many ELRGs were up-regulated from germination to the first hyphal branching when cultured on MSM, but were down-regulated during the same stages when cultured on BM. For example, NCU01258 (*cyn-1*), which encodes cyanate lyase in nitrogen metabolism, exhibited opposite expression patterns between the cultures on nitrogen-rich BM and cultures on nitrogen-poor MSM. Up-regulation of

cyn-1 indicates that the fungus turned to complex compounds such as cyanate as an alternative nitrogen supply in MSM. LLRGs were generally up-regulated during germ-tube extension and hyphal growth, but there was a dramatic down-regulation of these genes before germ tube appearance in conidia on BM, including a near 70-fold drop for *inv* (invertase, NCU04265), which catalyzes degradation of sucrose to glucose.

Coordinated metabolic and developmental networks during conidial germination—Key genes annotated in nitrogen metabolism and genes with known roles in conidia germination and the asexual-sexual switch were investigated for their associations in co-expression networks using a Bayesian criteria on edge support (**Fig. 6, Table S6**). Interestingly, associations among asexual development, including conidiation genes *con-8* and *con-13*, and genes *cot-2*, *cot-1*, and *cot-5* regulating the asexual growth and development, were conserved between cultures on the BM and MSM. Associations among genes playing roles in the initiation of sexual development, including light sensors *nop-1* and *phy-2* as well as genes *per-1*, *pp-1*, *sd*, and *tnr-1*, were also conserved between the two cultural conditions. However, for cultures on BM, the asexual development subnetwork was tightly modular, with nitrogen pathways represented by nitrate transporters *nit-10*, *nit-3* and *nit-6*, while the sexual initiation subnetwork was positioned downstream, perhaps responsive to expression regulation within the asexual development subnetwork—specifically, to nitrogen metabolism. Consistent with the higher levels of nitrogenous nutrition in BM (Table S1), nitrate transporters were modular BM cultures, in line with intensive activation of nitrogen intake and metabolism. Multiple interactions between nitrogen metabolism and a similarly modular conidiation pathway suggest a coordinated response nitrogen metabolism and conidiation to high-nitrogen media, associated with the promotion of asexual growth and

inhibition of sexual development. For cultures on the MSM, which supplies only trace amounts of nitrogen and facilitates entry into both asexual and sexual development, the asexual and sexual initiation modules were distinct but appeared less hierarchically organized, and nitrogen metabolism genes were integrated with those pathways much more than they were integrated with each other.

Alternative splicing—898 genes have been annotated with at least two isoforms (<http://genome.jgi.doe.gov/Neucr2/>), most of which are proteins involved in metabolism. By analysis of our paired-end sequencing, we differentiated among expression of these isoforms during the conidia germination (**Table S7-1**). Isoforms of 21 genes exhibited differential expression patterns between BM and MSM cultures, especially during the early and late stages (**Table S7-2**). Among the 17 isoforms exhibited a significant ($P < 0.01$) expression change for cultures on both media, isoforms of sexual reproduction essential gene *sub-3* (*submerged protoperithecia-3* NCU01154T1) exhibited 2-fold down-regulation for BM cultures but 2.5-fold upregulation for MSM cultures.

Knockout phenotypes during conidial germination—195 genes exhibited statistically ($P < 0.05$) and biologically (5-fold expression difference) significant differences between the two media, when comparing similar time points. Among these genes, 144 knockout strains ([Colot et al. 2006](#)) were available from the Fungal Genetic Stock Center (FGSC, 59) for phenotypic investigation. Compared to the strains with the same mating type strain as our wild type strain, we observed altered phenotypes in 22 of the knockout strains during conidial germination along with different expression regulations (**Figs. 7, S6**)., Of the 22 genes knocked out, nine genes

were not yet functionally annotated, and the other 13 genes were suggested to be involved in protein and amino acid metabolic processes, including genes coding for cation transporter (NCU00795), isovaleryl-CoA dehydrogenase (NCU02126), ergothioneine-1 (NCU04343), acetyltransferase (NCU04583), alpha-glucan branching enzyme (NCU05429), proteasome activator (NCU05620), carboxypeptidase S1 (NCU05980), glutamate decarboxylase (NCU06112), uracil phosphoribosyltransferase (NCU06261), glycogen synthase-1 (NCU06687), metallo-beta-lactamase (NCU07044), L-galactonate dehydratase (NCU07064) and nicotianamine synthase (NCU09855) (60). Expression of NCU07801, a hypothetical protein, was detected only in the early stages of germination, and the knockout of this gene exhibited significantly reduced germination rate and hyphal growth. Expression of NCU08095 was significantly down-regulated in both BM and MSM cultures. A knockout of NCU08095 exhibited a spore elongation stage, during which conidia spores extended their long axis to form a dumbbell-like structure (NCU08095, *cdg*, *conidia dumbbell germination*), before forming a normal germ-tube (**Fig. 8**). Orthologs of NCU08095 were found only in some genomes of Sordariomycetes, Leotiomycetes and Eurotiomycetes, and were absent in other ascomycetes and yeast genomes—and the ortholog in *Rustroemia* was annotated as a kynurenine formamidase (**Fig. S7**).

Discussion

Many fungi depend on production and distribution of asexual spores to efficiently colonize new substrates, and conidial germination is the critical first step of fungal colonization. We previously reported elements of sexual expression in asexual reproduction of *N. crassa* on BM (61). In this study, we investigated the conidial germination process and its responses to different nutritional conditions by profiling transcriptomics across four distinct morphological stages of

conidial germination on two different media. Commercially produced maple sap provides a readily available natural nutrient source enabling investigation of a more natural ecology and genome-wide transcriptomics, potentially more faithfully revealing core life-history regulatory mechanisms during diverse stages of *N. crassa* development, including conidial germination.

Fungal spores prepare for flexibility instead of efficiency—Nutrients are a major factor influencing the onset of *N. crassa* colonization, and extended maintenance on a singular artificial media is known to lead to degeneration of strains (62–64). Natural environments of *N. crassa* can be very diverse in terms of carbon and nitrogen level, and this fungus has been often found to flourish in high-carbon environments, in particular non-charred, sappy, woody remains of plants after forest fires. *N. crassa* has also been suggested to propagate as a plant endophyte (Kuo et al. 2014); MSM resembles the nutrition accessible to the fungus within the living plant environment. While parental original expression has been reported from fungal species (76–78), our data exhibited no impact of parental nutrient conditions on conidial germination. Although the tested wild type strains had been maintained on BM for multiple generations, the delayed germination of their conidiospores on BM is evidence that the conidia are equipped with a germination status to a more natural environmental setting. Such a lack of preference for parent nutrition conditions in conidial offsprings was supported by the dramatic change in transcriptomes and postponed expression up-regulation of large portion of metabolic pathway on BM. Our observation of postponed germination of conidia on BM also invites further investigation as to whether DNA methylation and strain degeneration might be occurring under constant growth on artificial media.

Genetic associations between development and the metabolism of nutrition—*Our observations are consistent with previous research on the impacts of nitrogen on sexual development, and further suggest that these effects begin in the very early stages of asexual growth during conidial germination. Nitrogen in the form of nitrate is the primary source of nitrogen nutrition and is often a limiting resource in natural environments* ([Follett 1995](#)). Therefore, fungal growth and development on the low-nitrogen natural medium used in this study is likely a good model for most fungal colonization of natural environments. *N. crassa* itself has a long history as a model in fungal genetics and developmental studies, and several standard media have been developed (65). Natural media such as carrot medium (66), have also been widely used in fungal research, especially in the case of pathogens whose growth conditions are challenging to be mimic with artificial media (18, 67). While these media are useful for investigating specific stages in *Neurospora* growth and development, they all have limitations when studying growth stages that require quick changes in nutrients under laboratory conditions. BM and MSM provide different nutrition (Table S1), especially in nitrogen level and carbon resources, for conidial germination, and MSM represent one of the likely environments for *Neurospora* that has been reported as a plant endophyte (Kuo et al. 2014). Low nitrogen in MSM induced the initiation of sexual development even at very early stage during conidia germination, and key regulatory genes in sexual development were activated in MSM cultures (Figs 3, 5, S1, S2 and S5).

Comparing the transcriptomics of cultures on different media provides insights as to how *N. crassa* growth and development responds to nutritional resources in natural environments. Asexual growth that respond promptly to carbon level showed no morphological differences between BM and MSM cultures, although the spores took long time to adopt to BM that made of mainly inorganic nutrition (Figs. 2, 4, S3 and S4). With regard to metabolism, we observed

expression regulation in response to carbon and nitrogen levels in the media. For example, expression of cyanate lyase *cyn-1* in nitrogen metabolism was upregulated for cultures in nitrogen poor MSM, and expression of invertase (*inv*) which catalyzes degradation of sucrose to glucose was down-regulated in glucose-rich BM (Table S3). In fact, two frequent expression patterns were identified on BM: continuously upregulated expression, and downregulated expression during the induction of germ tube formation followed by up-regulated expression afterward (Table S3). In contrast to these relatively homogeneous expression patterns attributed to a large number of genes in BM cultures, expression patterns identified in MSM cultures were more multifarious, indicating more intricacy of function and developmental regulation in cultures on the natural nutrients provided by maple sap.

Response of conidial germination to non-nutrient environmental factors—Regarding fungal responses to environmental signals, we observed much higher up-regulation of expression for early light-induced genes (ELIG) in MSM cultures than those on BM. ELIG are controlled by light-activated White Collar Complex (WCC) and regulate activities of late light-induced genes (LLIG) in growth, conidiation and sexual development in *N. crassa* (15, 16, 68–70). One explanation for the high up-regulation of ELIG in MSM cultures would be the boosted sexual development of *N. crassa* on MSM. Knockout of gene light-induced gene NCU01870 showed a female sterile phenotype (16), and this gene expressed increasingly in MSM cultures but was significantly down-regulated on BM, which inhibits sexual development in *N. crassa*. Using MSM helped us to reveal different aspects of gene regulation during the asexual-sexual switch by light responsive WCC and metabolism pathways, which have been impeded with specifically designed media like BM.

Our analysis showed that in the adaptively tuned decision to engage in asexual or sexual development, metabolic pathways, sensory responses to environmental stimuli, and developmental pathways are tightly associated even early during conidia germination. The asexual to sexual switch is an integrative process, linking the asexual and sexual modes of reproduction modes that respond to nearly opposite environmental settings (31, 61). In many ascomycetes, asexual reproduction is prolonged by high temperatures, high nutrition, high oxygen and ROS (reactive oxygen species), and by light exposure, and sexual reproduction can be induced by low temperature, low nutrition and oxygen, and reduced light intensity (71). Compared with artificial media such as BM, which is used specifically for inducing and maintaining asexual reproduction in *N. crassa*, MSM is closer to the natural nutrition in which this fungus is commonly encountered. Although our experiment only profiled expression during the very early stages of conidial germination and hyphal extension, genes modulating the asexual-sexual switch showed divergent regulating networks between the two culture conditions. Bayesian networks based on co-expression illustrated that both the asexual and sexual reproduction regulation were well modularized in both BM and MSM cultures. However, nitrogen metabolism regulation was tightly associated with asexual regulation only in BM cultures. The more diffuse organization of these gene interactions could be a consequence or cause of the more labile balance of the asexual-sexual switch in MSM. MSM cultures might induce a balanced regulation between asexual and sexual development.

Complex genetic regulation of conidial germination implied by KO phenotypes—Genes with critical roles in conidia germination were identified based on the comparative transcriptomics and focused knockout phenotyping. Many genes exhibited significant changes in expression

across the germination time course. 22 of them showed quantitative phenotypes in germination ratio and growth rate during conidial germination, some of which were already known to respond to environmental signals in fungal development. The knockout of NCU08095 (*cdg*) exhibited a germination phenotype resembling yeast budding morphology. BLAST searches against other fungal genomes suggested that this gene is conserved in Sordariomycetes and probably some other ascomycetes but that it is not present in yeast genomes. In fact, morphological transition from filamentous growth to yeast form are known to be associated with pathogenesis for some fungal species (Noble et al. 2017; Polvi et al. 2019).

Interestingly, no knockout strains showed distinct phenotypes on different media, although the targeted genes expressed significantly differently between the BM and MSM cultures. One explanation for this could be that expression of these genes are likely associated with developmental timing rather than being responsive to culture conditions. In other words, most genes that we chose based on their expression difference between the cultures on different media are likely quantitative regulators of developmental change, which led to quantitative differences in pace, rather than qualitative differences in development, between cultures on BM and MSM. Our results also call for greater attention to isoforms of genes and their distinct functions during fungal development. For example, isoforms of the gene *sub-3*, which is essential for sexual reproduction, exhibited a 2-fold down-regulation when cultured on BM. In contrast, isoforms of *sub-3* exhibited a 2.5-fold upregulation when cultured on MSM, on which sexual development can be expected to occur within a week after inoculation.

Conclusion—Synchronous metabolic and developmental processes underlying conidial germination, a rapid process in response to environmental signals including carbon and nitrogen

nutrition as well as light signals, were revealed by transcriptomics performed with synthetic or natural nutrition. Modularity among elements of early sexual development, asexual growth, and nitrogen metabolism were detected in conidial germination with a more diffuse set of network interactions in natural medium than in nitrogen-rich laboratory medium. The implication is that in natural environments a more tentative balance of asexual and sexual development is typical during growth and development of *N. crassa* colonies than has been previously implied by analyses relying on culture in media that suppress activation of the asexual-sexual switch. Nine genes that were previously unannotated for function that we have now identified as contributing to asexual growth after conidial germination may contribute significantly to modulating this balance and provide targets for future fungal growth control in pathogen prevention, biochemical fermentation optimization, and bioenergy generation.

Materials and methods:.

Strains and culture conditions—Germination studies were performed with *N. crassa* mat A (FGSC2489) macroconidia, which were harvested from 5-day cultures on solid (2% agar) Bird Medium ([Metzenberg 2004](#)). Macroconidia were collected with deionized distilled water containing Tween 20 (0.1%). They were then washed with autoclaved distilled water, filtered through a three-layer miracloth. 1×10^5 spores were placed onto the surface of cellophane-covered medium in standard Petri dishes.. MSM was based on maple sap (VerticalTM, Feronia Forests). When required, agar (2%) was added to the medium. Conidia were spread on both media and incubated at 25 C under constant white light. Light plays critical roles in *N. crassa* growth and development, and exposure to light is required for proper conidiation and production of conidia. The conidia used in this study were collected from culture under constant white light,

and avoid impacts from changes of light intensity during the conidia germination and RNA extraction process, the whole experiment was set under a constant stable light condition. Spore germination from dormant spores to branched hyphae was monitored at 15, 60, 120, 180, 240, 300 and 360 mins after inoculation. Cellophane membranes with fungal tissues were collected at 15, 120, 240, and 360 mins, when the majority of active spores (51%–92%) were at one of the following stages and beyond: fresh spores, showing evidence of polar growth, having doubled their long axis, and having commenced their first hyphal branching, on BM. The same time points were used for sampling tissues on MSM.. Tissue samples were flash frozen in liquid nitrogen and stored at -80 C. All tissues that were collected from multiple plates in one collection process were counted as one biological replicate. Three biological replicates were prepared for each sampled time-point on both BM and MSM.

RNA isolation and transcriptome profiling—Total RNA was extracted from homogenized tissue with TRI REAGENT (Molecular Research Center) as in Clark et al. (2008). Messenger RNA was purified using Dynabeads oligo(dT) magnetic separation (Invitrogen). Preparation of cDNA for sequencing followed the Illumina mRNA Sequencing Sample Preparation Guide. Complementary DNA was prepared using N₆ primers for samples of all time-points. The quality of cDNA samples was verified with a bioanalyzer (Agilent 423 Technologies) to ensure an insert size between 150–225 bp and by qPCR (Kapa Biosystems) to ensure an RNA concentration of at least of 0.5 ng/ ul before sequencing on an Illumina HiSeq 2500 at the Yale Center for Genomics Analysis (YCGA).

Data acquisition and analysis—Twenty-four sequencing libraries (3 replicates per condition) were produced from purified total RNA samples by the Illumina TruSeq stranded protocol. The libraries underwent 76 bp paired-end sequencing using Illumina HiSeq 2500 according to Illumina protocols, generating an average of 28 million paired-end reads per library. Adapter sequences, empty reads, and low-quality sequences were removed. For each read, we trimmed the first six nucleotides and the last nucleotides at the point where the Phred score of an examined base fell below 20 using in-house scripts. If, after trimming, the read was shorter than 45 bp, then the entire read was discarded. Trimmed reads were aligned to the *N. crassa* OR74A v12 genome from the Broad Institute (11) using Tophat v.2.0.12 with the default settings (Trapnell et al., 2009). Only the reads that mapped to a single unique location within the genome, with a maximum of two mismatches in the anchor region of the spliced alignment, were reported in these results. We tallied reads aligning to exons of genes with the program HTSeq v0.6.1p1. We also used HiSat2 and StringTie (72–74) to perform spliced alignments of the reads against the reference genome. A tally of the number of the reads that overlapped the exons of a gene was calculated using aligned reads and the gene structure annotation file for the reference genome (<http://genome.jgi.doe.gov/Neucr2/>). The tally for each sample was then processed with LOX v1.6 (75) to statistically analyze the gene expression levels. Each featuring at least one valid measurement at one of the four data points. Multiple hypothesis testing was applied when many genes were statistically evaluated, and a Bonferroni adjusted P value was provided for significance call ([Morgan 2007](#)). In addition, we also analyzed the differential expression at the isoform level using CuffDiff v 2.2.1, part of Cufflinks (76), providing the gene model annotation. More than 800 genes have been reported or predicted with isoforms

(<http://genome.jgi.doe.gov/Neucr2/>). We applied the fragment bias correction and strand specific parameters, leaving other options at their default setting.

Knockout strains and phenotype identification—Knockout strains for more than 9600 genes ([Colot et al. 2006](#)), including deletion cassettes for genes in either of the two mating types, were acquired from the Fungal Genetic Stock Center (FGSC) (59). knockout strains of genes that showed a significant (LOX P <0.01) expression difference across conidia germination in wild-type strains and regulation direction between the two media conditions (Tables S3, S4) were examined for altered phenotypes during conidia germination. Two mating types, *mat A* and *mat a*, regulate mating and sexual development in heterothallic *N. crassa*. Genotype *mat A* strains were assayed for their phenotype when available; otherwise, *mat a* strains were used. Genotype *mat a* strains were also assayed in parallel when *mat A* strains exhibited a distinct phenotype. Both *mat A* and *mat a* strains of wild type were monitored along with each knockout strain being phenotyped. All strains were cultured on BM and on MSM with three replicates under constant white light at 25 C. Germination of conidia was examined to reveal differences in development attributable to culture conditions. For each investigated strain, 3000–5000 conidia were plated onto 90 mm diameter plates and monitored. Germination and growth rate of 20 conidia picked at random were recorded. Each knockout strain that exhibited a significant morphological phenotype was crossed with the wild type strain, and cosegregation of the observed phenotype with deletion of the gene in the offspring was verified to ensure that the intended deletion was responsible for the mutant phenotype (18, 49, 77) .

Functional enrichment analyses—The Functional Catalogue annotation scheme (Ruepp *et al.*, 2004) was applied to group genes according to their cellular or molecular functions. The

statistical significance of over-representation of gene groups in functional categories relative to the whole genome was quantified by calculating P values via the hypergeometric distribution using the MIPS FunCat. Two kinds of P values are used to evaluate each functional category for whether the genes in each functional category were differentially expressed between stages at a higher frequency than expected based on the genome (Exact P) and based on background gene sets (Adjusted P). (Priebe et al. 2015). To achieve significance, we required both an Exact $P < 0.01$ and an Adjusted $P < 0.05$. Further functional annotation of statistically significantly differentially expressed genes in metabolic pathways was gathered via the biochemical pathway and annotation data in the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa and Goto, 2000). Functional annotation was further checked for genes of interest using the FungiDB database (60).

Bayesian network reconstruction—Biological networks were modeled using the Bayesian Network Web Server (78) supplied with conidia germination expression data for each culture condition. Input files contained fold changes between adjacent sample points across the experiment $((\log_2(\frac{F_{i+1}}{F_i}) - \log_2(\frac{F_{j+1}}{F_j})) / \sqrt{\frac{1}{n}(\frac{1}{F_i^2} + \frac{1}{F_j^2})})$. Global structure learning settings were retained at default settings. The models depicted are the 50% majority consensus of 100 models (edge selection threshold set to 0.5; the 100 highest-scoring networks were averaged), without imposing any structural constraints.

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Figure legends

Figure 1: *N. crassa* asexual spores cultured on BM (A-D) and MSM (E-H) at four distinctive morphological stages of germination: A,E) fresh conidia, B,F) polar growth, C,G) doubling of the longest axis, and D,H) at the time of the first hyphal branching. Arrows indicate first hyphal branches. Scale bar: 5 μ m.

Figure 2. Effects of media on conidial germination are genome-wide, and functional groups differentially respond to BM and MSM during the process. Examples include A) histone activities and genomic methylation, B) hyphal development genes annotated as functioning in conidial germination, C) transcription factors, D) early light responsive genes, and E) late light responsive genes. Ratios (BM / SM) of gene expression change (Tables S3, S4) from stage 1 to 2, 2 to 3, and 3 to 4.

Figure 3: Temporal analysis of growth and development of conidia of *N. crassa* cultured on BM and MSM. Plated conidia were examined at six time points across the process, enabling sector counts that revealed the A) proportions and B) stacked proportions of conidia at serial stages of germination cultured on BM, and the C) proportions and D) stacked proportions of conidia at serial stages of germination cultured on MSM. Measurements for conidia were color-coded at each stage of germination, including fresh conidia (blue), polar growth (red), doubling of the longest axis (green), and first hyphal branching (purple). An asterisk (*) indicates time points when RNAs were sampled. For staging, conidial germination was monitored for 20 conidia per plate, randomly picked on three plates; the error bars delineate one standard deviation of the mean.

Figure 4: Relative expression levels of G-protein coding genes exhibited differential regulation during conidial germination on BM and on MSM, including A) cAMP signaling genes that regulate responses to carbon sources, and B) expression of genes, *gpr-5* and *gpr-6* their potential coupled G-protein coding gene *gna-3*, that regulate responses to nitrogen starvation.

Figure 5: Relative expression levels of genes involved in carotenoid synthesis (pigmentation) pathways in culture on A) BM and B) MSM, colonial temperature-sensitive asexual development genes (*Cot*) in culture on C) BM and D) MSM, and selected genes whose expression is required for protoperithecial development, in culture on E) BM and F) MSM.

Figure 6: Bayesian networks of expression regulation show associations (arrows indicating directed edges) among genes involved in nitrogen metabolism (black), asexual growth and reproduction (blue), and light-regulated sexual development (red) during conidial germination and early hyphal growth A) on BM and B) on MSM.

Figure 7: Knockout strains exhibited higher and lower average growth at 2 h (blue) and 3 h (red) post inoculation compared with wild type strains of both A and a mating types (*wt-A* and *wt-a*). Phenotyping was performed on paired knockout and wild-type strains. Measurements of growth were conducted for three replicates of 20 randomly picked conidia for each strain. A high standard deviation is evident, arising primarily as a consequence of a high variance in the starting time of germination.

Figure 8: Phenotypes of knockouts of NCU08095 on BM at the stages of A) polar growth and B) doubling of the longest axis, exhibiting dumbbell-like (arrows) conidial extension before formation of the germ tube. Wild type germination on the same condition was shown in Fig.1.

Supplemental figure 1: Morphological development of *N. crassa* in (A–C) 3-day cultures and (D–F) 10-day cultures on Bird Medium (A, D), Maple Sap Medium (B, E), and Synthetic Crossing Medium (C, F). Abundant conidia were produced in the 3-day old cultures on BM and SCM (A, C). Protoperithecia were produced in the 10-day cultures on MSM and SCM media (E and F, inset). No protoperithecia were detected in the 10-day cultures on BM.

Supplemental figure 2: Expression differences of MAPK genes between BM and MSM cultures.

Supplemental figure 3: Expression differences of genes involved in hyphal growth between cultures on BM and cultures on MSM. A) genes involved in bud emergence and cell polarity; B) genes involved in bud site selection; C) genes involved in septins development and regulation; D) genes involved in syntenic homolog actin-ring formation; E) genes involved in syntenic homolog formation; and F) genes involved in transport and secretion during hyphal growth.

Supplemental figure 4: Cultures on BM and cultures on MSM exhibited differential regulation of nitrogen metabolism but similar regulation of polarity establishment, consistent with the observation of similar morphological development on different nitrogen supplies.

Supplemental figure 5: Differences in expression of light responsive genes during conidial germination. A) Expression of the Early Light Responsive Genes (ELRGs) was generally down-regulated in cultures on BM and MSM during the first two stages of conidial germination; B) Expression of the Late Light Responsive Genes (LLRGs) was generally down-regulated in cultures on BM but up-regulated in cultures on MSM.

Supplemental figure 6: Expression of genes showing knockout phenotypes during conidial germination in this study.

Supplemental figure 7: Phylogeny of orthologs of NCU08095 showing their existences in genomes of Leotiomycetes, Sordariomycetes and Eurotiomycetes. Branches with significant support (Posterior probability >0.95 from Bayesian analysis using MrBayes) were thicken.

Supplemental table 1: The compositions of BM and MSM.

Supplemental table 2: Summary of RNA seq data and mapping quality.

Supplemental table 3: Changes in gene expression levels during conidial germination on BM. analyzed using LOX.

Supplemental table 4: Changes in gene expression levels during conidial germination on MSM. analyzed using LOX..

Supplemental table 5: Results of functional enrichment analysis for genes classified with stage-specific expression patterns (Kegg).

Supplemental Table S6. Functional annotations of genes that were identified for the Bayesian Network of asexual-sexual developmental in responses to environmental factors during the conidial germination.

Supplemental table 7: 1) Significant expression changes between stages observed for isoforms in *Neurospora crassa* during germination of conidia on BM and MSM. 2) Isoforms whose expression was opposite and significantly different during germination of conidia on BM and on MSM.

