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Metabolic Impacts of Using Nitrogen and Copper-Regulated Promoters to Regulate Gene Expression in Neurospora crassa

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ABSTRACT The filamentous fungus Neurospora crassa is a long-studied eukaryotic microbial system amenable to heterologous expression of native and foreign proteins. However, relatively few highly tunable promoters have been developed for this species. In this study, we compare the tcu-1 and nit-6 promoters for controlled expression of a GFP reporter gene in N. crassa. Although the copper-regulated tcu-1 has been previously characterized, this is the first investigation exploring nitrogen-controlled nit-6 for expression of heterologous genes in N. crassa. We determined that fragments corresponding to 1.5-kb fragments upstream of the tcu-1 and nit-6 open reading frames are needed for optimal repression and expression of GFP mRNA and protein. nit-6 was repressed using concentrations of glutamine from 2 to 20 mM and induced in medium containing 0.5–20 mM nitrate as the nitrogen source. Highest levels of expression were achieved within 3 hr of induction for each promoter and GFP mRNA could not be detected within 1 hr after transfer to repressing conditions using the nit-6 promoter. We also performed metabolic profiling experiments using proton NMR to identify changes in metabolite levels under inducing and repressing conditions for each promoter. The results demonstrate that conditions used to regulate tcu-1 do not significantly change the primary metabolome and that the differences between inducing and repressing conditions for nit-6 can be accounted for by growth under nitrate or glutamine as a nitrogen source. Our findings demonstrate that nit-6 is a tunable promoter that joins tcu-1 as a choice for regulation of gene expression in N. crassa.

Regulatable promoters are powerful tools for genetic analysis of protein functions in both prokaryotic and eukaryotic organisms. These promoters have been used with great advantage for analysis of the functions of essential genes (Richardson et al. 1989; Miyajima et al. 1987). A drawback to this strategy is the time needed to dilute the essential protein due to cell growth and turnover after repressing mRNA production from the promoter. Regulatable promoters have also been used to express/overexpress genes at a particular time during growth or development and to study the resulting phenotypes. In fungi, perhaps the best-characterized and most-used regulatable promoter is the divergent promoter that regulates expression of GAL1 and GAL10 in the yeast Saccharomyces cerevisiae (Matsumoto et al. 1981; Guarente et al. 1982; Johnston and Davis 1984). This promoter is repressed during growth on glucose, but induced in medium without glucose and containing galactose (Matsumoto et al. 1981; Guarente et al. 1982). The GAL10 promoter has been used in a multitude of studies in yeast, including analysis of essential genes and regulated overexpression of genes (Richardson et al. 1989; Miyajima et al. 1987; Rose et al. 1987).

In recent years, there has been increasing interest in using microorganisms both for overexpression of native proteins and heterologous expression of proteins from other organisms (Medema et al. 2011). Examples of such applications in fungi are production of enzymes,
pharmacologically active proteins, natural products, and biofuels (Cherry and Fidantsef 2003; Cary et al. 2012; Garvey et al. 2013; Kubicek et al. 2009; Shin and Yoo 2013). In many cases, constitutive promoters have been used to drive expression of homologous or heterologous proteins in fungi, with follow-up analysis of metabolite levels using NMR or mass spectrometry approaches (Anasonitzis et al. 2014). However, for certain applications, regulatable promoters serve an important function in that they can be used to express toxic proteins or enzymes that produce metabolites that are themselves toxic to the cell (Scharf and Brakhage 2007; Shin and Yoo 2013). For example, a recent study in the filamentous fungus Aspergillus nidulans used the alcA promoter to achieve regulated expression of non-reducing polyketide synthases from Aspergillus terreus, with production of the expected products in good yield (Chiang et al. 2013).

Neurospora crassa is a model organism for the filamentous fungi, and available tools include a nearly complete gene knockout collection and more than 1000 mapped mutations (Perkins et al. 2001). To date, only a few regulatable promoters have been developed for use in N. crassa. The first such promoter, qa-2, is not highly induced and can only be turned on in low glucose (Campbell et al. 1994; Giles et al. 1985); ggc-1/ccg-1 is a glucose-repressible promoter (McNally and Free 1988) that has been used to drive expression of tyrosinase. However, the subsequent discovery that this promoter is also regulated by the circadian rhythm and blue light imposes additional requirements during harvesting of cells (Loros et al. 1989; Arpaia et al. 1995). Induction of the cys-16 promoter requires growth on limiting sulfur (0.25 mM methionine), and this promoter has not been used to drive expression of heterologous genes (Reeval and Paietta 2012). The light-regulated vvd promoter is highly tunable but requires stringent control of lighting conditions during tissue collection (Hurley et al. 2012). The copper-regulated tcu-1 promoter is also highly tunable and can operate in any genetic background (Lamb et al. 2013). The nit-6 gene promoter is an alternative candidate for regulated protein expression in N. crassa (Lafferty and Garrett 1974; Prodouz and Garrett 1981). nit-6 encodes NAD(P)H-nitrite reductase, the second step in nitrate assimilation (Lafferty and Garrett 1974; Prodouz and Garrett 1981). Expression of nit-6 mRNA is controlled by nitrogen catabolite repression through the action of the GATA transcription factor NIT-2 (Exley et al. 1993; Fu and Marzluf 1990) and by nitrate-specific control mediated by the NIT-4 fungal binuclear cluster transcription factor (Exley et al. 1993; Fu et al. 1995). These two modes of regulation result in repression of nit-6 during growth on glutamine or ammonium, but result in expression at high levels in medium containing nitrate as the sole nitrogen source (Exley et al. 1993).

In this study, we compare the tcu-1 and nit-6 promoters for regulated expression of genes in N. crassa. Although tcu-1 has been previously characterized (Lamb et al. 2013), this is the first implementation of nit-6 as a regulated promoter in N. crassa. We characterize the fragment sizes and conditions needed for induction and repression of these promoters. We also use proton (1H) NMR to perform metabolic profiling of cultures under inducing and repressing conditions for each promoter (Bundy et al. 2007; Barding et al. 2012; Larive et al. 2015). Our results provide a baseline for the metabolic changes that occur under the different growth conditions for each promoter.

### MATERIALS AND METHODS

#### Chemicals, media, and genetic procedures

Deuterium oxide (D2O, 99%) and ethylenediaminetetraacetic acid-d16 (EDTA) were purchased from Cambridge Isotope Laboratories (Andover, MA). DSS-d4 3-[trimethylsilyl]-1-propanesulfonic acid sodium salt was purchased from Isotec (Miamisburg, OH). Methanol-d4 (CD3OD, 99.8%) and fungal protease inhibitor cocktail (P8215) were obtained from Sigma-Aldrich (St. Louis, MO). Monobasic and dibasic sodium phosphate were purchased from Fisher Scientific (Pittsburgh, PA). TRizol RNA isolation reagent (#15596-026) was purchased from Life Technologies (Grand Island, NY).

N. crassa strains are listed in Table 1. N. crassa hyphal cultures were propagated in Vogel’s minimal medium (VM) (Vogel 1964). VM without ammonium nitrate or containing sodium nitrate or glutamine (Gln) was used to induce or repress, respectively, the nit-6 promoter (Exley et al. 1993). VM supplemented with 50 μM copper sulfate (Cu) was used to repress the tcu-1 promoter, whereas VM containing bathocuproine disulfonate (BCS; 81125-500MG; Sigma-Aldrich) was used to induce tcu-1 (Korrippal et al. 2010; Lamb et al. 2013). Sorbose-containing medium (FGS) (Davis and Deserres 1970) was used to facilitate colony formation on plates. N. crassa axenic spores (macronidia or conidia) were propagated using standard procedures (Davis and Deserres 1970). N. crassa transformations were performed by electroporation using conidia as the recipient (Ivey et al. 1996). Sexual crosses were performed using standard procedures and ascospore progeny were plated on FGS medium (Davis and Deserres 1970). When indicated, modified VM or FGS medium (Pall 1993) contained the antibiotic phosphinothricin (purified as described previously) (Hays and Selker 2000) at 400 μg/mL. Pantosthenate (pan) was used in media at 10 μg/mL.

N. crassa liquid submerged cultures were inoculated with conidia to a final density of approximately 10⁶/mL in 50 mL of VM-pan liquid medium and incubated at 30°C with shaking at 200 rpm for the indicated time in the dark. Tissue was collected on filter paper using a vacuum filtration system (EMD Millipore, Billerica, MA), flash-frozen in liquid nitrogen, and then pulverized in liquid nitrogen using a mortar and pestle.

#### Construction of plasmid vectors and N. crassa strains

Two different groups of expression vectors were constructed during this study. Both groups of vectors target V5 and GFP-tagged open reading frames of these promoters. We also use proton (1H) NMR to perform metabolic profiling of cultures under inducing and repressing conditions for each promoter (Bundy et al. 2007; Barding et al. 2012; Larive et al. 2015). Our results provide a baseline for the metabolic changes that occur under the different growth conditions for each promoter.

| Strain | Relevant Genotype | Source/Reference |
|--------|------------------|------------------|
| 74-OR23-IVA | Wild type, mat A | FGSC2489 |
| Δmus51-IV-8 | Δrid-1::mat, Δmus-51::nat, mat a | FGSC23148; (S. Ouyang, I. E. Cabrera, A. J. Campbell, K. A. Borkovich, unpublished data) |

**Table 1 Strains used in this study**
frames (ORFs) to the pan-2 locus, creating a pantothene auxotroph. However, the two vector groups implemented different promoters to drive expression of the V5-GFP fusion protein. One vector group contained the nitrogen-regulated nit-6 (Esley et al. 1993) promoter (pRS426PGV/ptcu-6), whereas the other contained the copper-regulated tcu-1 (Lamb et al. 2013) promoter (pRS426PGV/ptcu-1). The first step of vector construction was to use yeast recombinational cloning (Colot et al. 2006) to replace the ccg-1 promoter region in pRS426PGV (S. Ouyang, I. E. Cabrera, A. J. Campbell, K. A. Borkovich, unpublished data) with a fragment containing 0.5-, 1.0-, or 1.5-kb regions upstream of the nit-6 or tcu-1 genes. Then, two fragments were inserted into these modified vectors using yeast recombinational cloning. The first fragment included a multiple cloning sequence (MCS), a 5xGlycine linker, a V5-tag, and GFP sequence. The second fragment was the selectable marker gene bar, amplified from vector pTKJ1 (Jones et al. 2007). bar confers resistance to phosphinothricin (Pall 1993). All fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Primer sequences are listed in Table 2.

| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| Pan2-nit-6-1.5-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| Pan2-nit-6-1.0-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| Pan2-nit-6-0.5-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| pnti-6-RV | AACCCGGGGATCCACTAGTTCTAGATGCTGGCTGACGACAGAAAGACTAG |
| Pan2-ptcu1-1.5-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| Pan2-ptcu1-1.0-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| Pan2-ptcu1-0.5-FW | CTTGGCTATATTCTGGACCGGTACCGATGGGATAGAGAATGGCCGTTG |
| ptcu1-RV | AACCCGGGGATCCACTAGTTCTAGATGCTGGCTGACGACAGAAAGACTAG |
| Probe_GFP-FW | TGACCTCTGAGATCCTGAC |
| Probe_GFP-RV | AACCCGGGGATCCACTAGTTCTAGATGCTGGCTGACGACAGAAAGACTAG |

Table 2 Primer Sequences

Cultures used for induction and repression of the nit-6 and tcu-1 promoters

For experiments involving the nit-6 promoter, two identical sets of cultures were grown under repressing conditions (VM-Gln) for 14 hr. Cells were collected from each culture using a sterile filter paper/Buchner funnel assembly in a sterile hood and the cell pad was washed with sterile water. The cell pads were transferred to new flasks containing either VM-Gln (repressing conditions control) or VM-nitrate (inducing conditions) medium. Cultures were grown for the indicated times (1–6 hr), after which cells were collected as described above and flash-frozen in liquid nitrogen.

For the tcu-1 promoter, two sets of cultures were grown under repressing conditions (VM-Cu) for 14 hr. BCS (inducing conditions) was then added to a final concentration of 200 μM. An equal volume of water was used for the noninduced control. Both sets of cultures were then incubated with shaking for 3 hr. Cells were collected as described for the nit-6 promoter (see above).

Sample preparation for metabolomics experiments

Metabolite extractions were performed using a modification of a published procedure for Fusarium species (Lowe et al. 2010). Liquid cultures were harvested by vacuum filtration, washed three times in distilled water, and snap-frozen under liquid nitrogen. The material was ground using a mortar and pestle with liquid nitrogen and stored frozen at −80°C. The frozen samples were freeze-dried on a lyophilizer overnight. A 50 mM phosphate buffer at pH 7.0 containing 0.05% (w/v) of each DSS-d₆ and EDTA-d₆ and composed of 80% D₂O and 20% CD₃OD was used for sample extraction. The volume of pD was calculated
using the pH meter reading (pH) for a glass electrode calibrated with aqueous buffers using the equation pD = pH + 0.4 to correct for the deuterium isotope effect (Glasoe and Long 1960). A sample containing 15 mg of dried material was resuspended in 1 ml of the extraction solvent and then heated at 50°C for 10 min. After cooling, the samples were spun down in a microcentrifuge for 5 min. The supernatant was removed, transferred to a clean microcentrifuge tube, heated at 90°C for 2 min, cooled to 4°C for 45 min, and recentrifuged for 5 min. A 700-μl aliquot of the supernatant was transferred to a 5-mm NMR tube for 1H NMR.

**NMR measurements**

1H NMR spectra were measured using a Bruker 600 MHz Avance spectrometer equipped with a triple gradient inverse probe operating at 599.58 MHz. Six samples of nit-6(Gln) and nit-6(nitrate) and five samples of tcu-1(Cu) and tcu-1(BCS) were examined in 5 mm NMR tubes. Spectra were recorded at 25°C using presaturation for suppression of the residual water signal. A relaxation delay of 3.0 sec was used with an 8.50-μs 90° pulse at a power level of −5 dB. A spectral window of 7002.801 Hz was used, co-adding 256 scans into 42,014 complex points. Spectra were processed using Topspin 3.1 and zero-filled using 131,072 points with an applied 0.50 Hz line-broadening, with automated baseline correction to a fifth-order polynomial. Chemical shifts were referenced to DSS at 0.00 ppm. Resonance assignments were made by comparison to spectra measured for authentic standards and are consistent with previous metabolomics studies for *N. crassa* (Kim et al. 2011).

**Principal component analysis of NMR data**

NMR spectra were exported from Topspin in ASCII file format and data pretreatment and principal component analysis (PCA) performed using Matlab R2013b, the PLS Toolbox 5.5, and m-files written in-house. Spectral regions containing the residual water resonance (4.575–5.075 ppm) below 0.5 and above 9.0 ppm were set as dark regions prior to PCA. To equalize the impact of variables in PCA, unit variance scaling was applied, which uses the SD of each variable as the scaling factor (van den Berg et al. 2006). After unit variance scaling, all metabolites have an SD of 1; therefore, the data are analyzed on the basis of correlations instead of covariance.

Visualization of loadings is challenging with unit variance-scaled models because the line shapes of loadings are not interpretable in the same way as in unscaled models and therefore cannot be used directly to identify peaks dominating the variance in the data. The PC loading (p) can be expressed as follows (Johnson and Wichern 1988),

\[
    p_{k,n} = \frac{CC_{k,n} \sqrt{\sigma_{n}^2}}{\lambda_{n}}
\]

where \( \sigma_{n} \) and \( \lambda_{n} \) are variances of spectral (peak intensity) variable \( z_{k} \) and PC vector of scores \( t_{i} \), respectively, and \( cc \) denotes the correlation coefficient between \( z_{k} \) and \( t_{i} \). In the unscaled case, the line shapes of PC loadings are interpretable because a loading value is proportional to the SD of a spectral variable \( \sigma_{w} \), which is, respectively, lower and higher for variables that constitute minor and major intensities of a peak. This interpretation is destroyed when each variable is scaled to unit variance, *i.e.*, \( \sigma_{w} = 1 \). To aid in the interpretation of the dominant source of the variance contribution in unit-variance PCA score plots, a loading value can be multiplied by the SD of its original spectral variable. Similar to the unscaled case, the line shapes of transformed (or scaled) unit variance loadings will be distorted, showing the first-derivative shape of a metabolite peak in cases in which the variation caused by variable peak position contributes to a model (Cloarec et al. 2005).

**Data availability**

Strains and vectors are available upon request. The Supporting Information File contains detailed descriptions of all supplemental files.

**RESULTS**

**Construction of vectors and strains for regulated protein expression**

A recent study demonstrated that a 1.5-kb region upstream of the *tcu-1* gene could function as a tunable, copper-regulated promoter to drive expression of essential genes in *N. crassa* (Lamb et al. 2013). An aim of this work was to determine whether shorter regions of the *tcu-1* upstream sequence could function as a promoter. We also explored the region upstream of the *nitrate-6 (nit-6)* (Exley et al. 1993) gene for use as a regulated promoter for *N. crassa*. *nit-6* encodes the structural gene for nitrite reductase and is only expressed when *N. crassa* is cultured on medium containing nitrate and lacking a good nitrogen source (typically glutamine or ammonium) (Exley et al. 1993). The homologous promoter (*nitA*) has been demonstrated to be a tightly controlled promoter in the filamentous fungus *Aspergillus fumigatus* (Amaar and Moore 1998). We reasoned that *nit-6* might also be under tight regulation in *N. crassa* and would be useful for experiments requiring regulated protein expression.

We developed vectors for expression of proteins under control of regions corresponding to the *tcu-1* and *nit-6* promoters. The vectors were based on pRS426PVG (S. Ouyang, I. E. Cabrera, A. J. Campbell, K. A. Borkovich, unpublished data), developed to insert genes at the *pan-2* locus (Case and Giles 1958) in *N. crassa*. Mutation of *pan-2* leads to a requirement for pantothenic acid. The *cgg-1* promoter in pRS426PVG was replaced by fragments corresponding to upstream regions for the *nit-6* and *tcu-1* genes (Figure 1A). We amplified fragments corresponding to regions 0.5, 1.0, and 1.5 kb upstream of the start codon (Figure 1B). The *tcu-1* promoter group of vectors includes pRS426PVG/tcu-1_0.5 kb, pRS426PVG/tcu-1_1.0 kb, and pRS426PVG/tcu-1_1.5 kb, whereas the three vectors with the *nit-6* promoter fragments are pRS426PVG/nit-6_0.5 kb, pRS426PVG/nit-6_1.0 kb, and pRS426PVG/nit-6_1.5 kb. All vectors contained a multiple cloning site, a 5-glycine linker, and V5 and GFP tags 3' to the promoter fragment (Figure 1A). A bar cassette, conferring resistance to the antibiotic phosphinothricin (Pall 1993), was inserted 3' to the GFP gene. The vectors were then transformed into the *N. crassa* recipient strain *Anmus51-IV-8* (S. Ouyang, I. E. Cabrera, A. J. Campbell, K. A. Borkovich, unpublished data). A total of six strains were generated during this study (Table 1), each containing a different fragment of *tcu-1* or *nit-6* driving expression of GFP from the *pan-2* locus in *N. crassa*.

**Expression of GFP from the different promoter fragments.** We next investigated expression of GFP mRNA and protein in the six *N. crassa* strains carrying the different promoter fragments. For these studies, we used a strain in which the *cgg-1* promoter controls expression of GFP as a positive control (Figure 2A). The results showed that the growth regimens used for the different promoters did not influence expression of GFP mRNA or protein from *cgg-1* (Figure 2, A and B).

For experiments testing fragments from the 5’ upstream region of *tcu-1*, we grew *N. crassa* cells under repressing conditions (VM-Cu) and then added the copper chelator BCS to a final concentration of 200 μM (Lamb et al. 2013). The same volume of water served as a negative
Both sets of vectors have the \( pRS426PVG/pnit-6 \) group of vectors contain fragments upstream of the \( tcu-1 \) promoters (\( \text{levels of GFP mRNA were comparable using the} \text{tcu-1}_{1.5} \text{strain and only after addition of BCS (Figure 2, A and B). The analysis showed that GFP mRNA and protein could only be detected in the} \text{tcu-1}_{1.5} \text{strain showed that GFP mRNA and protein levels peaked at 3 hr}\). Results from Northern and Western analysis revealed that the GFP transcript could not be detected in the \text{tcu-1}_{1.5} \text{strain and VM-BCS for the} \text{ptcu-1}_{1.5} \text{strain. The blot shown is representative of at least three experiments. (B) Western analysis of protein extracts from strains with different promoter fragments. Whole cell extracts were isolated from the strains with different promoter fragments and samples containing 50 \( \mu \)g total protein subjected to Western analysis using GFP antiserum (see Materials and Methods for details). The asterisk indicates a higher-molecular-weight, nonspecific background band. Strains are the same as in (A). The blot shown is representative of at least three experiments. (C) Time course expression of GFP proteins driven by 1.5-kb promoter fragments for \text{tcu-1} \text{and} \text{nit-6}. Whole cell extracts were isolated and subjected to Western analysis as described for (B). The asterisk indicates a nonspecific background band. Strains are wild-type 74-OR23-IVA (WT), \text{pcgc-1}_{-0.5}, \text{pcgc-1}_{-1.0}, \text{pcgc-1}_{-1.5}, \text{ptcu-1}_{-0.5}, \text{ptcu-1}_{-1.0}, \text{and} \text{ptcu-1}_{-1.5}. The blot shown is representative of at least three experiments.}

\[\text{pcgc-1}_{-0.5}, \text{pcgc-1}_{-1.0}, \text{pcgc-1}_{-1.5}, \text{ptcu-1}_{-0.5}, \text{ptcu-1}_{-1.0}, \text{and} \text{ptcu-1}_{-1.5}.\]

In initial experiments, cells were incubated for 3 hr after induction (Figure 2, A and B). Results from Northern and Western analysis showed that GFP mRNA and protein could only be detected in the \text{tcu-1}_{1.5} \text{strain and only after addition of BCS (Figure 2, A and B). The levels of GFP mRNA were comparable using the} \text{ccg-1} \text{and 1.5 kb} \text{tcu-1} \text{promoters, but protein levels were higher using} \text{ccg-1} \text{(Figure 2, B and C), perhaps suggesting some aspect of post-transcriptional control for the} \text{tcu-1} \text{driven transcript. Subsequent experiments using only the} \text{tcu-1}_{1.5} \text{strain showed that GFP mRNA and protein levels peaked at 3 hr of induction and were greatly reduced at 6 hr (Figure 2, A and C). Thus, we concluded that use of the 1.5-kb promoter fragment and 3 hr of induction were sufficient to drive regulated expression of GFP under the} \text{tcu-1} \text{promoter in} \text{N. crassa}. \text{These results contrast with those from the previous study, in which induction of the} \text{tcu-1} \text{mRNA (not a fusion gene) peaked between 8 and 24 hr (Lamb et al. 2013). A possible explanation for this difference may be that GFP mRNA peaks faster but is less stable relative to} \text{tcu-1} \text{mRNA in} \text{N. crassa}. \text{For the} \text{nit-6} \text{promoter fragments, we first cultured} \text{N. crassa} \text{with 20 mM Gln as the nitrogen source (repressing conditions), collected the cells, and transferred to fresh medium containing 20 mM sodium nitrate to induce expression for 3 hr (Exley et al. 1993). Northern blot analysis revealed that the GFP transcript could not be detected in the...
protein were examined in cultures grown in nitrate (inducing condi-
tions) after long-term growth in nitrate. Levels of GFP mRNA and
protein extracts were isolated as described above. Western blot
analysis showed that GFP protein could be detected using both the
1.0-kb and 1.5-kb promoter fragments, with higher levels using the 1.5-
kbp promoter (Figure 2B). Levels of GFP mRNA were higher using the
1.5-kb nit-6 promoter fragment than with ccg-1 (Figure 2A), whereas
GFP protein levels exhibited a less dramatic difference (Figure 2B).
Later experiments with the nit-6_1.5 strain showed that levels of
GFP mRNA were elevated at 1 hr of induction, increased slightly at 3
hr, and then greatly diminished at 6 hr (Figure 2A). Less of a difference
was observed in the amount of GFP protein produced under these
conditions, with significant levels detected at time points 1 hr, 3 hr,
and 6 hr, and with the greatest amount observed after 3 hr and 6 hr of
induction (Figure 2C). These experiments suggested that the 1.5-kb
fragment was necessary to promote significant expression of heterolo-
gous genes using the nit-6 promoter and that mRNA and protein levels
peaked by 3 hr after induction. In addition, GFP mRNA and protein
levels were higher in experiments using the 1.5-kb nit-6 promoter than
the 1.5-kb tcu-1 promoter (Figure 2).

We compared the induction profile of GFP driven by the 1.5-kb tcu-1
and nit-6 promoter fragments to that of the endogenous tcu-1 and nit-6
ORFs by stripping and reprobing the blots used in Figure 2 with tcu-1
or nit-6 ORF probes (Figure S1). The results demonstrated that tcu-1
mRNA could be detected after 1 hr, increasing to much higher levels at
3 hr, and then greatly decreasing at 6 hr after addition of BCS to the
medium (Figure S1A, top panel). An identical pattern of expression was
observed for the endogenous nit-6 gene (Figure S1A, bottom panel).
These results showed that expression of the GFP reporter mirrors that
of the endogenous tcu-1 gene. The timing of expression of the native
nit-6 gene was similar to that of GFP, but levels of GFP mRNA were
greater than those of nit-6 at 1 hr.

Regulation of the nit-6 promoter by nitrate and glutamine

We further explored the conditions needed for induction and repression
of the 1.5-kb nit-6 promoter fragment. Initial experiments examined the
concentration of nitrate needed to induce expression after long-term
growth in 20 mM Gln. After overnight growth in VM-Gln, cells from
strain pnit-6_1.5 were transferred to VM containing 0.5–30 mM so-
dium nitrate (Figure S2). Cultures were incubated for an additional 3 hr
and total RNA and protein extracts were isolated as described above.
Northern blot results indicated that GFP mRNA levels were similar
under all concentrations of sodium nitrate (Figure S2). This result
indicates that the nit-6 promoter is extremely sensitive to nitrate, with
full induction of mRNA at 0.5 mM. Results from Western blot analysis
of protein extracts isolated from the same tissue revealed that GFP
levels increased slightly with higher concentrations of sodium nitrate,
peaking at ~20 mM nitrate (Figure S2).

We next investigated the kinetics of repression of the nit-6 promoter
by Gln after long-term growth in nitrate. Levels of GFP mRNA and
protein were examined in cultures grown in nitrate (inducing condi-
tions) for 14 hr and then transferred to medium containing 20 mM Gln
for different time periods (Figure 3). The strain with the ccg-1 promoter
was used as a control. The results demonstrated that levels of GFP
transcript are lower using the nit-6 promoter (inducing conditions)
vs. ccg-1 after overnight growth. These findings are consistent with
those showing that GFP transcript levels peak at 3 hr and then are
greatly reduced at 6 hr after induction (Figure 2A). GFP transcript
levels were decreased approximately two-fold within 30 min after trans-
fer to glutamine and GFP mRNA could not be detected after 1 hr
(Figure 3). In contrast, GFP protein levels declined more gradually,
decreasing at all time points until protein could not be detected (3 hr;
Figure 3). Of course, the relative stability of different proteins will
determine how rapidly they are cleared from the cell after the transfer
to repressing conditions.

We compared the repression of the endogenous nit-6 gene by glut-
amine to that of GFP by stripping and reprobing the blot used in
Figure 3. The results showed that expression of nit-6 is greatly reduced
after 15 min of exposure to glutamine, with the mRNA not detectable
by 1 hr of treatment (Figure S1B). This repression profile is similar to
that observed for GFP driven by the 1.5 kb nit-6 promoter fragment
(Figure 3). Taken together, these results suggest that nit-6 is a viable
promoter to use for experiments involving both induction and repres-
sion of genes in N. crassa.

1H NMR metabolite profiles

1H NMR provides an untargeted view of metabolic shifts resulting from
expression of the inserted gene. Figure 4 presents representative
1H NMR spectra for each sample type with key resonances labeled. We
were not surprised that use of Gln as a nitrogen source (Figure 4A)
significantly increases the levels of Glu and glutamate (Glu) compared
with the expression on media containing sodium nitrate. In contrast,
the resonances of trehalose and mannitol are lower in the extracts of
pnit-6_1.5 grown on Gln. The levels of alanine (Ala) also decrease in the
pnit-6-1.5(Gln) samples, which can be more readily observed in the
expansion of the spectra presented in Figure S3 at a lower vertical scale.
Taken together, the lower levels of trehalose, Ala, and mannitol suggest
a shift away from glycolytic metabolism compared to growth of the
pnit-6_1.5 promoter strain on VM-nitrate (Dijkema et al. 1985). Expansions of
NMR spectra measured for biological replicates (Figure S4A [pnit-6-1.5(Gln)]
and Figure S4B [pnit-6-1.5(nitrate)]) demonstrate that the cell culture and extraction methods are highly reproducible and allow for a more detailed examination of the impact of culture
conditions on the metabolite profile. It is important to note that the dif-
ferences in the metabolic profiles for pnut-6-1.5(Gln) and pnut-6-1.5
(nitrate) largely reflect the different growth conditions required to repress
or induce the nit-6 promoter, and not the products of the expressed gene.
Although obvious differences can be discerned by visual inspection of the PCA analysis highlighted in blue. containing medium (Figure S6B) is the presence of the BCS resonances, PCA score plots indicate the extent to which individual 1H NMR spectra that can be introduced for the most intense signals. In this study, the Unit variance scaling was used in our calculations to reduce the bias that can be introduced for the most intense signals. In this study, the PCA score plots indicate the extent to which individual 1H NMR spectra within a treatment group are similar and the spectra between treatment groups differ. Although statistical significance cannot be deduced from groupings in PCA scores plots, sample groupings do provide insights into differences between samples (Barding et al. 2012).

A clustering of the samples by treatment type is observed in the PCA score plots for the ptnit-6_1.5(Gln) and ptnit-6_1.5(nitrate) samples (Figure 5A). Together, PC1 and PC2 account for 57% of the variance in this sample set. Although the samples are not tightly grouped, there is clear segregation of the samples of ptnit-6_1.5 grown on VM-Gln (samples 1–6) and grown on media containing nitrate (samples 7–12). The PCA loadings highlight the variables, in this case metabolite NMR resonances, responsible for the greatest variance for a particular principal component (e.g., PC1 or PC2). Analysis of the loadings plot (Figure 5B) confirms our observations that the levels of Gln, Glu, Ala, mannitol, and trehalose differ significantly in the NMR spectra measured for the two treatments. In addition, the PCA loadings highlight the resonances between 0.5 and 1.5 ppm due to amino acids (e.g., valine, leucine, isoleucine, and threonine) that we did not identify as important in our analysis of the NMR spectra. Although the intensity of the PCA loading for a particular variable is an indicator of its relative importance, it should be recognized that loadings reflect the variance over all samples and cannot necessarily be assigned to individual treatments. This is especially the case when, as in Figure 5A, the sample groupings separate along both PC1 and PC2. In addition, in interpreting these results it should be noted that the signs of the loadings are arbitrary and are not correlated to an increase or decrease in resonance intensity.

Figure 6 presents the PCA results for the ptcu-1_1.5(BCS) and ptcu-1_1.5(Cu) samples. In Figure 6A the scores were calculated using all the variables, i.e., the full 1H NMR spectra except for the dark regions, which included the residual water resonance (4.575–5.075 ppm) and baseline (below 0.5 and above 9.0 ppm). Although the most variance is accounted for by PC1 (29.83%), the segregation of the sample groupings is observed along PC2. Examination of the loadings (data not shown) indicated the BCS resonances were the largest factor in the sample grouping in Figure 6, consistent with our visual observations of the NMR spectra for these samples. To further test this idea, the dataset was modified by removing the regions of the 1H NMR spectra containing the BCS resonances and PCA was performed again. The results of this analysis (Figure 6B) show a reduction in the extent of segregation of the samples compared with the scores plot in Figure 6A. In contrast, if the analysis is performed using only the regions of the spectra containing the BCS resonances (Figure 6C), the segregation of the samples by treatment is increased. Taken together, these results confirm that the ptcu-1_1.5 copper expression trigger results in a minimal metabolic perturbation as reflected by 1H NMR.

**DISCUSSION**

In this study, we develop nit-6 as an inducible/repressible promoter for *N. crassa*. We demonstrate that a region corresponding to 1.5 kb upstream of the nit-6 ORF achieves tight repression by glutamine and
induction by nitrate. We show that the same sized region is required for regulation of the recently described tcu-1 promoter (Lamb et al. 2013). We compare nit-6 to tcu-1 with regard to the amplitude and timing of induction of a GFP reporter. The results showed that nit-6 leads to higher levels of mRNA and protein than tcu-1, but with similar kinetics. Levels of GFP message and protein were comparable using the ccg-1 promoter and inducing conditions for the nit-6 promoter. Furthermore, the timing of expression of GFP was similar to that of the endogenous tcu-1 and nit-6 genes in the same strains.

By testing promoter fragments corresponding to 0.5, 1.0, and 1.5 kb upstream of the tcu-1 and nit-6 ORFs, we determined that 0.5 and 1.0 kb fragments were insufficient to drive high levels of expression of GFP mRNA and protein. The results for tcu-1 can be explained by the large 5′ untranslated region (UTR) present on the mRNA (657 nts; Broad Institute Neurospora crassa Database; http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html); use of the 1-kb fragment would only leave 343 nts for a promoter. In the case of nit-6, the Broad Database shows a relatively small 193-nt UTR. However, an in silico analysis of the upstream region of nit-6 (Chiang and Marzluf 1995) revealed three possible NIT-4 binding sites at −695, −677, and −547 and eight GATA elements at −713, −445, and −235. The authors did not report whether they searched further upstream for additional NIT-2 binding sites, but these results suggest that at least 713 nts upstream of the ORF would be necessary for proper regulation of nit-6.

The impact of the growth conditions used for promoter induction on N. crassa metabolism was evaluated using 1H NMR, a well-established technique for metabolic profiling (Larive et al. 2015). The metabolites detected are summarized in Table S1. For both the nit-6_1.5 and ptcu-1_1.5 strains, no metabolic perturbation was detected as a direct result of GFP expression. The growth conditions used for induction of tcu-1 are truly minimally perturbing, as detected by 1H NMR. Although the
different growth conditions used for induction/repression of nit-6 produced minor (and anticipated) changes in the levels of some primary metabolites, the metabolic response was similar to that observed for the wild-type.

There are several examples of carbon-regulated promoters used in fungi, including the GAL1/GAL10 promoter in S. cerevisiae (Matsumoto et al. 1981; Guarente et al. 1982; Johnston and Davis 1984) and alcA in A. nidulans (Waring et al. 1989). Both of these promoters are repressed during growth on glucose and induced using either galactose (GAL1/GAL10) (Matsumoto et al. 1981; Guarente et al. 1982; Johnston and Davis 1984) or ethanol/threonine (alcA) (Waring et al. 1989; Bailey and Arst 1975). Relevant to use of carbon-regulated promoters in fungi and effects on the metabolome, we previously analyzed the metabolic profile of N. crassa after growth on medium containing high (1.5%) or limiting (0.15%) glucose (Kim et al. 2011). Our results demonstrated that carbon availability influences the global metabolic profile in wild-type N. crassa, with differences in levels of several amino acids, intracellular glucose, mannitol, and other compounds (Kim et al. 2011). Our results in this study using nitrate vs. Gln for regulation of nit-6 demonstrated that tca-1 causes minimal perturbation of the metabolome and would be most useful for applications whereby maintenance of a consistent metabolic state is required. We also anticipate that the use of metabolic profiling in conjunction with gene expression will be increasingly important in future studies in which the expression products have a significant metabolic impact because of their direct or indirect effect on specific metabolite pathways or because of unanticipated toxic effects.

The A. fumigatus niiA promoter (Amaar and Moore 1998), homologous to nit-6, has been used successfully to regulate gene expression in this organism, including that ofessential genes (Hu et al. 2007). In both N. crassa and A. fumigatus, nit-6/niiA is tightly regulated by nitrogen source. This is crucial for study of essential gene functions and for expression of potentially toxic proteins from heterologous sources. Our work demonstrates the metabolic consequences of using this promoter, which may warrant consideration for certain applications, particularly those involving expression of genes in nitrogen-regulated pathways in both A. fumigatus and N. crassa.

In summary, our findings demonstrate that nit-6 is a tunable promoter that joins tca-1 as an option for regulated expression of genes in N. crassa. In addition, 1H NMR metabolic profiling proved to be useful in assessing the impacts of the inserted promoters on the levels of primary metabolites and identifying changes that resulted from differences in the growth conditions used to induce or repress expression.

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LITERATURE CITED

Amaar, Y. G., and M. M. Moore, 1998 Mapping of the nitrate-assimilation gene cluster (crrA-niiA-niiD) and characterization of the nitrite reductase gene (niiA) in the opportunistic fungal pathogen Aspergillus fumigatus. Curr. Genet. 33: 206–215.

Asanosztisz, G. E., E. Kourtoglou, D. Mamma, S. G. Villas-Boas, D. G. Hatzinikolaou et al., 2014 Constitutive homologous expression of phosphoglucomutase and transaldolase increases the metabolic flux of Fusarium oxysporum. Microb. Cell Fact. 13: 43.

Arpaia, G., J. J. Loros, J. C. Dunlap, G. Morelli, and G. Macino, 1995 Light induction of the clock-controlled gene ccg-1 is not transduced through the circadian clock in Neurospora crassa. Mol. Gen. Genet. 247: 157–163.

Bailey, C., and H. N. Arst, Jr., 1975 Carbon catabolite repression in Aspergillus nidulans. Eur. J. Biochem. 51: 573–577.

Barding, G. A., Jr, R. Saldivis, and K. C. Larive, 2012 Quantitative NMR for bioanalysis and metabolomics. Anal. Bioanal. Chem. 404: 1165–1179.

Bundy, J. G., B. Papp, R. Harmston, R. A. Browne, E. M. Clayton et al., 2007 Evaluation of predicted network modules in yeast metabolism using NMR-based metabolite profiling. Genome Res. 17: 510–519.

Campbell, J. W., C. W. Enderlin, and C. P. Selitrennikoff, 1994 Vectors for expression and modification of cDNA sequences in Neurospora crassa. Fungal Genet. Newsl. 41: 20–21.

Cary, J. W., P. Y. Harris-Coward, K. C. Ehrlich, B. M. Mack, S. P. Kale et al., 2012 NsdC and NsdD affect Aspergillus flavus morphogenesis and aflatoxin production. Eukaryot. Cell 11: 1104–1111.

Case, M. E., and N. H. Giles, 1958 Recombination mechanisms at the pan-2 locus in Neurospora crassa. Cold Spring Harb. Symp. Quant. Biol. 23: 119–135.

Cherry, J. R., and A. L. Fidantsef, 2003 Directed evolution of industrial enzymes: an update. Curr. Opin. Biotechnol. 14: 438–443.

Chiang, T. Y., and G. A. Marzluf, 1995 Binding affinity and functional significance of NIT2 and NIT4 binding sites in the promoter of the highly regulated nit-3 gene, which encodes nitrate reductase in Neurospora crassa. J. Bacteriol. 177: 6093–6099.

Chiang, Y. M., C. E. Oakley, M. Ahuja, R. Entwistle, A. Schultz et al., 2013 An efficient system for heterologous expression of secondary metabolite genes in Aspergillus nidulans. J. Am. Chem. Soc. 135: 7720–7731.

Cloarec, O., M. E. Dumas, J. Trygg, A. Craig, R. H. Barton et al., 2005 Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in 1H NMR spectroscopic metabolic studies. Anal. Chem. 77: 517–526.

Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew et al., 2006 A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. USA 103: 10352–10357.

Davis, R. H., and F. J. deSerres, 1970 Genetic and microbiological research limitations caused by chemical shift variability and improved visualization of biomarker changes in 1H NMR spectroscopic metabolic studies. Anal. Chem. 77: 517–526.

Dijkema, C., H. C. Kester, and J. Visser, 1985 13C NMR studies of carbon metabolism in the hyphal fungus Aspergillus nidulans. Proc. Natl. Acad. Sci. USA 82: 14–18.

Exley, G. E., J. D. Colandene, and R. H. Garrett, 1993 Molecular cloning, characterization, and nucleotide sequence of nit-6, the structural gene for nitrite reductase in Neurospora crassa. J. Bacteriol. 175: 2379–2392.

Freitag, M., R. L. Williams, G. O. Kothe, and E. U. Selker, 2002 A cytosine methyltransferase homologue is essential for repeat-induced point mutation in Neurospora crassa. Proc. Natl. Acad. Sci. USA 99: 8802–8807.

Fu, Y. H., B. Feng, S. Evans, and G. A. Marzluf, 1995 Sequence-specific DNA binding by NIT4, the pathway-specific regulatory protein that mediates nitrate induction in Neurospora. Mol. Microbiol. 15: 935–942.

Fu, Y. H., and G. A. Marzluf, 1990 nit-2, the major positive-acting nitrogen regulatory gene of Neurospora crassa, encodes a sequence-specific DNA-binding protein. Proc. Natl. Acad. Sci. USA 87: 5331–5335.

Garvey, M., H. Klose, R. Fischer, C. Lambertz, and U. Commandeur, 2013 Cellulases for biomass degradation: comparing recombinant cellulase expression platforms. Trends Biotechnol. 31: 581–593.

Giles, N. H., M. E. Case, J. Baum, R. Geever, L. Huiet et al., 1985 Gene organization and regulation in the qa (quinic acid) gene cluster of Neurospora crassa. Microbiol. Rev. 49: 338–358.

Glasoe, P. K., and F. A. Long, 1960 Use of glass electrodes to measure acidities in deuterium oxide. J. Phys. Chem. 64: 188–190.

Guarente, L., R. R. Yocum, and P. Gifford, 1982 A GAL10–CYC1 hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site. Proc. Natl. Acad. Sci. USA 79: 7410–7414.

Hays, S., and E. U. Selker, 2000 Making the selectable marker bar tighter and more economical. Fungal Genet. Newsl. 47: 107.

Hu, W., S. Sillaots, S. Lemieux, J. Davison, S. Kaufman et al., 2007 Essential gene identification and drug target prioritization in Aspergillus fumigatus. PLoS Pathog. 3: e24.
Korripally, P., A. Tiwari, A. Haritha, P. Kiranmayi, and M. Bhanoori, Kubicek, C. P., M. Mikus, A. Schuster, M. Schmoll, and B. Seiboth, Lafferty, M. A., and R. H. Garrett, 1974 Puri Matsumoto, K., A. Toh-e, and Y. Oshima, 1981 Isolation and character-
ization of dominant mutations resistant to carbon catabolite repression of Neurospora crassa. Mol. Biol. 1: 140–1448.

Jones, C. A., S. E. Greer-Phillips, and K. A. Borkovich, 2007 The response regulator RRG-1 functions upstream of a mitogen-activated protein ki-
nase pathway impacting asexual development, female fertility, osmotic stress, and fungicide resistance in Neurospora crassa. Mol. Biol. Cell 18: 2123–2136.

Kim, H., and K. A. Borkovich, 2004 A pheromone receptor gene, pre-1, is essential for mating type-specific directional growth and fusion of tricho-
gynes and female fertility in Neurospora crassa. Mol. Microbiol. 52: 1781–1798.

Kim, H., and K. A. Borkovich, 2006 Pheromones are essential for male fertili
ty and sufficient to direct chemotropic polarized growth of tricho-
gynes during mating in Neurospora crassa. Eukaryot. Cell 5: 544–554.

Kim, J. D., K. Kaiser, C. K. Larive, and K. A. Borkovich, 2011 Use of 3H nuclear magnetic resonance to measure intracellular metabolite levels during growth and asexual sporulation in Neurospora crassa. Eukaryot. Cell 10: 820–831.

Korripally, P., A. Tiwari, A. Haritha, P. Kiranmayi, and M. Bhanoori, 2010 Characterization of Ctr family genes and the elucidation of their role in the life cycle of Neurospora crassa. Fungal Genet. Biol. 47: 237–245.

Krystofova, S., and K. A. Borkovich, 2005 The heterotrimeric G-protein subunits GNG-1 and GNB-1 form a Gbetagamma dimer required for normal female fertility, asexual development, and Galpha protein levels in Neurospora crassa. Eukaryot. Cell 4: 365–378.

Kubicek, C. P., M. Mikus, A. Schuster, M. Schnoll, and B. Seiboth, 2009 Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. Biotechnol. Biofuels 2: 19.

Lafferty, M. A., and R. H. Garrett, 1974 Purification and properties of the Neurospora crassa assimilatory nitrite reductase. J. Biol. Chem. 249: 7555–7567.

Lamb, T. M., J. Vickery, and D. Bell-Pedersen, 2013 Regulation of gene expression in Neurospora crassa with a copper responsive promoter. G3 (Bethesda) 3: 2273–2280.

Larive, C. K., G. A. Barding, Jr, and M. M. Dinges, 2015 NMR spectroscopy for metabolomics and metabolic profiling. Anal. Chem. 87: 133–146.

Loros, J. J., S. A. Denome, and J. C. Dunlap, 1989 Molecular cloning of genes under control of the circadian clock in Neurospora. Science 243: 385–388.

Lowe, R. G., J. W. Allwood, A. M. Galster, M. Urban, A. Daudi et al., 2010 A combined 3H nuclear magnetic resonance and electrospray ionization-mass spectrometry analysis to understand the basal metabo-
lism of plant-pathogenic Fusaria spp. Mol. Plant Microbe Interact. 23: 1605–1618.

Matsumoto, K., A. Toh-e, and Y. Oshima, 1981 Isolation and character-
ization of dominant mutations resistant to carbon catabolite repression of galactokinase synthesis in Saccharomyces cerevisiae. Mol. Cell. Biol. 1: 83–93.

McNally, M. T., and S. J. Free, 1988 Isolation and characterization of a Neurospora glucose-repressible gene. Curr. Genet. 14: 545–551.

Medema, M. H., R. Breitling, R. Bovenberg, and E. Takano, 2011 Exploiting plug-and-play synthetic biology for drug discovery and production in microorganisms. Nat. Rev. Microbiol. 9: 131–137.

Miyajima, I., M. Nakafuku, N. Nakayama, C. Brenner, A. Miyajima et al., 1987 GPA1, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor signal transduction. Cell 50: 1011–1019.

Ouyang, S., G. Park, H. S. Atamian, C. S. Han, J. E. Stajich et al., 2014 MicroRNAs suppress NB domain genes in tomato that confer resistance to Fusarium oxysporum. PLoS Pathog. 10: e1004464.

Pall, M., 1993 The use of Ignite (basta; glufosinate; phosphinothricin) to select transformants of bar-containing plasmids in Neurospora crassa. Fungal Genet. NewsL. 40: 57.

Perkins, D. D., A. Radford, and M. S. Sachs, 2001 The Neurospora compendium. Academic Press, San Diego, CA.

Prodoust, K. N., and R. H. Garrett, 1981 Neurospora crassa NAD(P)H-
nitrite reductase. Studies on its composition and structure. J. Biol. Chem. 256: 9711–9717.

Ramadan, Z., D. Jacobs, M. Grigorov, and S. Kochhar, 2006 Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. Talanta 68: 1683–1691.

Reveal, B. S., and J. V. Paietta, 2012 Analysis of the sulfur-regulated control of the cystathionine gamma-lyase gene of Neurospora crassa. BMC Res. Notes 5: 339.

Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed, 1989 An essential G1 function for cyclin-like proteins in yeast. Cell 59: 1127–1133.

Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink, 1987 A Saccharomyces cerevisiae genomic plasmid bank based on a centromere-containing shuttle vector. Gene 60: 237–243.

Scharf, D. H., and A. A. Brakhage, 2013 Engineering fungal secondary metabolism: a roadmap to novel compounds. J. Biotechnol. 163: 179–183.

Selker, E. U., 1990 Premiebiotic instability of repeated sequences in Neurospora crassa. Annu. Rev. Genet. 24: 579–613.

Shin, M. K., and H. S. Yoo, 2013 Animal vaccines based on orally presented yeast recombinants. Vaccine 31: 4287–4292.

Tsu, H.-C. T., A. J. Pease, T. M. Koehler, and M. E. Winkler, 1994 Detection and quantitation of RNA transcribed from bacterial chromosomes and plasmids, pp. 197–200 in Methods in Molecular Ge-
netics, edited by K. W. Adolph. Academic Press, Inc., San Diego, CA.

van den Berg, R. A., H. C. Hoefsloot, J. A. Westerhuis, A. K. Smilde, and M. J. van der Werf, 2006 Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 7: 142.

Vogel, H. J., 1964 Distribution of lysine pathways among fungi: Evolution-
ary implications. Am. Nat. 98: 435–446.

Waring, R. B., G. S. May, and N. R. Morris, 1989 Characterization of an inducible expression system in Aspergillus nidulans using alcA and tubulin-coding genes. Gene 79(1): 119–130.

Worley, B., and R. Powers, 2013 Multivariate analysis in metabolomics. Curr. Metabolomics 1: 92–107.