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Exploring the bZIP transcription factor regulatory network in Neurospora crassa.

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INTRODUCTION

The fungal kingdom is an excellent model for the study of transcription factor (TF) diversity and evolution, primarily due to the functional characterization and identification of direct target genes of TFs in the yeast *Saccharomyces cerevisiae*. The duplication of TFs (paralogues) and subsequent regulatory network divergence are major forces in the evolution of phenotypic complexity. In *S. cerevisiae*, the basic leucine zipper (bZIP) family of TFs is an excellent example of gene duplication and divergence of function. In *S. cerevisiae*, the bZIP TF family contains 14 genes and can be divided into three groups. The largest is the *YAP1* group, which includes eight members. Five *YAP1* family members (*YAP1*, *YAP2*, *YAP3*, *YAP4*, and *YAP5*) have been implicated in the oxidative stress and DNA-damage responses (Tan et al., 2008; Workman et al., 2006). The differences in gene regulation among the Yap members can be attributed to two important mechanisms: sequence differences in the DNA-binding motifs that are targeted by the different Yap proteins (Cohen et al., 2002), and variations in the regulation of the Yap proteins themselves, including post-translational modifications such as phosphorylation and disulfide bond formation (Kuge et al., 2001). The integration of expression profiles and chromatin-binding studies of the Yap proteins in response to DNA damage have shown a significant overlap of Yap regulons, especially between Yap1 and Yap2 (Tan et al., 2008). The second largest group of bZIP members in *S. cerevisiae* includes SKO1, ACA1 and CST6 (ACA2). Strains containing a mutation in SKO1 are affected in osmotic and oxidative stress, while mutations in ACA1 and CST6 result in strains that are affected in the utilization of alternative carbon sources (Rep et al., 2001). The third group of bZIP TFs in *S. cerevisiae* shows both conservation and divergence. These data indicate how *N. crassa* responds to stress and provide information on pathway evolution.
S. cerevisiae contains a single gene encoding Gcn4, which regulates genes in response to amino acid starvation (Hinnebusch, 2005).

Genome sequences of about 50 additional fungal genomes are currently available, and a number of these organisms, such as Aspergillus nidulans, Magnaporthe grisea and Neurospora crassa, have genetic tools available to assess the functions of predicted TF genes. In the filamentous fungus N. crassa, there are about 200 predicted general TFs (with and without DNA-binding domains) (Borkovich et al., 2004; Galagan et al., 2003; Shi et al., 2005). The predicted DNA-binding TFs in N. crassa mostly fall into six families, i.e. the basic helix–loop–helix (bHLH), bZIP, C2H2 zinc finger, GATA factor, Myb and Zn binuclear cluster families. Recently, whole-genome microarrays for N. crassa have been validated and have provided expression data associated with asexual spore germination and vegetative growth (Kasuga et al., 2005; Kasuga & Glass, 2008). From a National Institutes of Health (NIH)-funded Neurospora program project, a large number of gene deletion strains have been constructed, including a viable deletion set of 103 predicted TF genes (Colot et al., 2006; Dunlap et al., 2007). The availability of such tools makes it possible to dissect TF networks and to compare regulatory networks that have evolved among paralogous TFs (homologues within one species) and between orthologous TFs (homologues between different species). Previously, we showed that the regulons of the bZIP TF Gcn4 of S. cerevisiae, which is involved in regulating genes in response to amino acid starvation (Hinnebusch, 2005), and its orthologue in N. crassa, CPC-1 (Ebbole et al., 1991), show conservation in the functional categories of genes that respond to amino acid starvation and which are dependent on functional Gcn4 and CPC-1 (Tian et al., 2007). Although the overall cellular response to amino acid starvation is similar between S. cerevisiae and N. crassa, there is substantial divergence in the direct target genes of Gcn4 in S. cerevisiae versus the predicted CPC-1 target genes in N. crassa.

In addition to cpc-1, there are eight genes in N. crassa with predicted bZIP DNA-binding domains, including the sulfur metabolism regulator cys-3 (Fu & Marzluf, 1990). While this study was in progress, an additional bZIP family member, NCU03905 (nap-1; here named NcAp-1, see Results), was shown by Takahashi et al. (2010) to be involved in mendicane resistance and was postulated to be a YAP1 orthologue. The functions of the remaining members of this family are currently unknown. In an effort to understand the bZIP regulatory network in N. crassa, we determined the transcriptional profiles of seven viable bZIP mutants during vegetative growth. This study revealed a genome-wide map of transcriptional regulation via members of the bZIP family in N. crassa and showed both cooperativity and specificity among the bZIP TF family in a filamentous ascomycete species. We subsequently assessed the sensitivity of the bZIP mutants to a variety of environmental stresses and also identified NCU03905 (Neurospora crassa AP 1-like TF, NcAp-1) as the functional orthologue of S. cerevisiae YAP1. By performing transcriptional profiling experiments of both the wild-type (WT) and a ΔNcAp-1 mutant in response to oxidative stress, we identified the NcAp-1 regulon. Functional and transcriptional profiling comparisons of the S. cerevisiae Yap1, Schizosaccharomyces pombe Pap1, C. albicans Cap1 and N. crassa NcAp1 regulatory networks in response to oxidative stress (this study) and in response to exposure to menadione (Takahashi et al., 2010) revealed both conservation and divergence of TF regulons.

METHODS

Strains and culture conditions. Strains containing deletion mutations in the predicted bZIP TF genes included FGSC 11121 (ANCU00499), FGSC 11343 (ANCU01994), FGSC 11131 (ANCU03905), FGSC 11133 (ANCU04211) and FGSC 11269 (ANCU08055) (Colot et al., 2006), and were kindly provided by Dr K. Borkovich (University of California, Riverside), while the cpc-1 mutant (FGSC 4264), cys-3 mutant (FGSC 4028) and the WT sequenced strain (FGSC 2489) were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey, 2003). Strains were inoculated onto slants containing Vogel’s minimal medium (VMM) (Vogel, 1956) and grown at 30°C for 2 days, followed by incubation at 25°C under constant light for 7 days. Conidia were harvested with water and inoculated as a horizontal strip onto 150 ml Bird’s minimal medium (BMM) (Metzenberg, 2004) on a large (25 cm × 25 cm) Petri plate layerd with a sheet of cellophane. Conidia were germinated under constant light at 25°C for 24 h. A 4 mm wide mycelial section was subsequently inoculated as a horizontal strip onto fresh solid BMM. Twenty-four hours post-inoculation, a 1 cm wide section of mycelia at the periphery of the colony, which corresponded to hyphae 1–3 h old, and a 1 cm internal section of the colony (corresponding to hyphae 12–15 h old) were harvested, immediately plunged into liquid nitrogen and stored at −80°C for RNA isolation.

For transcriptional profiling of the WT and the ΔNcAp-1 strain in response to hydrogen peroxide (H2O2), strains were treated as above, with the exception that large (25 cm × 25 cm) Petri plates containing a solid physical barrier in the middle of the plate were used. One half of the plate contained BMM, while the other half contained BMM supplemented with 6 mM H2O2 (added when the medium was at about 40°C). A preliminary comparison of expression data for the WT exposed to 2 and 6 mM H2O2 versus minimal medium revealed that there were no significant differences in expression when N. crassa was exposed to 2 mM H2O2, but significant differences were detected upon exposure to 6 mM H2O2. A horizontal strip containing hyphae was isolated from the BMM, 3 cm from the barrier. After 12 h, the periphery of the colony reached the barrier and grew into the BMM + H2O2. After 6 h of growth, an ~1 cm strip of the colony periphery was isolated from the BMM + H2O2 plate, plunged into liquid nitrogen and stored at −80°C until use.

For transcriptional profiling of the WT and ΔNcAp-1 mutant in response to cadmium chloride (CdCl2) treatment, conidia were harvested and inoculated in 100 ml liquid minimal medium for 24 h at 30°C with shaking (225 r.p.m.). CdCl2 was added to the cultures to a final concentration of 10 mM. After 30 min treatment, the cadmium-containing cultures were spun down, filtered through Whatman No. 1 filter paper and frozen immediately in liquid nitrogen.
RNA isolation and cDNA labelling. Total RNA from frozen samples was isolated using zirconia/silica beads (0.2 g, 0.5 mm diameter, Biospec) and a Mini-BeadBeater (Biospec) with 1 ml TRizol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA (100 µg) was further purified using an RNeasy kit (Qiagen). RNA integrity was checked with a Nanodrop spectrophotometer and by agarose gel electrophoresis. cDNA synthesis and labelling followed the protocol of the ChipShot Indirect Labelling kit from Promega (catalogue no. Z4000). The dyes Cy3 and Cy5 (Amersham, catalogue no. RPN5661) were incorporated into cDNA by adding Cy3 or Cy5 monofunctional N-hydroxysuccinimide ester dye to the cDNA solution for 1 h at 22 °C. The cDNA was subsequently cleaned by using a ChipShot membrane column and dried under vacuum.

Microarray hybridization and data analysis. The methods for microarray hybridization and data analysis were as described in Tian et al. (2007). Briefly, 70-mer oligonucleotides representing all predicted N. crassa genes and additional intergenic regions were printed onto GAP slides (Corning, catalogue no. 40005). We used a closed loop for experimental design that increases statistical power (Townsend & Taylor, 2005). Each RNA sample was used in at least four individual hybridizations. Images from the Axon 4000B microarray scanner were analysed by GenePix 6.0 software; statistical support for differential expression levels was obtained by BAGEL analyses (Townsend & Hartl, 2002). cis-Element analysis was performed as previously described (Tian et al., 2007). The 1000 bp region upstream of the predicted translational start site of nine common targets between H2O2 and CdCl2 conditions was retrieved and evaluated by MDscan (Liu et al., 2002), BioProspector (Liu et al., 2001) and MEME (Bailey & Elkan, 1994) for motif prediction.

Quantitative real-time RT-PCR (qRT-PCR). qRT-PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems) using a Qiagen SYBR kit (catalogue no. 204143). Amplification of mRNA from the actin gene was used as an internal control for normalization. Methods were as described previously (Dementhon et al., 2006).

NcAp-1–GFP plasmid construction and Neurospora transformation. The ORF of NCU03905 was inserted into the pMF272 vector, which includes a GFP cassette (Freitag et al., 2004). For the construct using the NcAp-1 native promoter to drive expression, the NcAp-1 upstream sequence and ORF were amplified using primers containing NotI and PacI sites (forward primer 5'-CTG CCG CGG CCG CCC'TCT CTT TCT TCT ATC TGA ATC G-3', reverse primer 5'-CTT TAA TTA ACA GTG TGG CAG CTG TCT TTC G-3'). For the construct using the cgl promoter, the NcAp-1 gene was amplified using primers containing SpeI and PacI sites (forward primer 5'-CTA GTA TGA CGT CGA CTG ACC CTG CCT-3', reverse primer 5'-CTT TAA TTA ACA GTG TGG CAG CTG TCT TTC G-3'). The NcAp-1–gfp constructs were transformed to a NCU03905 strain containing a his-3 marker (R21-07) using Neurospora transformation methods (Margolin et al., 1997). Verification of full-length NcAp-1–gfp insertion at the his-3 locus was confirmed by PCR. To assess localization of NcAp-1–gfp upon oxidative stress, 16 h-old hyphae from the NCU03905 (NcAp-1–gfp) strains (CT-C17 and CT-N9) were treated with 30 mM H2O2 and 10 mM CdCl2. After 30 min of exposure, NcAp-1–GFP localization was assessed via fluorescence microscopy using a Zeiss Axioskop II microscope and GFP fluorescence filter. Photographs of hyphae were taken using a Zeiss Axioskop II microscope with a Hamamatsu digital charge-coupled device (CCD) camera, and further processed with Adobe Photoshop 7.0.

Stress screening of conidia. Fresh conidia were harvested from 7 day-old slants and suspended in 500 µl H2O to a final concentration of 108 µl-1. Seven microlitres of a conidial dilution series (107, 106, 105, 104 and 103 µl-1) was spotted onto a BDES plate (Davis & De Serres, 1970) or BDES plates plus one of the following compounds: H2O2, 1 or 2 mM; CdCl2, 50 or 100 µM; sorbitol, 0.8 M; β-mercaptoethanol (β-ME), 1 or 4 mM; 3-aminotriazole (3-AT), 1 or 2 mM; NaCl, 0.8 mM. Plates were incubated at 25 °C for 2 days and then growth was evaluated.

Identification of orthologues and regulogs in N. crassa and other fungi. The sequences of predicted ORFs in the N. crassa genome were downloaded from Broad Institute version 7 (http://www.broad.mit.edu/annotation/genome/neurospora/Downloads.html). Neurospora discreta and Neurospora tetrapera genomic data were obtained from http://genomes.jgi-psf.org/Neud1/Neud1.download.html and http://genomes.jgi-psf.org/Neut1/Neut1.download.html, respectively. S. cerevisiae ORFs were downloaded from the SGD database (http://download.yeastgenome.org/sequence/genomic_sequence/orf_dna/). Aspergillus fumigatus genome data were downloaded from TIGR (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/A_fumigatus/annotation_dbs/). Candida albicans genome data were downloaded from http://www.candidagenome.org/DownloadContents.shtml Sch. pombe ORFs were downloaded from http://www.sanger.ac.uk/Projects/S_pombe/protein_download.shtml.

A. nidulans and M. grisea ORF protein sequences were downloaded from The Broad Institute (http://www.broad.mit.edu/annotation/giai/). Yarrowia lipolytica ORF protein sequences were downloaded from http://www.genolevures.org/download.html. Orthologous genes were identified as best bidirectional hits using BLASTP, with a cut-off value of 1e-6. The bZIP member prediction was performed by hidden Markov model (HMM) searches; the bZIP TF HMM model (PF00170.10) was downloaded from Pfam (http://pfam.sanger.ac.uk/). The sequence was defined as a DNA-binding motif if the HMM search P value was <1×10^-8.

Transcriptional profiling data for S. cerevisiae in response to oxidative stress were obtained from Gasch et al. (2000), and the chromatin-immunoprecipitation (ChIP) data were downloaded from Harbison et al. (2004). C. albicans oxidant stress response data were downloaded from Wang et al. (2006). The Cap1 ChIP data were downloaded from Znaidi et al. (2009). Sch. pombe oxidant stress profiling data were downloaded from Chen et al. (2008). The A. fumigatus proteomics data for the H2O2 stress response were downloaded from Lessing et al. (2007). The N. crassa profiling data on mendicane were downloaded from Takahashi et al. (2010).

RESULTS

TF gene expression in N. crassa during vegetative growth

In N. crassa, there are about 200 predicted general TFs (with and without a DNA-binding domain) (Borkovich et al., 2004; Galagan et al., 2003; Shiu et al., 2005). The class of TFs with predicted DNA-binding domains comprises six families, i.e. the bHLH, bZIP, C2H2 zinc finger, Myb, GATA factor and Zn(II)2 binuclear cluster groups, plus a group of miscellaneous TFs. We used an HMM to revise this list based on the sequence of the DNA-binding domain (see Methods). We identified 178 TFs (Supplementary Table S1, worksheet p1) with a clear DNA-binding motif; 166 of these belonged to one of the six defined gene families listed above. The remaining 12 genes encoded TFs classified as miscellaneous (various DNA-binding domains). These 178 TFs can be thought of as the core
set for *N. crassa*, although additional TF genes may be present that contain a non-typical DNA-binding motif(s).

Since TFs play a critical role in the regulation of gene expression patterns, we evaluated the expression pattern of the 178 predicted TFs during vegetative growth of *N. crassa* at two developmental time points. We evaluated the expression of predicted TFs using full-genome *Neurospora* microarrays (Kasuga et al., 2005; Kasuga & Glass, 2008; Tian et al., 2007) from hyphae at the periphery of a colony (a section of the colony that is 0–3 h old) and a second section from the interior of a 12–15 h-old colony. Different sections of a colony of *N. crassa* have been shown to vary in expression profile (Kasuga & Glass, 2008), and in addition the hyphae in these sections show morphological and developmental differences. For example, hyphae at the periphery exhibit foraging behaviour, while hyphae in the interior of a colony form asexual reproductive structures, including aerial hyphae and conidia. Expression patterns for 98 (55%) of the predicted TFs were detected. With a minimum difference of 1.5-fold, 35 TFs showed a significantly different expression pattern between the periphery and the interior of a colony (Table 1). Most of these TFs (33/35) showed an increase in relative expression level in the colony periphery, while only two increased in expression level in the interior of a colony [NCU01459 and NCU08726; NCU08726 encodes *fl* (fluffy), a TF involved in asexual spore development] (Bailey & Ebbole, 1998). Strains containing mutations in 15 of the 35 predicted TFs showed phenotypes different from a WT strain (Table 1), including nine with vegetative growth defects.

### Table 1. Genes encoding TFs with significant differences in expression between the periphery and the interior of a colony

| TF             | Family | Tip/interior* | Growth (mm day$^{-1}$)† | Annotation                                      |
|----------------|--------|---------------|--------------------------|-------------------------------------------------|
| NCU00749       | BHLH   | 2.0           | 60–65                    | Predicted protein                               |
| NCU004731      | BHLH   | 1.9           | 65–70                    | *sah-2*, short aerial hyphae-2                  |
| NCU002724      | BHLH   | 1.6           | 70–75                    | Helix–loop–helix DNA-binding domain              |
| NCU00499       | bZIP   | 1.6           | 25–30                    | *ada-1*, all development altered-1              |
| NCU01459       | bZIP   | 0.6           |                          | *ast-2*, ascospore lethal-2                      |
| NCU00090       | C2H2   | 6.0           | 60–65                    | Related to PacC protein                         |
| NCU00340       | C2H2   | 3.5           | 20–30                    | *pp-1*, Ste12-like TF                           |
| NCU00694       | C2H2   | 3.0           | 70–75                    | Putative protein                                |
| NCU02671       | C2H2   | 2.4           | 20–25                    | Cutinase G-box binding protein                  |
| NCU03043       | C2H2   | 2.0           | 60–65                    | Related to Krüppel protein                      |
| NCU03421       | C2H2   | 7.7           | 60–65                    | Conserved hypothetical protein                  |
| NCU03699       | C2H2   | 2.4           | 70–75                    | Conserved hypothetical protein                  |
| NCU06487       | C2H2   | 2.1           | 75–80                    | Predicted protein                               |
| NCU06907       | C2H2   | 1.8           | 70–75                    | Conserved hypothetical protein                  |
| NCU06503       | C2H2   | 1.8           | 55–60                    | Related to DNA-binding protein                  |
| NCU08807       | C2H2   | 1.7           |                          | *cre-1*, carbon repressor protein 1             |
| NCU09252       | C2H2   | 1.5           | 75–80                    | Conserved hypothetical protein                  |
| NCU01154       | GATA   | 2.3           |                          | *sub1*, submerged protoperithecia-1             |
| NCU09008       | GATA   | 2.3           | 60–65                    | Conserved hypothetical protein                  |
| NCU05685       | Myb    | 1.6           |                          | Conserved hypothetical protein                  |
| NCU00155       | Zn2Cys6| 2.0           | 70–75                    | Transcriptional activator Mut3p                 |
| NCU00289       | Zn2Cys6| 2.5           | 65–70                    | *tah-1*, all aerial hyphae-1                    |
| NCU01097       | Zn2Cys6| 3.6           | 70–75                    | Related to NITA protein                         |
| NCU04866       | Zn2Cys6| 2.0           | 50–55                    | Related to FacB DNA-binding protein             |
| NCU06799       | Zn2Cys6| 3.0           | 60–65                    | Hypothetical protein                            |
| NCU07199       | Zn2Cys6| 5.0           | 80–85                    | Conserved hypothetical protein                  |
| NCU07788       | Zn2Cys6| 2.7           | 55–60                    | *col-26*, colonial-26                          |
| NCU08063       | Zn2Cys6| 2.1           | 65–70                    | Putative protein                                |
| NCU08307       | Zn2Cys6| 3.0           |                          | Hypothetical protein                            |
| NCU02576       | Zn2Cys6| 1.9           |                          | Related to cutinase TF                          |
| NCU07392       | Zn2Cys6| 1.5           | 60–65                    | *adv-1*, all development altered                |
| NCU06407       | Zn2Cys6| 1.5           | 55–60                    | *vd-3*, vegetative asexual development-3        |
| NCU02307       | Zn2Cys6| 1.5           | 70–75                    | Conserved hypothetical protein                  |
| NCU08726       | Zn2Cys6| 0.2           | 20–25                    | *fl*, conidial development protein fluffy       |
| NCU03593       | Zn2Cys6| 1.7           | 10–15                    | Probable homeoprotein                           |

*Significance cut-off (BAGEL) of at least 1.5-fold.
†Growth data from the Broad Institute (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).
Phylogenetic analysis of \textit{N. crassa} bZIP family members

In \textit{S. cerevisiae}, the bZIP TF family has been well studied and consists of 14 members. The \textit{YAP} family of bZIP TFs, of which there are eight members, are thought to be a result of at least six duplication events from a common ancestor (\textit{GCN4}) (Kellis et al., 2004), and can be divided into four subfamilies: \textit{YAP1} and \textit{YAP2}, \textit{YAP4} and \textit{YAP6}, \textit{YAP5} and \textit{YAP7}, and \textit{YAP3} and \textit{YAP8} (Tan et al., 2008) (Fig. 1). We performed phylogenetic analysis of predicted bZIP domain proteins from the hemiascomycete species \textit{S. cerevisiae} (14 members) and \textit{C. albicans} (seven members), a species at the base of the hemiascomycete clade, \textit{Yarrowia lipolytica} (10 members), the archiascomycete species \textit{Sch. pombe} (six members), and the euascomycete species \textit{A. nidulans} (nine members), \textit{M. grisea} (10 members) and \textit{N. crassa} (nine members). The phylogenetic tree of the bZIP domains showed divergence into two separate clades (Fig. 1). The lower clade includes all known members of the \textit{YAP} family (\textit{YAP} clade), while the upper clade includes all other bZIP domain proteins, including \textit{S. cerevisiae GCN4}, \textit{SKO1}, \textit{HAC1}, \textit{MET28}, \textit{ACA1} and \textit{CST6} (Fig. 1) (\textit{GCN4} clade). In \textit{N. crassa}, the \textit{GCN4} clade bZIP TFs include \textit{cpc-1} (NCU04050), \textit{cys-3} (NCU03536), \textit{ada-1} (NCU00499), \textit{asl-1} (NCU01345) and NCU01459. While the \textit{YAP} family clade includes eight members from \textit{S. cerevisiae}, \textit{Sch. pombe} contains only one homologue, \textit{pap1} (Fig. 1). The four \textit{YAP}-like TFs in \textit{A. nidulans} and the three \textit{YAP}-like TFs in \textit{M. grisea} and \textit{N. crassa} clustered within the \textit{YAP} bZIP clade, although none of the predicted euascomycete \textit{YAP}-like proteins clustered closely with the \textit{S. cerevisiae} \textit{Yap} proteins.

Although phylogenetic analysis clearly showed that gene duplication of bZIP genes occurred in \textit{S. cerevisiae} (\textit{ACA1} and \textit{CST6}, and \textit{YAP} members) and \textit{Sch. pombe} (\textit{ATF21} and \textit{PCR1}), within the euascomycete species only a single recent gene duplication event was detected in \textit{M. grisea} (MG02006.4 and MG04009.4), which clustered with a bZIP protein of unknown function (NCU01459) in \textit{N. crassa}. Viable homokaryotic deletion mutations in NCU01459 are not recoverable (Colot et al., 2006). A more ancient duplication within the \textit{GCN4} clade was identified in \textit{A. nidulans} (AN8643.2 and AN6849.2); these bZIP domains do not cluster closely with any other bZIP proteins. No recent duplication of any bZIP domain gene was detected in \textit{N. crassa}, consistent with the observation that gene duplications are not tolerated (Galagan & Selker, 2004). Thus, the major expansion of the bZIP gene family in fungi occurred before the divergence of euascomycete species from \textit{Sch. pombe} and \textit{S. cerevisiae}, approximately 300 million years ago.

Transcriptional profiling of bZIP mutants

In \textit{N. crassa}, two bZIP genes have been well characterized. The first encodes an orthologue of \textit{GCN4}, called \textit{cpc-1}, which is involved in regulating genes in response to amino acid starvation (Ebbole et al., 1991; Hinnebusch, 2005; Tian et al., 2007). The second bZIP gene encodes a transcriptional regulator, \textit{CYS-3}, which regulates genes in response to sulfur sources (Paietta, 1992). While this study was in progress, a third member, NCU03905 (\textit{nap-1}), was shown to be important for resistance to treatment with menadione and proposed to be a \textit{YAP1} orthologue.
(Takahashi et al., 2010). Here we refer to NCU03905 as NcAp-1, as nap refers to a neutral amino acid permease locus in N. crassa (Perkins et al., 2001; Rao & DeBusk, 1973). Homokaryotic mutant strains are available for seven of nine predicted bZIP genes [Δada-1 (NCU00499), ΔNcu01994, ΔNcAp-1 (NCU03905), ΔNCU04211, cpc-1 (NCU04050), ΔNCU08055 and cys-3 (NCU03536)]. The Δada-1 mutant (all development altered; NCU00499), showed a reduced growth rate (2.5 cm day$^{-1}$ compared with 7.5 cm day$^{-1}$ for the WT) and very short aerial hyphae. Strains containing mutations in the other six bZIP TF genes (ΔNCU01994, ΔNcAp-1, ΔNCU04211, cpc-1, ΔNCU08055 and cys-3) showed growth and asexual reproduction phenotypes indistinguishable from those of the WT strain. Strains containing mutations in two remaining bZIP genes, asl-1 (NCU01345) and asl-2 (NCU01459), showed an ascospore lethality phenotype; homokaryotic strains containing mutations in these genes are not available (Colot et al., 2006).

To identify putative target genes of the bZIP TF mutants, we compared transcriptional profiles of the seven bZIP mutants from hyphae at the colony periphery (0–3 h) with those from the interior of a colony (12–15 h). In the WT, Functional Category (FunCat) analysis (Ruepp et al., 2004) of 509 genes that had higher expression levels in the colony periphery showed an enrichment for genes involved in cell type differentiation, cell wall proteins, carbon compound and polysaccharide metabolism, cell growth/morphogenesis (regulation of directional cell growth), cellular export and secretion, and cell signalling (P<0.001; Supplementary Table S2, worksheet p1). FunCat analysis of the 363 genes that had an increased expression level in the interior of the colony showed an enrichment for genes involved in stress response and detoxification, energy-related genes (glycolysis and pentose phosphate pathway), and amino acid and carbon metabolism (P<0.001; Supplementary Table S2, worksheet p4). In the colony periphery, the number of genes in the seven bZIP mutants that showed altered expression profiles compared with the WT ranged from 21 to 509 (Table 2 and Supplementary Table S3), including genes that increased in relative expression level (from three in ΔNcAp-1 to 290 in Δada-1) and genes that showed a decrease in relative expression level (from five in Δcys-3 to 219 in Δada-1) (Table 2). Expression patterns of genes in the interior of a colony in the bZIP TF deletion strains also showed differences from the WT (Table 2 and Supplementary Table S4), ranging from three affected genes in ΔNcAp-1 to 139 in Δcys-3. The strain carrying a deletion of ada-1 showed the greatest number of expression differences from the WT, consistent with its growth defect.

FunCat analysis of affected genes in the Δada-1 mutant that showed reduced expression in the colony periphery showed enrichment for carbon compound and carbohydrate metabolism (P=2.3×10$^{-7}$). Genes involved in carbon compound and carbohydrate metabolism also showed enrichment in other bZIP mutants, including ΔNCU01994, ΔNCU04211 and ΔNCU08055 (P=0.001 to 0.01).

Similar to data from the colony periphery, affected genes displaying decreased expression in the colony interior in the Δada-1 mutant showed an enrichment for carbon compound and carbohydrate metabolism, and the pentose phosphate pathway (P=1.03×10$^{-6}$). In the ΔNCU01994 and cys-3 strains, genes involved in amino acid metabolism, carbon compound and carbohydrate metabolism, lipid, fatty acid and isoprenoid metabolism, and metabolism of vitamins, cofactors and prosthetic groups, were enriched (P=0.002 to 6.3×10$^{-9}$), while NAD/NADP binding was enriched in the ΔNCU04211 and ΔNCU08055 strains. Enriched categories of genes that showed an increased expression level in the colony interior included transport facility and cell defence genes in Δada-1, polysaccharide metabolism and cell wall genes in ΔNCU01994 and ΔNCU08055, and virulence and disease factor genes in ΔNCU04211.

Consistent with their phylogenetic relationships, strains containing mutations in bZIP TF genes that were more closely related showed an overlap in affected gene sets, with an overlap of up to 24% in strains containing mutations in the closely related paralogues NCU08055 and cys-3 (Supplementary Tables S3 and S4). By contrast, only a 1% overlap in expression profiles was identified between strains containing deletions of distant bZIP paralogues, for example, NcAp-1 (NCU03905) and ada-1 (NCU00499). These data suggest that the regulation of some genes and processes has been conserved in the bZIP TF regulons.

| TF mutant | Genes downregulated in periphery | Genes upregulated in periphery | Genes downregulated in interior | Genes upregulated in interior |
|-----------|----------------------------------|--------------------------------|-------------------------------|-------------------------------|
| ΔNCU00499 | 219                              | 290                            | 80                            | 56                            |
| ΔNCU01994 | 12                               | 9                              | 49                            | 77                            |
| ΔNCU08055 | 47                               | 3                              | 6                             | 3                             |
| ΔNCU04211 | 45                               | 5                              | 65                            | 28                            |
| cpc-1     | 22                               | 19                             | 5                             | 52                            |
| ΔNCU08055 | 32                               | 6                              | 50                            | 107                           |
| cys-3     | 5                                | 56                             | 24                            | 139                           |
following duplication and divergence. Similarly, in *S. cerevisiae*, the number of genes co-regulated by YAP family members ranges between 9 and 15 %, and increases to 25 % upon exposure to DNA-damaging agents (Tan *et al.*, 2008).

**NcAp-1 functional characterization and localization**

To elucidate the molecular function of the bZIP TFs in *N. crassa*, we screened the bZIP TF mutants for sensitivity to a variety of chemical stresses, including exposure to sorbitol, NaCl, 3-AT, cadmium, β-ME and H₂O₂. The ΔNcAp-1 mutant was sensitive to all the stresses tested except for β-ME (Supplementary Fig. S1). However, only slight sensitivity to H₂O₂, and no sensitivity to NaCl has been observed in an independently derived NCU03905 (*nap-1*) mutant during hyphal growth (Takahashi *et al.*, 2010). The Δcys-3 mutant showed a mild sensitivity to sorbose (Supplementary Fig. S1). The other bZIP TF deletion mutants did not show any obvious sensitivity under any of the conditions tested.

The predicted NcAp-1 protein (NCU03905) has a PAP1 domain (Fig. 2c), which is an important H₂O₂ response domain in *Sch. pombe* (Chen *et al.*, 2008; Cohen *et al.*, 2002; Vivancos *et al.*, 2006). In *N. crassa*, the closest paralogue to NcAp-1 is NCU01994, although the predicted

![Fig. 2.](http://mic.sgmjournals.org) Complementation of ΔNcAp-1 and localization of NcAp-1–GFP. (a) Comparison of the phenotype of the WT (FGSC 2489), the NcAp-1 mutant (ΔNCU03905) and ΔNcAp-1 complemented strains (NcAp-1–gfp and cccg1 NcAp-1–gfp) exposed to 1 mM H₂O₂ or 100 μM Cd²⁺ using a dilution series of conidial suspensions (from left to right: 10⁵, 10⁴, 10³, 10², 10¹). Plates were incubated at 25 °C for 2 days and then evaluated for growth. (b) NcAp-1–GFP localization in a ΔNcAp-1 (his3-3::NcAp-1–gfp) strain grown on minimal medium (untreated, upper panels), after treatment with 30 mM H₂O₂ (H₂O₂ treated, centre panels) or 10 mM CdCl₂ (Cd²⁺ treated, lower panels). The left-hand panels show GFP fluorescence (NCAp-1–GFP), while the centre panels show nuclear 4,6-diamidino-2-phenylindole (DAPI) staining. The right-hand panels show merged GFP and DAPI images. (c) Domain prediction of Yap1, Yap2, Pap1, NCU03905 (NcAp-1) and its closest paralogue NCU01994. Green, bZIP motif; yellow, PAP1 domain.
protein product of NCU01994 lacks a PAP1 domain (Fig. 2c). However, a strain containing both ΔNcAp-1 and ΔNCU01994 deletions resembles the ΔNcAp-1 mutant with respect to sensitivity to stress (Supplementary Fig. S1).

The redistribution of S. cerevisiae Yap1 from the cytoplasm to the nucleus in response to oxidative stress is a major regulatory mechanism (Kuge et al., 1997), and also occurs in Sch. pombe (PAP1) (Vivancos et al., 2004), C. albicans (CAP1) (Zhang et al., 2000) and A. fumigatus (AfYap1) (Lessing et al., 2007). In N. crassa, NcAp-1–GFP in a WT strain shows redistribution from the cytoplasm to the nucleus upon exposure to menadione (Takahashi et al., 2010). To observe the behaviour of NcAp-1 during oxidative and heavy metal stress in N. crassa, we tagged NcAp-1 with GFP at the C terminus. The introduction of the NcAp-1–gfp construct into the ΔNcAp-1 strain restored cadmium and H2O2 resistance, indicating that the construct encodes a functional NcAp-1 protein (Fig. 2a). Under conditions of no (or low) stress (minimal medium), NcAp-1 localized to the cytoplasm (Fig. 2b). However, when the ΔNcAp-1 (NcAp-1–gfp) strain was exposed to stress (30 mM H2O2 for 5–30 min or 10 mM CdCl2 for 5–10 min, NcAp-1–GFP accumulated in nuclei across the fungal colony (Fig. 2b). These data indicate that the redistribution of Yap1-like proteins to the nucleus upon exposure to oxidants or heavy metals is a highly conserved mechanism of transcriptional regulation in ascomycete fungi in response to a variety of stresses.

**Identification of the NcAp-1 regulon under oxidative stress**

To determine the NcAp-1 stress regulon, we performed transcriptional profiling of the WT and the ΔNcAp-1 mutant grown in minimal medium with and without oxidative stress imposed by treatment with 6 mM H2O2 (see Methods). There were 45 genes induced by H2O2 (Fig. 3a, sets C, D and E) that were dependent on NcAp-1 for transcription. FunCat analysis (Ruepp et al., 2004) of these 45 putative target genes showed that stress responses, oxygen detoxification and metabolism of peptide-derived compounds (such as thioredoxin) were enriched (Table 3). Of the 45 genes (Supplementary Table S5, worksheet P1), 11 have an annotated function in relation to the stress response, especially the oxidant stress response pathway, including two glutathione S-transferases, gst-1 (NCU05780) and gst-2 (NCU04109); four NAD(P)(H)-dependent dehydrogenase/flavin oxidoreductases, mig-2 [menadione-induced gene (Takahashi et al., 2010)] (NCU07452), mig-3 (NCU04452), mig-6 (NCU09285) and NCU08402; an oxidase (NCU05164) and a glutathione peroxidase (NCU05780); a probable reductase (gst-2, NCU09040); a glutathione peroxidase (NCU09534); and a predicted ABC transporter (NCU03776) (Table 4). We confirmed the dependence of expression on NcAp-1 for four genes, cat-2 (NCU05770), gpx-3 (NCU09534), NADH oxidase (mig-3, NCU04452) and glutathione transferase-1 (gst-1, NCU05780) by qRT-PCR (Supplementary Fig. S2). Five genes from this 11-gene oxidant stress set have been shown to be NcAp-1-dependent upon exposure to menadione (Takahashi et al., 2010), including two predicted glutathione S-transferases [gst-1 (NCU05780) and gst-2 (NCU04109)] and three NAD(P)(H)-dependent dehydrogenase/flavin oxidoreductases [mig-2 (NCU07452), mig-3 (NCU04452) and mig-6 (NCU09285)]. These data indicate that the glutathione S-transferase and NADPH dehydrogenases are important for resistance to multiple types of oxidant stress in N. crassa.

**Identification of the NcAp-1 regulon under cadmium stress**

In addition to sensitivity to H2O2, the ΔNcAp-1 mutant was also sensitive to other stress conditions, including exposure to heavy metals such as cadmium (Supplementary Fig. S1). We therefore compared expression profiles of the WT and ΔNcAp-1 mutant upon exposure to CdCl2 versus H2O2 (see Methods and Supplementary Table S1). A total of 34 genes induced by exposure to Cd2+ were dependent on NcAp-1 for transcription (Fig. 3b, sets D and E, Supplementary Table S5, worksheet P2). Nine of these 34 genes overlapped with the 45 targets identified upon exposure to H2O2 and were dependent upon functional NcAp-1. These nine genes form the core targets of NcAp-1 (Table 4) and include the two glutathione S-transferase genes, gst-1 and gst-2 (NCU04109 and NCU05780, respectively), four NAD(P)(H)-dependent dehydrogenase/oxidoreductase genes, mig-2, mig-3, mig-6 and NCU08402, a predicted methoxylase (NCU08402), an oxidase (NCU05164) and a glutathione peroxidase (NCU09534). Six of these 34 genes are induced by menadione treatment (Takahashi et al., 2010) (Supplementary Table S5, worksheet P2), including a predicted glutathione S-transferase, gst-4 (NCU10521), plus five of the nine core genes identified above (gst-1, gst-2, mig-2, mig-3 and mig-6). The 24 Cd2+ condition-specific target genes included several stress/oxidant stress-related genes, including a predicted thioredoxin (NCU06556) and uricase (NCU07853). Interestingly, the bZIP TF cys-3 and nitrogen and sulfur metabolism genes were also enriched in this list (Supplementary Table S5, worksheet P2), suggesting that sulfur metabolism regulated by CYS-3 may cross-talk with the metal response pathway regulated by NcAp-1.

**cis-Element analysis of NcAp-1 targets**

As indicated by FunCat analysis, the NcAp-1 target gene set (45 under H2O2 and 34 under Cd2+) was enriched for genes involved in oxidative stress responses (Table 4). An earlier study evaluating promoter regions of genes that were induced upon treatment with menadione and which were NcAp-1-dependent failed to identify upstream regulatory elements (Takahashi et al., 2010). To identify potential NcAp-1 cis-regulatory elements in this study, we analysed 1000 bp upstream of the nine common target genes identified between the H2O2 and Cd2+ responses.
(see Methods). We identified the *N. crassa* cis-regulatory element TTA(t/c)(t/c)AAT, which was similar to cis-regulatory elements identified for Yap1 (TTACTAA), Cap1 (TTAc/gTAA), AfYap1 [TTA(G/C)TAA] and Pap1 (TTACGTAA), and which occurred in seven of the nine common target genes ($P = 0.0006$), 14 of 45 H$_2$O$_2$-responsive target genes ($P = 0.056$), and 15 of 34 cadmium-responsive target genes ($P = 0.000006$) (Fig. 3c, d). This cis-regulatory element was enriched in NcAp-1 putative target genes relative to its prevalence in the *N. crassa* genome. This set of genes is predicted to be directly regulated by NcAp-1. Of the nine common target genes, conservation of the NcAp-1 cis sequence was identified in the promoter regions of four orthologues in the related species *N. discreta* (NCU00847, NCU04452, NCU09285 and NCU05164) and in five orthologues in *N. tetrasperma* (NCU00847, NCU04452, NCU07452, NCU09285 and NCU09534).

### Table 3. FunCat analysis of NcAp-1 targets

Enriched groups with $P < 0.001$ are shown; groups enriched under both Cd$^{2+}$ and H$_2$O$_2$ conditions are in bold type.

| Functional category                                      | Cd$^{2+}$ $P$ value | H$_2$O$_2$ $P$ value |
|--------------------------------------------------------|---------------------|----------------------|
| 01 METABOLISM                                          |                     |                      |
| 01.02 Nitrogen, sulfur and selenium metabolism         |                     |                      |
| 01.02.07 Regulation of nitrogen, sulfur and selenium metabolism |                     |                      |
| 01.20 Secondary metabolism                             |                     |                      |
| 01.20.37 Metabolism of peptide-derived compounds       |                     |                      |
| 01.20.37.01 Metabolism of thioredoxin, glutaredoxin, glutathione |                     |                      |
| 02 ENERGY                                              |                     |                      |
| 02.16 Fermentation                                     |                     |                      |
| 02.16.01 Alcohol fermentation                          |                     |                      |
| 32 CELL RESCUE, DEFENCE AND VIRULENCE                 |                     |                      |
| 32.07 Detoxification                                   |                     |                      |
| 32.07.03 Glutathione conjugation reaction              |                     |                      |

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In this study, we evaluated both the phenotype and the expression patterns associated with strains containing mutations in predicted bZIP TF genes in *N. crassa*. We identified a single bZIP mutant, containing a deletion of NCU03905, which showed sensitivity to multiple stresses. We further characterized the transcriptional response of strain ΔNcAp-1 (NCU03905) upon exposure to oxidative and heavy metal stresses in comparison with the WT, and identified a number of core stress-response genes. We subsequently compared our data with those from a recent report of an independently derived Δnap-1 mutant and its effect on transcription in response to exposure to menadione (Takahashi et al., 2010). We further compared our datasets with those obtained under different growth conditions and with expression profiling datasets from hemiascomycete species. These comparisons indicated that a small number of genes, which are primarily involved in an oxidant response, are regulated by AP1-like TFs and have been evolutionarily conserved.

Our screening showed that the ΔNcAp-1 strain was sensitive to multiple stresses, including oxidant, heavy metal, osmotic and amino acid starvation stress, while strains containing mutations in six additional bZIP genes did not show increased sensitivity to the tested conditions. These data indicate that NcAp-1 is a critical stress response regulator in *N. crassa*. Our expression profiling results of the WT versus the ΔNcAp-1 mutant under H₂O₂ and Cd²⁺ exposure support this prediction, as multiple pathways were affected in the ΔNcAp-1 mutant, including, as expected, the oxidant response pathway, but also nitrogen metabolism genes. In addition, cys-3 (which is a sulfur metabolism regulator) and sulfur metabolism genes were induced by exposure to Cd²⁺ and were NcAp-1-dependent, suggesting crosstalk between the NcAp-1 and cys-3 regulons. In support of this hypothesis, genes involved in sulfate metabolism are also induced by Cd²⁺ exposure in *C. albicans* (Enjalbert et al., 2006).

Genome-wide stress-response profiling data have been published for the genes orthologous to NcAp-1 in *S. cerevisiae* (Yap1) (Gasch et al., 2000; Cohen et al., 2002), *Sch. pombe* (Pap1) (Chen et al., 2008) and *C. albicans* (Cap1) (Wang et al., 2006). In addition, ChIP data are also available for Yap1 (Harbison et al., 2004) and Cap1 (Znaidi et al., 2009), and proteomics data are available for AfYap1 in *A. fumigatus* (Lessing et al., 2007). The stress response, and in particular, the oxidant stress response, was enriched in the Yap1, Cap1, Pap1, NcAp-1 and AfYap1 datasets. Out
of the nine core NcAp-1-dependent stress response genes (Table 4), eight have orthologues in S. cerevisiae, and four of them (OYE3, YKLO71W, OYE2 and ADH5) are regulated by Yap1 (Cohen et al., 2002; Gasch et al., 2000; Harbison et al., 2004). Furthermore, Yap1 binds to the promoter region of OYE2, based on the ChIP data (Harbison et al., 2004). Three of seven orthologues of the NcAp-1-dependent genes are bound and regulated by CAP1 in C. albicans (Wang et al., 2006; Znaidi et al., 2009), while in Sch. pombe, the regulation of six of the eight orthologues is dependent on PAP1 (Table 4). In A. fumigatus, the protein abundance of protoporphyrinogen oxidase (Afu4g00910) is dependent on AfYap1 (Lessing et al., 2007) and the protein is orthologous to the NcAp-1 target NCU05164. In summary, eight of nine NcAp-1 core target genes (Table 4) have been shown to be regulated by Ap1-like proteins in at least one other ascomycete species. The ninth gene, NCU00847 (related to 7-alpha-cephem-methoxylase), has no clear orthologue in S. cerevisiae, Sch. pombe and C. albicans, and is possibly a filamentous fungal-specific oxidant stress response gene. In addition, genes encoding catalases are another group that show regulation under H2O2 conditions by Ap1-like TFs, including catalase-2 (NCU05770) (NcAp-1), catalase-2 (AfYap1), catalase-1 (Cap1) and catalase-T (Yap1). In a recent publication, Takahashi and co-workers found that catalase-3 (NCU00355) rather than catalase-2 is induced by menadione treatment in N. crassa, and is nap-1 (NcAp-1)-dependent (Takahashi et al., 2010). However, in our study, upon treatment with H2O2, NCU00355 was induced in both the WT (3.3-fold) and the ΔNcAp1 mutant (2.5-fold).

The identification of the NcAp-1 regulon and pathway comparisons among ascomycete species suggest that the oxidant stress response pathway in fungi has been conserved over 300 million years of evolution and that Ap1-like TFs are the main regulators of this response pathway. Both catalases and flavin oxidoreductases are regulated by Ap1-like TFs in S. cerevisiae, C. albicans, Sch. pombe and N. crassa. We identified a common gene set that was induced by amino acid starvation, and oxidant and heavy metal stress, and whose predicted function was oxygen detoxification. These data suggest that reactive oxygen species (ROS) might be a general signal for stress. In all five ascomycete species (S. cerevisiae, Sch. pombe, C. albicans, A. fumigatus and N. crassa), the Ap1-like protein localized to nuclei from the cytoplasm following exposure to a variety of stresses.

However, there is also evidence that the Ap1 pathway has diverged among the ascomycete species. In S. cerevisiae, the functions of the duplicated Yap proteins are partially redundant, especially those of Yap1 and Yap2 (Cohen et al., 2002). In C. albicans, the oxidant stress response has multiple distinct pathways: the Cap1 pathway and the Hog1 mitogen-activated protein kinase (MAP kinase) pathway (Enjalbert et al., 2006). The Cap1 pathway is activated upon exposure to both low and high concentrations of H2O2, while the Hog1 pathway is only activated at higher concentrations of H2O2. In Sch. pombe, the Pap1 pathway is activated upon exposure to low H2O2 concentrations, while the Sty1 (Hog1 orthologue)–Atf1 (Sko1 orthologue) pathway is activated upon exposure to high concentrations of H2O2 (Chen et al., 2008; Vivancos et al., 2006). However, the Hog1 and Sko1 pathways in S. cerevisiae mainly regulate responses to osmotic stress (Rep et al., 2001), although Skn7 is also involved in oxidative stress, independently of the Yap1 pathway (Estuch, 2000). In N. crassa, the HOG1 orthologue is os-2 (NCU07024) and the SKN7 orthologue is rrg-2 (NCU02413); both genes may be involved in the oxidant stress response pathway, as mutants are sensitive to H2O2 (Banno et al., 2007). The Atf1/Sko1 orthologue in N. crassa is NCU01345, but is ascospore-lethal, and so homokaryotic strains are not available. Based on these data, in N. crassa, at least three oxidant stress response pathways may exist, the OS-2, RRG-2 and NcAp-1 pathways. In the future, it will be informative to study the relationships between various stresses and the pathways regulated by these three TFs.

**Evolution of the bZIP TF family regulatory network**

How an organism coordinates its transcriptional repertoire in response to developmental or environmental signals, and how evolution affects the topology of these TF networks, are still mostly unanswered questions. One way to study the regulatory networks is to focus on a single biological function. For example, over 30 TFs are involved in DNA damage in S. cerevisiae (Workman et al., 2006). An alternative is to examine regulatory relationships using a phylogenetic framework, such as analysing the regulatory network within the Yap subfamily (Tan et al., 2008). In our study, we analysed seven of the nine members of the bZIP TF family in N. crassa. Phylogenetic analyses indicated that the bZIP TF family is an ancient duplicated gene family, with no recent genome duplications in any fungal species, with the exception of S. cerevisiae. Although our data showed that the regulons of the bZIP TFs have diverged, up to 25% of the target genes were shared between some TF pairs, for example, the affected gene sets from the colony interiors of the cy5-3 and ΔNCU08055 mutants. However, profile comparisons showed differences between the peripheral and interior sections of the colony among the different bZIP mutants. For example, although 25% of the affected genes in the interior of a colony were identical between the ΔNCU08055 and cy5-3 mutants, only 6% were identical between the ΔNCU08055 and cy5-3 datasets from the periphery of the colony. From our stress screening and phenotype analyses, most bZIP TF mutants did not show increased sensitivity to oxidant, metal or osmotic stress. In addition, none of the mutants showed a growth phenotype under minimal media conditions, with the exception of the Δada-1 (NCU00499) mutant. Transcriptional profiling of four bZIP mutants (Δada-1, ΔNCU01994, ΔNCU04211 and ΔNCU08055) revealed a role for these TF genes in carbon compound and carbohydrate metabolism, provid-
being a further testable hypothesis for metabolic processes regulated by these TFs. Three bZIP TF mutants could not be assayed, because homokaryotic mutants are not available, suggesting that these bZIP TFs regulate essential processes in \textit{N. crassa}. Further genetic, phenotypic and molecular analyses to identify direct targets of the \textit{N. crassa} bZIP TF family and epistasis relationships will reveal the commonalities and complexities of transcriptional networks regulated by this group of TFs. These data will also enable further comparative analyses to decipher mechanisms associated with the evolution of regulatory networks in \textit{N. crassa} and other fungi.

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