Comparative Transcriptome Analysis of the CO₂ Sensing Pathway Via Differential Expression of Carbonic Anhydrase in Cryptococcus neoformans

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ABSTRACT

Carbon dioxide (CO₂) sensing and metabolism via carbonic anhydrases (CAs) play pivotal roles in survival and proliferation of pathogenic fungi infecting human hosts from natural environments due to the drastic difference in CO₂ levels. In Cryptococcus neoformans, which causes fatal fungal meningoencephalitis, the Can2 CA plays essential roles during both cellular growth in air and sexual differentiation of the pathogen. However the signaling networks downstream of Can2 are largely unknown. To address this question, the present study employed comparative transcriptome DNA microarray analysis of a C. neoformans strain in which CAN2 expression is artificially controlled by the CTR4 (copper transporter) promoter. The P_CTR4::CAN2 strain showed growth defects in a CO₂-dependent manner when CAN2 was repressed but resumed normal growth when CAN2 was overexpressed. The Can2-dependent genes identified by the transcriptome analysis include FAS1 (fatty acid synthase 1) and GPB1 (G-protein β subunit), supporting the roles of Can2 in fatty acid biosynthesis and sexual differentiation. Cas3, a capsular structure designer protein, was also discovered to be Can2-dependent and yet was not involved in CO₂-mediated capsule induction. Most notably, a majority of Can2-dependent genes were environmental stress-regulated (ESR) genes. Supporting this, the CAN2 overexpression strain was hypersensitive to oxidative and genotoxic stress as well as antifungal drugs, such as polyene and azole drugs, potentially due to defective membrane integrity. Finally, an oxidative stress-responsive Atf1 transcription factor was also found to be Can2-dependent. Atf1 not only plays an important role in diverse stress responses, including thermotolerance and antifungal drug resistance, but also represses melanin and capsule production in C. neoformans. In conclusion, this study provides insights into the comprehensive signaling networks orchestrated by CA/CO₂-sensing pathways in pathogenic fungi.

CARBON dioxide (CO₂) sensing, transport, and metabolism are critical in survival, proliferation, and differentiation in diverse microbes, including pathogenic fungi (BAHN and MÜHLSCHLEGEL 2006). Opportunistic fungal pathogens exposed to ambient air in a natural environmental setting must overcome dramatic changes in CO₂ levels during infection (150-fold change from 0.036 to 5%). In Candida albicans, which causes superficial and systemic candidiasis in immunocompromised patients, high CO₂ levels induce the morphological transition from yeast to hypha that is an important virulence attribute contributing to tissue adhesion and invasion of the pathogen (STMS 1986; MOCK et al. 1990). Recently it has been reported that CO₂ sensing by C. albicans is mediated by adenyl cyclase, which produces cAMP, thereby activating protein kinase A (PKA) to trigger filamentous growth (KLENGEL et al. 2005). Another striking example of fungal CO₂-mediated signaling has also been demonstrated in Cryptococcus neoformans, which causes fatal fungal meningoencephalitis in both immunocompetent and immunocompromised individuals. High CO₂ levels enhance antiphagocytic polysaccharide capsule production during infection (GRANGER et al. 1985; VARTIVARIAN et al. 1993) and inhibit sexual differentiation by blocking cell-cell fusion via repression of pheromone production (BAHN et al. 2005).

During CO₂ sensing and metabolism, carbonic anhydrase (CA), a zinc-containing metalloenzyme, is the key enzyme that catalyzes hydration of CO₂ and produces bicarbonate (HCO₃⁻) and a proton (H⁺). Since the soluble form of CO₂, bicarbonate, is likely to be a key intracellular CO₂ signaling molecule as well as a substrate for diverse carboxylating enzymes essential for lipid, amino acid, and pyrimidine synthesis, CAs are
considered to play pivotal roles in the metabolic process. CA is ubiquitously found in all organisms, catalyzing diverse cellular processes including carbon fixation in plants, and tumor development, respiration, pH and ion homeostasis, and electrical activity in the retinal and nervous systems in mammals (Bahn and Müller-Schlegel 2006). The CA enzyme family can be broadly grouped into four classes: α-CA, β-CA, γ-CA, and δ-CA (for reviews, see Henry 1996; Tripp et al. 2001). The α-CAs are widely distributed in bacteria, plants, and mammals. In humans, 14 α-class CAs have been identified including four membrane-associated (IV, IX, XII, and XIV) and extracellularly secreted (VI) CAs. Particularly, CA IV and XIV were found to be extracellular membrane-bound CAs expressed in the lung and brain (Wang et al. 1997; Parkkila et al. 2001).

β-CAs are found in bacteria, algae, plants, and fungi, but not in mammals (Henry 1996; Watson et al. 2003). Fungal β-CA was first characterized in the nonpathogenic model yeast, Saccharomyces cerevisiae, called Nce103, and found to be essential for growth in ambient CO2 levels (Gotz et al. 1999; Clark et al. 2004; Amoroso et al. 2005). The requirement of Nce103 for growth can be relieved by enriching air with 5% CO2 (Amoroso et al. 2005), which appears to result in spontaneous hydration of CO2 into bicarbonate. The role of fungal CA in pathogenic fungi has been recently uncovered in C. albicans and C. neoformans (Bahn et al. 2005; Klenk et al. 2005; Mogensen et al. 2006). C. albicans contains a single functional CA, called Nce103 (Klenk et al. 2005), whereas C. neoformans possesses two CAs, named Can1 and Can2, although the latter is the major form of CA (Bahn et al. 2005; Mogensen et al. 2006). Disruption of genes encoding Nce103 and Can2 causes growth arrest of C. albicans and C. neoformans, respectively, under ambient air conditions (Bahn et al. 2005; Klenk et al. 2005; Mogensen et al. 2006). Normal growth of those CA mutants can be restored by incubating them in either high CO2 conditions or an animal host (Bahn et al. 2005; Klenk et al. 2005). Interestingly, these studies raised the hypothesis that CAs might be directly or indirectly involved in fungal CO2 signaling pathways. In C. albicans, the activity of adenylyl cyclase (AC) to produce cAMP and thus activate the cAMP-signaling pathway is enhanced by bicarbonate (Klenk et al. 2005; Mogensen et al. 2006). In C. neoformans, the mating inhibition by high CO2 can be partially reversed by mutation of the CAN2 gene (Bahn et al. 2005). Therefore, fungal CA is not only crucial for cell survival and proliferation, but also mediates various CO2-related signaling cascades that are important for virulence and differentiation of pathogenic fungi. On the basis of recent analysis of crystal structure of Can2 (Schlicker et al. 2009), Can2 belongs to the “plant-type” β-CA and carries a unique N-terminal extension consisting of four antiparallel α-helices. The N-terminal extension of Can2 appears to interact with the active site entrance of the dimer (Schlicker et al. 2009).

Although lipid biosynthesis was found to be one of the essential cellular processes affected by mutation of the CA gene (Bahn et al. 2005), downstream genes and signaling networks regulated by CO2 and CAs remained to be elucidated.

To elucidate downstream target genes of a signaling network, genome-wide comparative transcriptome analysis by using the wild-type (WT) strain and the corresponding mutant strain is normally employed. However, this type of comparative analysis is not appropriate for identifying target genes downstream of Can2 since the can2Δ deletion mutant exhibits severe growth defects under ambient air conditions, and growth restoration of the can2Δ mutant by enrichment with high CO2 will bypass the requirement of Can2 by spontaneous hydration of CO2 into bicarbonate (HCO3−). To circumvent this problem, we constructed a C. neoformans strain in which expression levels of the CAN2 gene are artificially controlled by an alternative promoter system. For this purpose, we used the copper-regulated CTR4 promoter system that has been successfully utilized to conditionally modulate expression levels of genes of interest (Ory et al. 2004; Chayakulkeeree et al. 2007). We employed this strain to elucidate its transcriptome patterns by DNA microarray to identify and characterize genes whose transcript levels are regulated directly or indirectly by Can2 in C. neoformans. This transcriptome analysis discovered a number of CA-dependent genes, including those involved in fatty acid biosynthesis (FAS1), organization of polysaccharide capsule (CAS3), and sexual differentiation (GPB1), although a majority of them do not have any known function. Furthermore, among the Can2-dependent genes, a number of environmental stress-response (ESR) genes and the oxidative stress-responsive Atf1 transcription factor were included, suggesting that differential CA expression could be correlated with stress-response regulation in C. neoformans. Therefore, this study not only elucidates CA-dependent genes on a genome-wide scale for the first time, but also provides further insights into the signaling network of CA/CO2-sensing pathway in pathogenic fungi.

MATERIALS AND METHODS

Strains and media: The strains used in this study are listed in supporting information, Table S1. All C. neoformans strains were cultured in YPD (yeast extract-peptone-dextrose) medium unless indicated separately. L-DOPA or Niger seed medium for melanin production and agar-based Dulbecco’s modified Eagle’s (DME) medium for capsule production were modified Eagle’s (DME) medium unless indicated separately. L-DOPA or Niger seed medium for capsule production were modified Eagle’s (DME) medium unless indicated separately. L-DOPA or Niger seed medium for capsule production were modified Eagle’s (DME) medium unless indicated separately.
RNA samples that were isolated from each WT H99 and bathocuproinedisulfonic acid (BCS) for induction into 200 ml of fresh YNB medium containing either 200 μg of gold microcarrier beads (0.6 μm, BioRad), and introduced into the serotype A *C. neoformans* strain H99 strain via biolistic transformation (Davidson et al., 2002). Stable transformants were selected on YPD medium containing 200 μg of geneticin (100 μg/ml). The P_{CTR4} promoter replacement strains were screened by diagnostic PCR and Southern blot analysis using a CAN2-specific probe generated by PCR with primers B359 and B93.

Total RNA isolation: For total RNA isolation used in DNA microarray analyses, the WT H99 and P_{CTR4}::CAN2 strains were cultured in 50 ml YPD medium at 30°C for 24 hr, washed with sterile PBS buffer twice, and resuspended with sterile water. Then 1 ml of resuspended cells was inoculated into 100 ml of YNB medium (optical density at 600 nm is ~0.2) and incubated for 12 hr. For zero-time samples, 50 ml out of the 100-ml culture was used. Then 20 ml of the resuspended culture was inoculated into 200 ml of fresh YNB medium containing either 200 μM bathocuproinedisulfonic acid (BCS) for induction of CAN2 or 25 μM copper sulfate (CuSO_{4}) for repression of CAN2, further incubated for up to 36 hr at 30°C. During incubation, 50 ml of the culture was sampled after 6, 12, 24, and 36 hr and used for total RNA isolation. As biological replicates for DNA microarray, three independent cultures for each strain and growth condition were prepared for total RNA isolation. Total RNAs were isolated by using TRIzol reagent as previously described (Ko et al., 2009).

DNA microarray and data analysis: For an experimental set of total RNAs (Cy5 labeling), we used three independent total RNA samples that were isolated from each WT H99 and P_{CTR4}::CAN2 strain grown for 12 hr in YNB containing either BCS or CdSO_{4}. For control total RNA (Cy3 labeling), all of the reference RNAs. For cDNA synthesis, Cy5/Cy3 labeling, prehybridization, hybridization, and slide washing, we followed the protocols described previously (Ko et al., 2009). For DNA microarray slides, we utilized *C. neoformans* serotype D (JEC21) 70-mer oligonucleotide microarray slides containing 7936 spots (Duke University). Three independent DNA microarrays with three independent biological replicates were performed.

The DNA microarray slides were scanned with a GenePix 4100A scanner (Axon Instrument) and the scanned images were analyzed with GenePix Pro (ver. 6.0) and g3 file (UCSC/ genomold.wustl.edu/activity/ma/cneformans/assay.spec. cgi). For array data analysis, we used the CDF algorithm and a dataset that has been mapped to each corresponding 70-mer oligonucleotide sequence printed on the array slides as previously described (Ko et al., 2009). The JEC21 oligomer chip covers 6302 genes of the total 6980 genes in the H99 strain with an e-value 1 e^{-}\text{90% coverage} by blast search. Each *S. cerevisiae* gene name or ID listed in Table S1, Table S2, Table S3, Table S4, and Table S5 was identified by blast search with the serotype A gene sequence, (e-value cutoff: e^{-}\text{95% filtering}). For hierarchical and statistical analysis, data transported from GenePix software were analyzed with Acuity software by employing LOWESS normalization, reliable gene filtering (>95% filtering), hierarchical clustering, zero transformation, ANOVA analysis (P < 0.05), and Excel software (Microsoft).

**Construction of the cas3A and atf1A mutant strains:** The gene disruption cassettes for the CAS3 and ATFI genes were constructed by double joint PCR with NAT split marker or overlap PCR by using primers listed in Table S1 as previously described (Davidson et al., 2002; Kim et al., 2009). For amplification of NAT marker, plasmid pNATSTM#220 for ATFI and pNATSTM#122 for CAS3 were used as templates (Table S1). Purified gene disruption cassettes were introduced into the H99 strain by biolistic transformation. Each mutant was screened and confirmed by both diagnostic PCR and by Southern blot analysis.

**Northern hybridization:** Northern blot analysis was performed with 10 μg of total RNA from each strain that was used for DNA microarray analysis. Electrophoresis, probe preparation and radiolabeling, hybridization, and development were carried out by following the standard protocols previously described (Ausubel et al., 1994; Ko et al., 2009).

**Growth and stress-response assay:** For quantitative growth assays, the WT H99 and P_{CTR4}::CAN2 (YSB734) strains were cultured overnight at 30°C in liquid YPD medium, inoculated into YNB liquid medium containing 200 μM BCS and 25 μM CuSO_{4}, and further incubated at 30°C for 6, 12, and 24 hr. After the indicated time of incubation, a portion of the cultures was sampled, serially diluted, spread onto duplicate YPD agar plates, and incubated at 30°C. After 2 days, colony-forming units (CFUs) per each plate were determined. Stress and antifungal drug sensitivity tests were performed as previously described (Ko et al., 2009; Maeng et al., 2010).

**Capsule and melanin assays:** Qualitative visualization and quantitative measurement of capsule size and melanin production were performed as described previously (Bahn et al., 2004). For additional quantitative measurement of capsule size, packed cell volume was also measured by using hematocrit capillary tubes. Briefly, cells grown on DME medium for 2 days were scraped, washed with PBS buffer to remove released polysaccharide, and fixed with 10% formalin. Cell concentration was determined by using hemocytometer and adjusted to 1 × 10^3 cells/ml with PBS buffer. Forty microliters of the cell suspension was loaded into microhematocrit capillary tubes (Hirschmann Labogéräte, no. 91000257), of which tips were sealed with clay and paraffin to prevent evaporation of medium during incubation. The capillary tubes were placed vertically overnight at room temperature to allow cell packing by gravity. The packed volume of cells was measured by ratio length of packed cell volume phase/length of total volume phase. Two or three independent experiments were performed in triplicate. Statistical difference in capsule size between strains was determined by Bonferroni’s multiple comparison test by using Prism 4 software (GraphPad).

**Microarray data accession number:** The microarray data generated by this study was submitted to the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE21192.

**RESULTS**

Construction of the P_{CTR4}::CAN2 strain for artificially controlling CAN2 expression: To construct the CAN2 promoter replacement strain, the 375-bp CAN2 promoter region was replaced with the 2-kb fragment of the CTR4 promoter and the NAT dominant selectable marker (Figure 1A). We verified the correct genomic organization of the P_{CTR4}::CAN2 allele by Southern blot analysis with XbaI/Xhol- or HindIII-digested genomic DNAs (Figure 1B and data not shown). Four indepen-
dent P\textsubscript{CTR4}\textasciitilde CAN2 strains were confirmed and employed for further analyses. We further verified whether CAN2 expression levels in the P\textsubscript{CTR4}\textasciitilde CAN2 strains are modulated by adding ‘‘BCS (a copper chelator) or CuSO\textsubscript{4},’’ which activates or represses the CTR4 promoter, respectively. Northern blot analysis showed that CAN2 expression levels in the P\textsubscript{CTR4}\textasciitilde CAN2 strains were strongly induced by addition of BCS and repressed by addition of CuSO\textsubscript{4} (Figure 1C), indicating that CAN2 expression levels can be modulated by controlling copper levels in the growth media. CAN2 expression levels in the P\textsubscript{CTR4}\textasciitilde CAN2 strains grown in the presence of BCS were even higher than native CAN2 expression levels in the WT strain grown in the presence of BCS (Figure 1C), suggesting that the CTR4 promoter is stronger than the CAN2 promoter under copper-depleted conditions. The P\textsubscript{CTR4}\textasciitilde CAN2 strain exhibited conditional growth defects in response to copper levels: We addressed whether the growth of P\textsubscript{CTR4}\textasciitilde CAN2 strains could be controlled under ambient air conditions by changing copper levels in the medium. The P\textsubscript{CTR4}\textasciitilde CAN2 strains did not show any growth defects in YNB liquid medium containing 200 μM BCS (+BCS) and 25 μM CuSO\textsubscript{4} (+Cu). After a 12 hr-incubation, a portion of cultures was sampled and its total RNAs was isolated for Northern blot analysis as described in MATERIALS AND METHODS. The P\textsubscript{CTR4}\textasciitilde CAN2 strain exhibited conditional growth defects in response to copper levels: We addressed whether the growth of P\textsubscript{CTR4}\textasciitilde CAN2 strains could be controlled under ambient air conditions by changing copper levels in the medium. The P\textsubscript{CTR4}\textasciitilde CAN2 strains did not show any growth defects in YNB liquid medium containing 200 μM BCS (+BCS) and 25 μM CuSO\textsubscript{4} (+Cu). After a 12 hr-incubation, a portion of cultures was sampled and its total RNAs was isolated for Northern blot analysis as described in MATERIALS AND METHODS.
in liquid YNB medium containing either BCS or CuSO₄ at different time points. In agreement with results described above, the P_{CTR4::CAN2} strains also showed growth defects in the liquid YNB medium containing CuSO₄, but not BCS (Figure 2B). Taken together, our results demonstrate that CAN2 expression is tightly controlled by the replaced CTR4 promoter, which enables the precise control of C. neoformans growth under ambient air conditions, depending on copper levels in the culture medium.

**Identification of Can2-dependent genes by comparative transcriptome analysis of the P_{CTR4::CAN2} strain and the wild-type C. neoformans.** To identify CA-regulated genes, we performed genome-wide comparative transcriptome analysis of the WT and P_{CTR4::CAN2} strains grown in the presence of BCS or CuSO₄ by using DNA microarrays as described in MATERIALS AND METHODS.

First we compared genome-wide expression profiles of the P_{CTR4::CAN2} strains under copper-depleted conditions (+BCS), which showed normal growth patterns with induced CAN2 expression, with those under copper-enriched conditions (+Cu), which showed retarded growth patterns with repressed CAN2 expression. A total of 133 genes (120 genes with serotype A gene ID) exhibited significantly different expression levels (ANOVA, P < 0.05) (Figure 3A). Among these, 9 genes were differentially regulated in the WT strain between BCS and CuSO₄-containing media, indicating that these genes are copper-dependent genes. These genes include the SOD1, LAC1, and CTR3 genes. The CTR3 gene that encodes a copper transporter was most downregulated, mainly due to the increased copper concentrations in the growth medium. The SOD1 and LAC1 genes are also known to be induced by copper (CULOTTA et al. 1995; JIANG et al. 2009). Therefore, a total of 123 genes appeared to be CAN2-dependent genes. Among these, only 10 genes exhibited >1.5-fold induction or reduction when CAN2 is repressed. As expected, the CAN2 gene was the most highly repressed gene (Figure 3B), further verifying the quality of our microarray results. Upregulation of the FUR1 gene, encoding uracil phosphoribosyltransferase, upon repression of the CAN2 gene is notable. Previously we have hypothesized that the can2 mutation inhibits cellular growth by affecting a variety of decarboxylating enzymes that use bicarbonate as a substrate and are required for biosynthesis of lipid, amino acids, and pyrimidines (BAHN et al. 2005). Therefore, it is conceivable that reduced pyrimidine biosynthesis by CAN2 repression allows cells to induce the FUR1 gene, which is one of the genes in the pyrimidine salvage pathway.

CAN2 expression levels induced by the CTR4 promoter were higher than those by the CAN2 native promoter in the presence of BCS (Figure 1C). Therefore, we compared transcriptome profiles of the WT and CAN2-promoter replacement strains in the presence of BCS. A greater number of genes (524 genes) were significantly and differentially regulated (ANOVA, P < 0.05) (Figure 4A). Among these, a total of 136 genes (128 genes with serotype A gene ID) showed >1.5-fold induction or reduction and only 10 genes showed more than twofold regulation (Figure 4B). Except the CAN2 gene, only two genes, CNAG_04307.2 (uricase, named PRX1), and CNAG_06917.2 (peroxiredoxin, named URI1), have predicted functions based on sequence homology (Figure 4C). Regulation of the uricase gene appears to be related to CA and CO₂ metabolism. Uricase catalyzes the oxidation of uric acid to 5-hydroxyisourate, which can interact with H₂O₂ to produce allantoin and carbon dioxide.

On the basis of these array results, we further defined a true Can2-dependent gene based on the following criteria. First a Can2-dependent gene should be identi-
fied from both array data sets with statistical significance (ANOVA, P < 0.05). Second, the Can2-dependent gene should exhibit opposite expression patterns in each data set. Following these criteria, we have selected 17 Can2-dependent genes except Can2 itself (Table 1). These include MEU1, GBPI, NAPI, FAS1, CFO2, CAS3, and ATFI; a majority of these (9 of 17 genes) do not have orthologs in other fungi. The MEU1 gene encodes fatty acid synthase and has been reported to be essential for survival of C. neoformans (Chayakulkeeree et al. 2007). The MEU1 gene encodes methylthioadenosine phosphorylase, which is the initial enzyme catalyzing the methionine salvage pathway (Subhi et al. 2003). The GBPI gene encodes the G8-subunit in the pheromone response MAPK pathway and positively controls sexual differentiation of C. neoformans (Wang et al. 2000). These data further confirm that the CO2-sensing carbonic anhydrase signaling pathway is closely connected to the fatty acid and amino acid biosynthetic pathways and sexual differentiation of C. neoformans as previously proposed (Bahn et al. 2005).

The role of Cas3 in capsule production of C. neoformans: CAS3 is one of a few genes whose expression is positively regulated by CAN2 overexpression in our array analysis (Table 1). Although it is homologous to Cap64, which is required for capsule production, Cas3 is not directly involved in capsule formation but instead is required for O-acetylation of the capsule in C. neoformans (Moyrand et al. 2004). However it is still possible that Cas3 is indirectly involved in capsule production through regulation of other capsule genes as the authors speculated (Moyrand et al. 2004). Here we tested the possibility that Cas3 might be involved in CO2-mediated capsule induction since it has been previously reported that capsule production of C. neoformans is increased by high CO2 concentrations (Granger et al. 1985; Vartivarian et al. 1993).

To address this hypothesis, we generated two independent cas3Δ mutants and tested their ability to produce capsule in either ambient air or high CO2 concentrations (Figure S1). Based on quantitative measurements of capsule production, the WT strain exhibited greatly enhanced capsule production in the presence of high CO2 (5%) conditions (Figure 5A). In contrast, the cac1Δ mutant that is highly defective in capsule production under ambient air did not show any enhanced levels of capsule in response to high CO2 concentrations (Figure 5A), indicating that CO2-mediated capsule induction is required for activation of adenylyl cyclase, which has been proposed before (Bahn and Mühlischlegel 2006). The two independent cas3Δ mutants exhibited WT levels of capsule under ambient air conditions (Figure 5A), as previously reported (Moyrand et al. 2004). In response to high CO2 concentration, the cas3Δ mutants also showed enhanced capsule production like the WT H99 strain (Figure 5A), indicating that Cas3 is not required for CO2-mediated capsule induction.

We also tested whether overexpression of CAN2 may enhance capsule production by growing the PCTR4::CAN2 strains in DME + BCS medium. We hypothesized that increased production of intracellular bicarbonate by CAN2 overexpression might activate adenylyl cyclase, resulting in enhanced capsule induction. Unexpectedly, however, the PCTR4::CAN2 strains exhibited WT levels of capsule in DME + BCS medium.
under either ambient air or high CO₂ conditions (Figure 5B). In conclusion, CAN2 overexpression does not enhance capsule induction in *C. neoformans*, although adenylyl cyclase is required for CO₂-mediated capsule induction.

**ESR genes are regulated by Can2:** A recent genome-wide transcriptome analysis of the stress-activated HOG pathway uncovered a number of ESR genes, whose expression is affected by osmotic shock, antifungal drug, or oxidative stress in *C. neoformans* (Ko *et al.* 2009). Interestingly, a majority of the Can2-dependent genes (11 of 17 genes) were found to be ESR genes of *C. neoformans* (Table 1). This finding suggests that CAN2 overexpression can be sensed as stress because increased levels of bicarbonate and protons may change normal cellular physiological conditions. To test this hypothesis, we performed diverse stress sensitivity tests with the *PCTR4::CAN2* strains grown on YNB + BCS medium (Figure 6). The *PCTR4::CAN2* strains exhibited hypersensitivity to several stress-inducing agents, including H₂O₂, CdSO₄, and methylmethane sulfonate (MMS) compared to the WT strain. This indicates that increased antifungal drug susceptibility of the *PCTR4::CAN2* strains appears to be independent of the ergosterol biosynthesis because the phenotypes of the *PCTR4::CAN2* strains were distinct from those of *hog1Δ* mutants that are defective in ergosterol biosynthesis (Figure 6). Instead, hypersensitivity of the *PCTR4::CAN2* strain to antifungal drugs may result from its defective membrane integrity because the *PCTR4::CAN2* strains exhibited increased sensitivity to SDS. Taken together, CAN2 overexpression alters normal cellular physiology, which results in defective responses to diverse environmental stresses and antifungal drugs.

**The role of the transcription factor Atf1 regulated by Can2:** Among the Can2-dependent genes, the one encoding the transcription factor Atf1 was notable, although it does not belong to ESR genes (Table 1). Northern blot analysis confirmed that *ATF1* expression was slightly upregulated when CAN2 is repressed, but slightly downregulated when CAN2 is overexpressed (Figure S2), similar to the microarray results. Atf1 is a major transcription factor activated by the stress-activated Sty1 (a Hog1 MAPK homolog) MAPK in *Schizosaccharomyces pombe* (Shiozaki and Russell 1996; Wilkinson *et al.* 1996; Degols and Russell 1997). In *C. neoformans*, the thioredoxin system is controlled by Atf1, indicating that Atf1 is involved in oxidative stress response (Missall and Lodge 2005). However, the function of Atf1 in diverse stress responses and its relationship with the HOG pathway in *C. neoformans* still remains unknown.
Therefore, we independently constructed the \textit{atf1Δ} mutant strain in the WT H99 strain that retains full virulence (Figure S3) and tested various stress sensitivities and virulence factor production compared with the mutants of the stress-activated HOG pathway and other stress-related signaling pathways such as the Ras and cAMP pathways. As control strains, we used the \textit{atf1Δ} mutant (here designated as \textit{atf1ΔE}), which was previously constructed in the H99-Eunuch strain background (H99E) (Missall and Lodge 2005).

The two independently generated \textit{atf1Δ} mutants exhibited the expected hypersensitivity to tert-butyl hydroperoxide (tBOOH) similar to the \textit{atf1ΔE} mutant (Figure 7) as previously reported (Missall and Lodge 2005). The \textit{atf1Δ} mutants also exhibited hypersensitivity to H₂O₂, but hyperresistance to diamide (Figure 7). Furthermore, the \textit{atf1Δ} mutant showed increased sensitivity to high temperature (40°) and the toxin methylglyoxal (Figure 7). These stress-response phenotypes of the \textit{atf1Δ} mutant are similar to those of the \textit{hog1Δ} mutant. In contrast, the \textit{atf1Δ} mutant did not show any increased sensitivity to osmotic shock, cell wall/membrane destabilizing agents such as SDS, and genotoxic agents, such as hydroxyurea and methylethamethane sulfonate (Figure 7 and Figure S4). As previously described (Ko et al. 2009), the \textit{hog1Δ} and \textit{skl1Δ} mutants were more sensitive to osmotic shock under carbon-starvation conditions and the presence of hydroxyurea. Furthermore, the \textit{atf1Δ} mutant exhibited rather unique antifungal drug resistance patterns, which were also distinguished from those of the \textit{hog1Δ}, \textit{ras1Δ}, and \textit{cac1Δ} mutants (Figure 7). As previously reported, the \textit{hog1Δ} mutant exhibited hypersensitivity to amphotericin B, but increased resistance to fluconazole and fludioxonil (Figure 7). However, the \textit{atf1Δ} mutant exhibited increased resistance to both amphotericin B and fluconazole, but showed slightly enhanced sensitivity to fludioxonil (Figure 7). Notably, the \textit{atf1Δ} mutant was much more sensitive to flucytosine treatment than the WT strain. Similarly, the \textit{ras1Δ} mutant also exhibited hypersensitivity to flucytosine. In contrast, the \textit{hog1Δ}, but not \textit{cac1Δ} mutant exhibited only slightly increased sensitivity to flucytosine (Figure 7). The stress and antifungal drug response phenotypes of the \textit{atf1ΔE} mutant in the H99E background were generally identical to those of the \textit{atf1Δ} mutants in the WT H99 background, although we have found several phenotypic differences between H99 and H99E strains (Figure 7). Since the H99E strain was more thermosensitive than the H99 strain, thermosensitivity of the \textit{atf1ΔE} strain was not observed (Figure 7).

The \textit{atf1Δ} mutant exhibited increased melanin and capsule production (Figure 8), which is similar to the \textit{hog1Δ} mutant. In capsule production, both \textit{atf1Δ} and \textit{atf1ΔE} mutants exhibited higher levels of capsule than the H99 and H99E strains, respectively (Figure 8A). Similarly, melanin production was more enhanced in

**TABLE 1**

| SeroA ID | C. ne gene name | S. ce gene name | S. po gene name | Fold changes (log₂ scale) | Function | Crypto-ESR |
|---------|----------------|----------------|----------------|--------------------------|----------|------------|
| 00054   | None           | None           | None           | 0.308 -0.397             | DUF1264 domain-containing protein | OxR-up, OxR-dw |
| 00165   | None           | MEU1           | None           | 0.313 -0.613             | Methylthioadenosine phosphorylase |
| 00906   | None           | None           | None           | 0.365 -0.661             | Nucleolar protein |
| 01262   | GPB1           | STE4           | Git5           | 0.333 -0.482             | G-protein beta subunit GPB1 | OxR-dw |
| 02091   | NAPI           | NAPI           | None           | 0.343 -0.59              | Nucleosome assembly protein | OxR-up |
| 02099   | FAS1           | FAS1           | FAS1           | 0.453 -0.67              | Fatty acid synthase beta subunit | OxR-dw, FxR-dw |
| 02741   | None           | OCT1           | None           | 0.399 -0.831             | Mitochondrial intermediate peptidase |
| 02958   | CFO2           | None           | None           | 0.549 -0.286             | Ferrooxidase | OxR-dw |
| 03544   | None           | None           | None           | 0.259 -0.52              | Conserved hypothetical protein | OxR-dw |
| 03644   | CAS3           | None           | None           | -0.461 0.34              | Cap64 homologs |
| 04090   | ATF1           | None           | ATF1           | 0.347 -0.483             | Activating transcription factor 2 |
| 04837   | None           | None           | None           | 0.422 -0.461             | Conserved hypothetical protein |
| 05976   | None           | NOP58          | None           | -0.338 0.262             | SnoRNA binding domain containing protein | OxR-dw, OxR-up |
| 06556   | None           | None           | None           | 0.189 -0.447             | Mitochondrial protein | OxR-dw |
| 06668   | None           | AFI38          | None           | 0.538 -0.284             | Mitochondrial protein | FxR-dw |
| 06906   | None           | None           | None           | 0.154 -0.493             | Hypothetical protein | OxR-dw |
| 07465   | None           | None           | None           | -0.449 0.72              | Conserved hypothetical protein | OxR-dw |

\textit{C. neoformans}; \textit{S. ce}, \textit{Saccharomyces cerevisiae}; \textit{S. po}, \textit{Schizosaccharomyces pombe}; ESR, environmental stress regulated; OxR-up/dw, up- or down-regulated by oxidative stress; OsR-up/dw, up- or down-regulated by osmotic stress; FxR-up/dw, up- or down-regulated by fludioxonil.
To achieve this goal, we constructed the conditional differentiation of regulated by CA, which is essential for growth and significance ($P < 0.001$ and NS, not significant ($P > 0.05$).)

![Figure 5](Image)

**Figure 5.** The role of Cas3 and Can2 in CO$_2$-mediated capsule induction. Capsule synthesis levels of the following *C. neoformans* strains were quantitatively measured by using hematoctrit as described in MATERIALS AND METHODS. The Y-axis indicates the relative capsule volume, which is percent ratio of length of packed cell volume phase vs. length of total loading volume phase. (A) *C. neoformans* strains [the wild-type H99 and *cas1Δ* (YSB42) and *cas3Δ* (YSB667 and YSB668) mutant strains] were grown in DME medium under either ambient air (−CO$_2$) or high CO$_2$ (5%, +CO$_2$) conditions for capsule induction. (B) *C. neoformans* strains [the wild-type H99 and *cas1Δ* (YSB42) and P$_{CTR4}^{Can2}$ (YSB734) mutant strains] were grown in DME medium containing 200 μM BCS under either ambient air (−CO$_2$) or high CO$_2$ (5%, +CO$_2$) conditions for capsule induction. Three independent experiments with triplicates were performed. Statistical analysis was performed by using Bonferroni multiple comparison test. *$P < 0.001$ and NS, not significant ($P > 0.05$)."

both *atf1Δ* and *atf1ΔE* mutants than the H99 and H99E strains, respectively (Figure 8B). Interestingly, the H99E strain was found to be highly defective in melanin production, which is similar to the *cas1Δ* mutant, compared to the H99 strain (Figure 8B). In both Nigher seed and L-DOPA media, the *atf1Δ* mutant exhibited increased melanin production (Figure 8B). Taken together, Atf1 appears to play important roles in response to environmental stress and antifungal drug treatment and production of melanin and capsule in a manner that is shared with and distinct from the HOG pathway in *C. neoformans*.

**DISCUSSION**

In this study, we aimed to elucidate the genes regulated by CA, which is essential for growth and differentiation of *C. neoformans*, on a genome-wide scale. To achieve this goal, we constructed the conditional *CAN2* expression mutant in which the native *CAN2* promoter is replaced with the *CTR4* promoter, which is controlled by the copper levels in the growth medium (Ory et al. 2004). The P$_{CTR4}^{Can2}$ strain exhibited growth defects when *CAN2* expression is repressed by addition of CuSO$_4$, but grew normally when the *CAN2* gene is overexpressed by addition of high CO$_2$. As an additional benefit from this study, we could monitor *CAN2* overexpression phenotypes in media containing BCS because *CAN2* expression levels induced by the *CTR4* promoter were much higher than the native *CAN2* expression levels.

Through the comparative transcriptome analysis by DNA microarray with the P$_{CTR4}^{Can2}$ strain, we found several Can2-dependent genes in *C. neoformans*, which have not been reported before. To the best of our knowledge, this is the first genome-wide transcriptome analysis to discover the target genes directly or indirectly regulated by carbonic anhydrase in the CO$_2$-sensing and metabolic pathway. The majority of Can2-dependent genes appeared to be uniquely found in *C. neoformans*. However, the list of Can2-dependent genes that have predicted functions based on sequence homology, including *FUR1*, *URI1*, *PRX1*, *FAS1*, *GBP1*, *CAS3*, *CFO1*, *ATF1*, *MEU1*, *NAP1*, *OCT1*, *NOP58*, and *AIM38*, provided insight into the downstream signaling network modulated by CO$_2$ and CA in *C. neoformans*.

The *FAS1* (fatty acid synthase 1) gene was upregulated when *CAN2* is repressed, but downregulated when *CAN2* is overexpressed. This result may explain how fatty acid biosynthesis is regulated by CA. Previously we have shown that the growth defect of the *can1Δ* mutant can be partially restored to normal by exogenous addition of fatty acid, such as palmitate and myristate, indicating the role of Can2 in fatty acid biosynthesis (Bahn et al. 2005). Therefore expression of the FAS1 gene may be controlled as a compensatory mechanism for different CA activity and intracellular bicarbonate levels. Both Fas1 and Fas2 are known to be required for fatty acid biosynthesis and essential for growth of *C. neoformans* (Chayakulkeeree et al. 2007).

Differential expression of *GBP1*, a Gβ-subunit of the pheromone-responsive Cpk1 MAPK pathway, also helps us to understand the role of Can2 in sexual differentiation of *C. neoformans*. Can2 is involved in both initial and terminal stages of sexual cycles of *C. neoformans* in negative and positive manners, respectively (Bahn et al. 2005). Our array data showing that basal expression levels of *GBP1* is induced when Can2 is repressed indicate that the Gpb1-mediated Cpk1 MAPK pathway can be controlled by Can2.

In *C. neoformans*, high CO$_2$ enhances capsule production (Granger et al. 1985; Vartivarian et al. 1993). Originally we hypothesized that Can2-mediated CAs3 induction may be responsible for enhancement of capsule production by high CO$_2$ concentration. Cas3 has been identified as one of a seven-member family of
Capsular structure designer proteins, although it is not directly involved in capsule formation but instead required for O-acetylation of the capsule in *C. neoformans* (Moynard et al. 2004). Our data showed that Cas3 is not required for CO₂-mediated capsule induction. Mogensen and co-workers demonstrated that bicarbonate directly activates Cac1 adenylyl cyclase *in vitro*, which is the key component of the cAMP-signaling pathway, which plays a major role in capsule biosynthesis (Mogensen et al. 2006). Supporting this, we also found that CO₂-mediated capsule induction requires functional adenylyl cyclase *in vivo*. However, we have found that CAN2 overexpression did not enhance capsule production in either ambient air or high CO₂ conditions. Therefore, it still remains elusive whether increased bicarbonate or activated Can2 directly activates adenylyl cyclase for capsule induction *in vivo*. It is also possible that unnatural CAN2 overexpression by the CTR4 promoter may affect other factors that counteract normal capsule induction.

One of the interesting findings of our array analysis is that a majority of Can2-dependent genes were ESR genes, whose expression is differentially regulated in response to environmental stresses, such as osmotic shock and oxidative stresses (Ko et al. 2009). The reason for regulation of the ESR genes by the CAN2 gene is not clear. Differential expression of the CAN2 gene will change intracellular levels of bicarbonate and proton, which affect intracellular pH and osmotic balance. These intracellular physiological changes can be sensed as stress, which may trigger cells to induce or repress ESR genes to counterbalance the stress. Supporting this, the CAN2 overexpression strain was hypersensitive to oxidative stress (H₂O₂) and genotoxic stress (MMS).

Related to this finding, the transcription factor Atf1 was found to be differentially regulated by Can2. Atf1 is orthologous to *S. pombe* Atf1, which is a key downstream transcription factor of the stress-activated Sty1 MAPK (Shiozaki and Russell 1996; Wilkinson et al. 1996; Degols and Russell 1997). Although Missall and Lodge previously reported that Atf1 is involved in regulation of the thioredoxin system during oxidative stress response, here we further characterized the role of Atf1 in diverse stress response by using independently constructed *atf1*Δ mutants in the WT H99 strain background. The *atf1*Δ mutants that we generated generally exhibited identical phenotypes compared to the *atf1*ΔE mutants that Missall and Lodge generated in the H99E strain background. Minor phenotypic differences appear to result from the strain background difference. At this point, it is not clear whether Atf1 is the transcription factor working downstream of the HOG pathway. The *atf1*Δ mutant shared some of stress-response phenotypes with the *hog1*Δ mutant and yet exhibited phenotypes distinct from those of the *hog1*Δ mutant, including genotoxic stress and membrane-destabilizing response and antifungal drug resistance. It is possible that Atf1 may be responsible for a subset of HOG-dependent phenotypes. Whether Atf1

![Figure 6](image-url)
is a direct downstream effector for the Hog1 MAPK needs to be further addressed in future study.

Recently, Sheth and co-workers monitored global transcriptional response of *C. albicans* under 5.5% CO₂, which mimics host physiological conditions. Interestingly, among the 14 genes whose expressions are significantly changed by high CO₂, three of them (HSP12, AAH1, and OPT1) are known to be regulated by the HOG pathway (Sheth et al. 2008), indicating that CO₂ sensing and metabolism could also be related to the stress-response signaling pathway in *C. albicans*. Particularly, transcript abundance of HSP12, which encodes a small heat-shock protein involved in protein folding under stress conditions, are lowered by 5.5% CO₂ in an adenylyl cyclase/cAMP-dependent manner, suggesting that *C. albicans* considers ambient air having 0.036% CO₂ as stress. Supporting this finding, our recent transcriptome analysis of the cAMP-signaling pathway in *C. neoformans* demonstrated that two HSP12 orthologous genes, HSP12 and HSP122, are both...
regulated by cAMP and HOG pathways in the pathogen (Maeng et al., 2010).

The last notable finding of this study is a potential of Can2 and Atf1 as targets for combination antifungal therapy along with commercially known antifungal drugs. First, the CAN2 overexpression strains were extremely sensitive to amphotericin B (AmpB) andazole drugs. Therefore, a molecule(s) that is able to induce activity of Can2 or expression of CAN2 could be used for treatment of cryptococcosis along with AmpB and azole drugs. The synergistic mechanism between CAN2 overexpression and AmpB or azole drugs is not clear at this point. It is possible that decreased FAS1 expression in the CAN2 overexpression strain may affect cell membrane integrity, which results in increased sensitivity to AmpB, which destroys membrane integrity or increased intracellular uptake of the antifungal drugs. Another benefit for the Can2-activating molecule could be decreased stress sensitivity of C. neoformans, which may result in rapid clearance during host infection. Second, the atf1Δ mutant was much more sensitive to fluconazole than the wild-type strain. Interestingly, the ras1Δ mutant was also found to be hypersensitive to fluconazole. These data indicate that an inhibitor of Atf1 or Ras1 in combination with fluconazole could be effective for treatment of cryptococcosis. The reason for this synergism is not understood at this point.

In conclusion, our CA-transcriptome analysis not only discovered a number of Can2-dependent genes in C. neoformans, but also made the finding that perturbed CO2/bicarbonate homeostasis may cause impaired cellular response to diverse environmental stresses. Therefore, modulation of the signaling network of the CA/CO2-sensing pathway may provide a novel therapeutic method for treatment of pathogenic fungi.

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Comparative Transcriptome Analysis of the CO₂ Sensing Pathway Via Differential Expression of Carbonic Anhydrase in *Cryptococcus neoformans*

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**FIGURE S1.**—Deletion of the CAS3 gene. (A) The overlap PCR transformation strategy for disruption of the CAS3 gene. Primers for overlap PCR and diagnostic PCR are indicated as bent arrows. (D) Southern hybridization was performed to verify positive cas3Δ mutants.
Figure S2.—Transcript levels of $ATF1$ by overexpression or suppression of $CAN2$ expression. Northern blot (top panel) and quantitative RT-PCR (qRT-PCR, bottom panel) show transcript levels of $ATF1$ in the wild-type and the $P_{CTR4}$::$CAN2$ strains grown in YNB+BCS medium (left panel) or the $P_{CTR4}$::$CAN2$ grown in YNB+BCS and YNB+CuSO$_4$ medium. For qRT-PCR, data obtained from three independent biological replicates with three technical replicates were normalized by using $ACT1$ as a control. Relative gene expression indicates normalized $ATF1$ expression levels to those of the wild-type strain (left panel) or the $P_{CTR4}$::$CAN2$ strain in YNB+BCS medium (right panel).
FIGURE S3.—Deletion of the *ATF1* gene. (A) The *NAT* split-marker transformation strategy for disruption of the *ATF1* gene. Primers for the double-joint PCR and diagnostic PCR are indicated as bent arrows. (B) Southern hybridization was performed to verify positive *atf1Δ* mutants.
FIGURE S4.—The role of Atf1 in osmotic and genotoxic stress response. Each *C. neoformans* strain (the wild-type H99 strain and *hog1Δ* [YSB64], *ssk1Δ* [YSB261], *skn7Δ* [YSB349], *atf1Δ* [YSB676, YSB678, and YSB679] mutant strains) was grown overnight at 30°C in liquid YPD medium, 10-fold serially diluted (1–10⁴ dilutions), and spotted (3 μl of dilution) on YPD agar containing the indicated concentrations of NaCl, KCl, sorbitol, methylmethane sulfonate (MMS), and hydroxyurea (HÚ). Cells were incubated at 30°C for 72 h and photographed.
# TABLE S1

**C. neoformans** strains and primers used in this study

| Strain       | Genotype | Parent | Reference                      |
|--------------|----------|--------|--------------------------------|
| H99          | MATα     |        | (Perfect et al., 1993)         |
| H99-Eunich   | MATα     | H99    |                                |
| BE7-151      | MATα atf1Δ::HYG | H99-Eunich |                        |
| YSB64        | MATα hog1Δ::NAT-STM#177 | H99 | (Bahn et al., 2005)         |
| YSB261       | MATα ssk1Δ::NAT-STM#205 | H99 | (Bahn et al., 2006)         |
| YSB349       | MATα skn7Δ::NAT-STM#201 | H99 | (Bahn et al., 2006)         |
| YSB733       | MATα PCTR::CAN2 | H99 | This study                    |
| YSB734       | MATα PCTR::CAN2 | H99 | This study                    |
| YSB735       | MATα PCTR::CAN2 | H99 | This study                    |
| YSB667       | MATα cas3Δ::NAT-STM#122 | H99 | This study                    |
| YSB668       | MATα cas3Δ::NAT-STM#122 | H99 | This study                    |
| YSB669       | MATα cas3Δ::NAT-STM#122 | H99 | This study                    |
| YSB676       | MATα atf1Δ::NAT-STM#220 | H99 | This study                    |
| YSB678       | MATα atf1Δ::NAT-STM#220 | H99 | This study                    |
| YSB679       | MATα atf1Δ::NAT-STM#220 | H99 | This study                    |

| Primer Name | Sequence                         | Comment                        |
|-------------|----------------------------------|--------------------------------|
| B79         | TGTGGATGCTGGCCGGAGGATA           | ACT1 promoter screening oligo  |
| B93         | CTATGCTGTAATGACTGAGCC            | CAN2 CR2                       |
| B354        | GCATGCAGGATTCGAGTG               | NAT/CTR Left                   |
| B355        | GATTGGTGAAAGTCGTGTTGTCG          | NAT/CTR Right                  |
| B356        | CATTTCCGTTGCCATAAGTG             | CAN2 CSo1                      |
| B357        | CACCTTCTTGATTGTAGGGG             | CAN2 CL1                       |
| B358        | CACCTCAGACTTCACCAATGCTGCCCTTTCCACGCTGAACC | CAN2 CL2                     |
| B1026       | GTAAAACGAGCCAGTGACC              | M13F extended                  |
| B1027       | CAGGAAACAGCTATGACCATG            | M13R extended                  |
| B1243       | GAGTCAGGAGTTGAGTTGGG             | ATF1-L1 for the left flanking region |
| B1244       | GCTGACTGCGCGGTGTTTTATTTCAATGATGAGGC | ATF1 L2 for the left flanking region |
| B1245       | CATGGTCAATAGCTGTTCTGTTGGG       | ATF1 R1 for the right flanking region |
| B1246       | GAAAGAAGAAGAATACACTTGGTGC       | ATF1 R2 for the right flanking region |
| B1247       | GTCTTTTTGCTCCTTGAAACC           | ATF1 screening oligo paired with B79 |
| B1377       | ATTAGGTCAGGCAGACCC              | ATF1 southern probe paired with B1780 |
| NAT-STM# | Sequence                              | Description                      |
|---------|---------------------------------------|----------------------------------|
| B1454   | AAGGTGTCCCGACGACGAATCG                | NAT Split marker Right (NSR)     |
| B1455   | AACTCCGTCGGAGCCCCCATCAAC              | NAT Split Marker Left (NSL)      |
| B1780   | ACCAGGTGTAAACCCATCC                  | ATF1 southern probe paired with  |
|         |                                       | B1377                            |
| B1817   | AAAACCTGGGCGGAACACAG                 | CAS3 screening oligo paired with |
|         |                                       | B79                              |
| B1818   | CGTCGGACATTCAATCTAC                  | CAS3 L1 for the left flanking region |
| B1819   | GCTGACTGGCCGTGTTTACATTGGCAGGAAGAGATATG | CAS3 L2 for the left flanking region |
| B1820   | CATGGTCACTAGCTGGCTTCCTGGCATAGTGCTGACTTACAG | CAS3 R1 for the right flanking region |
| B1821   | ATGGTCCCACGTGACTATCC                 | CAS3 R2 for the right flanking region |
| B1829   | CCGAAGCCTTTGACTAC                    | CAS3 southern probe oligo        |

Each NAT-STM# indicates the Nat' marker with a unique signature tag.
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TABLE S2

Microarray data generated by this study

Table S2 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.118315/DC1.
TABLE S3

Genes regulated by differential expression of CAN2 in the CAN2 promoter replacement strain growing in either BCS or Copper-containing medium

Table S3 is available for download as a Word (.doc) file at http://www.genetics.org/cgi/content/full/genetics.110.118315/DC1.
TABLE S4

Genes regulated by differential expression of CAN2 between the wild-type strain and the CAN2 promoter replacement strain growing in BCS-containing medium

Table S4 is available for download as a Word (.doc) file at http://www.genetics.org/cgi/content/full/genetics.110.118315/DC1.
**TABLE S5**

*List of Can2-dependent genes in C. neoformans*

Table S5 is available for download as a Word (.doc) file at http://www.genetics.org/cgi/content/full/genetics.110.118315/DC1.

| Gene Name | Description |
|-----------|-------------|
| Gene1     | Description1 |
| Gene2     | Description2 |
| Gene3     | Description3 |

*Note: The table is not included in the image, but the description is provided.*