Carbon Dioxide is a Powerful Inducer of Monokaryotic Hyphae and Spore Development in *Cryptococcus gattii* and Carbonic Anhydrase Activity is Dispensable in This Dimorphic Transition

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Abstract

*Cryptococcus gattii* is unique among human pathogenic fungi with specialized ecological niche on trees. Since leaves concentrate CO₂, we investigated the role of this gaseous molecule in *C. gattii* biology and virulence. We focused on the genetic analyses of β-carbonic anhydrase (β-CA) encoded by *C. gattii* CAN1 and CAN2 as later is critical for CO₂ sensing in a closely related pathogen *C. neoformans*. High CO₂ conditions induced robust development of monokaryotic hyphae and spores in *C. gattii*. Conversely, high CO₂ completely repressed hyphae development in sexual mating. Both CAN1 and CAN2 were dispensable for CO₂ induced morphogenetic transitions. However, *C. gattii* CAN2 was essential for growth in ambient air similar to its reported role in *C. neoformans*. Both can1 and can2 mutants retained full pathogenic potential *in vitro* and *in vivo*. These results provide insight into *C. gattii* adaptation for arboreal growth and production of infectious propagules by β-CA independent mechanism(s).

Introduction

*Cryptococcus gattii*, a basidiomycetous yeast, is an emerging pathogen in North America causing fatal disease in both healthy and immunocompromised humans as well in a wide range of animals including birds, domestic and wild mammals [¹,²]. A large outbreak of *C. gattii* infection among humans and animals in
Vancouver Island, British Columbia, Canada, and the isolation of *C. gattii* from several genera of trees other than *Eucalyptus*, have indicated that this fungus must have broader geographic distribution including Pacific Northwest in the United States, and around the world [1, 3–8]. In extensive ecological investigations, *C. gattii* was isolated readily from soil, air, and water surrounding trees, in regions in the vicinity of Vancouver Island; evidently, *C. gattii* dispersal in the environment has been occurring through distribution of tree byproducts, aerosolization, water flow, and arthropogenic factors [9, 10].

Given the numerous possibilities for *C. gattii* dispersal, the organism’s de novo colonization mechanisms on trees and regions surrounding these trees are far from clear. Xue *et al* [11] have demonstrated that the young *Arabidopsis thaliana* plant surfaces represent a permissible environment, in which *C. gattii* and its closely related species *C. neoformans* can complete their sexual cycle (α-α mating). This intriguing finding raised the possibility that plants might serve as a critical host in the production of infectious propagules in the form of sexual spores (basidiospores). However, the predominance of α mating type both clinically and environmentally indicated that sexual mating in nature might be a limited and rare event. A number of studies raised the possibility that monokaryotic fruiting (α-α mating or same sex mating) might be a widespread phenomenon in *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and *C. gattii* [12–14]. Studies examining strains from outbreak investigations on Vancouver Island have found diploid isolates of α mating type with heterozygous alleles at their α-mating locus suggesting that the hypervirulent *C. gattii* VGII outbreak strains arose as a result of α-α mating [15]. Interestingly, the fruiting body (basidium) containing basidiospores as a result of α-α mating were not observed in *C. gattii* in the laboratory setting [16]. Therefore, it is possible that monokaryotic fruiting results from mating-dependent and mating-independent developmental pathways. A recent study from *C. neoformans* var. *neoformans* found cell cycle arrest induced mating-independent monokaryotic fruiting[17].

*C. gattii* is unique among human pathogenic fungi in its ecological niche; it predominantly inhabits trees by mechanisms not yet clearly understood. Since plants concentrate CO2 through the action of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCo), it is conceivable that *C. gattii* is sensing CO2 for its survival and propagation in the environment [18]. A number of reports provide insight into how pathogenic fungi sense environmental CO2 via carbonic anhydrase (CA) and fungal adenyl cyclase [19–22]. CO2 diffusion into or out of the cells is facilitated by its conversion to bicarbonate ions (HCO3−), which are utilized for several cellular processes in the cell. CO2-HCO3− inter-conversion is catalyzed by CAs, which are zinc metalloenzymes and are grouped into five evolutionarily unrelated families, α, β, γ, δ, and ε-CA [23–25]. Of these, β-CA is unique to fungi and reported to be essential for fungal growth in ambient air (CO2 ~ 0.036%) but not in a high CO2 (5%) environment [19–22].

In the present study, we focused on the genetic analyses of β-carboxic anhydrase (β-CA) encoded by *C. gattii* CAN1 and CAN2 as later is critical for CO2 sensing in a closely related pathogen *C. neoformans*. Our results provide insight
into \textit{C. gattii} adaptation for arboreal growth and the production of infectious propagules by β-CA independent mechanism(s).

**Methods**

**Strains and media**

The \textit{C. gattii} strains used in this study are listed in Table 1. These strains were routinely maintained on yeast extract peptone dextrose agar (YPD) slants, and were stored in 15% glycerol at \(-70^\circ\text{C}\). YPD containing nourseothricin (100 μg/ml) or hygromycin B (200 μg/ml) was used to screen \textit{can1}, and \textit{can2} single mutant and \textit{can1can2} double mutant strains [26]. The preparation of the various media - V8 medium for sexual (α-α) mating, filament agar for monokaryotic fruiting, Niger seed agar for melanin production, urea agar for urease production, and agar based Dulbecco’s modified Eagle’s (DME) medium for capsule production were used as described [27]. YPD containing menadione (3 μg/ml), or paraquat (1 mM) was used for oxidative stress, NaCl (1.4 M, and 1.8 M) for osmotic stress, and NaNO\textsubscript{2} (1 mM) for nitrosative stress were prepared as described [26]. Yeast nitrogen base (YNB) broth containing various sugars was prepared as described [28]. For determination of amino acid requirements, synthetic dextrose (SD) medium containing 0.17% YNB and 1% glucose was supplemented with adenine (20 mg/l), uracil (30 mg/l), L-arginine (20 mg/l), leucine (60 mg/l), histidine (20 mg/l), tryptophane (30 mg/l). For determination of fatty acid requirements, YPD agar supplemented with palmitate (1–10 mM) or myristate (1–10 mM) and with 1% Tween 80 as surfactant was prepared as described previously [20].

**Plasmids and oligonucleotides**

Plasmids and oligonucleotides used in this study are listed in Table 2. The full-length \textit{CAN1} and \textit{CAN2} gene sequences from \textit{C. neoformans} were BLAST searched in the NCBI database for \textit{C. gattii} (R265) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), which yielded R265 cont1.355, and R265 cont1.479, for \textit{CAN1} and \textit{CAN2}, respectively. Primers were designed to amplify approximately 1500-bp fragments of the \textit{CAN1} and \textit{CAN2} genes from genomic DNA of NIH 444 strain of \textit{C. gattii}. The nucleotide sequences for the \textit{CAN1} and \textit{CAN2} genes from NIH 444 have been submitted to the GenBank database (\textit{CAN1} = EU723699; \textit{CAN2} = EU723700). The \textit{C. neoformans} cDNA sequences from \textit{CAN1} and \textit{CAN2} were aligned with the \textit{C. gattii} \textit{CAN1} and \textit{CAN2} genomic sequences, using the GAP function of the GCG Wisconsin package to obtain exon/intron boundaries. cDNA sequences for \textit{C. gattii} retrieved through this analysis were used in multiple alignments for comparison with \textit{C. neoformans}. 
Disruption of *C. gattii* **CAN1** and **CAN2** genes

Gene disruption was carried out as described previously [26, 29]. Disruption cassettes for **CAN1** and **CAN2** were constructed by PCR fusion [30]. In brief,

### Table 1. *Cryptococcus gattii* strains used in this study.

| Strain        | Genotype | Source                                      |
|---------------|----------|---------------------------------------------|
| NIH 444 (ATCC 32609) | Wild type MATa (serotype B) | American Type Culture Collection (ATCC), Manassas, VA |
| NIH 198       | Wild type MATa (serotype B) | Kwon-Chung K.J. (NIH, Bethesda, Maryland) |
| can1-1        | MATa wild type can1::NAT      | This study |
| can1-2        | MATa wild type can1::NAT      | This study |
| can2          | MATa wild type can2::NAT      | This study |
| can2 + CAN2   | MATa wild type CAN2           | This study |
| can1can2-1    | MATa wild type can1::NAT;can2::HYG | This study |
| can1can2-2    | MATa wild type can1::NAT;can2::HYG | This study |

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### Table 2. Plasmids and oligonucleotides used in this study.

| Plasmids | Description                          | Source                                      |
|----------|--------------------------------------|---------------------------------------------|
| pCH333   | ACT1::NAT::TRP1 cloned into pCR2.1   | Heitman J. (Duke University)                |
| pJAF15   | ACT1::HYG::TRP1 cloned into pCR2.1   | Heitman J. (Duke University)                |

| Oligonucleotides | Sequences                               | Purpose                         |
|------------------|-----------------------------------------|---------------------------------|
| V1442            | 5'-ATCTGCAGAATTCGCCCTTA-3'              | 1.7-kb NAT amplification        |
| V1460            | 5'-GAATTCGCCCTTGAGAGAT-3'              | 1.7-kb NAT amplification        |
| V1467            | 5'-CAGTCGTGGTGCTTGATTGTC-3'            | CAN2 ORF                        |
| V1470            | 5'-ACCCAGTCCTTGATCACGTC-3'             | CAN2 ORF                        |
| V1496            | 5'-ACGCGGCTGATATAAACC-3'               | Diagnostic primer for can2 single and can1can2 double mutants |
| V1497            | 5'-CCTCAAGGACCACCACCTCAT-3'            | Diagnostic primers for can2 single and can1can2 double mutants |
| V1511            | 5'-CCACGGAGGCTCATCTTCAT-3'             | CAN2 upstream region            |
| V1650            | 5'-TCCCGCAGCCTAAGGGGGAATTCCTGCTGAGGTTTCTCAGCCCTCT-3' | CAN2 upstream region |
| V1514            | 5'-GCCACAGCTCAACTCTCTAACA-3'           | CAN2 downstream region          |
| V1651            | 5'-CTCTGTTTCTCATCTTCCTCAAGGGTGAATTCCAGCTCATCATCAGGTTGGAACG-3' | CAN2 downstream region |
| V1515            | 5'-GGTTGATTGTTGAGTTGATAGA-3'           | CAN1 upstream region            |
| V1516            | 5'-TCCCGCAGCCTAAGGGGGAATTCTCAGATGAGGCGGTTGAGGGCTTTGCTGAGG-3' | CAN1 upstream region |
| V1517            | 5'-CTCTGTTTCTCATCTTCTCAGGGGGAATTCTCAGATGAGGCGGTTGAGGGCTTTGCTGAGG-3' | CAN1 downstream region |
| V1518            | 5'-CAACCTGAAGCAGCTTACAGGCTGAGGCTGTAGA-3' | CAN1 downstream region |
| V1609            | 5'-AAACTCAGCTTACAGGGCGGTTGAGGGCTG-3'   | Diagnostic primers for can1 mutant screen |
| V1610            | 5'-CTCTGTTTCTCATCTTCATCAGGCTGAGGCGGTTGAGGGCTTTGCTGAGG-3' | Diagnostic primers for can1 mutant screen |
| V1685            | 5'-GGTTTATCTGTATATACACGCGG-3'          | 1.4-kb HYG amplification        |
| V1686            | 5'-GCTCGAGAGGATGTTGAGGCTG-3'           | 1.4-kb HYG amplification        |

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upstream and downstream regions flanking the \textit{CAN1} and \textit{CAN2} genes (approximately 1 kb on either side) and the full-length \textit{NAT} marker gene from pCH333 plasmid were PCR-amplified. PCR amplicons were gel-purified, added in a molar ratio of 1:3:1, as 5’-flanking (\textit{CAN1} or \textit{CAN2}):marker (\textit{NAT}):3’-flanking (\textit{CAN1} or \textit{CAN2}) amplicons, followed by reaction at 94°C for 2 min, and 15 cycles at 94°C for 30 s and 58°C for 10 min to allow fusion to occur. The fusion product was used as template in conventional PCR, to obtain \textit{can1}::\textit{NAT} and \textit{can2}::\textit{NAT} alleles. The constructs were directly used to transform \textit{C. gattii} NIH 444 wild type (WT) strain by biolistic delivery, and transformants were selected on YPD containing nourseothricin. The potential \textit{can1} and \textit{can2} mutants were screened by diagnostic PCR using primer pair V1609/V1610 designed from the \textit{CAN1} flanking \textit{NAT} gene and V1496/1497 from the \textit{CAN2} flanking \textit{NAT} gene. The \textit{can1} and \textit{can2} mutants were further confirmed for gene deletion and single integration events by reverse transcriptase (RT)-PCR and Southern blot analyses, respectively. The \textit{can1can2} double knockout mutants were created by disruption of the \textit{CAN2} gene in the \textit{can1} mutant using the \textit{can2}::\textit{HYG} allele, followed by diagnostic PCR and Southern blot analyses, as described for the \textit{can2} single mutant. Two of these clones termed \textit{can1can2-1}, and \textit{can1can2-2} were used for further studies.

For construction of the \textit{CAN2} reconstituted strain, a 2.9-kb fragment containing full-length \textit{CAN2} ORF was PCR-amplified from genomic DNA of \textit{C. gattii} WT strain using primers V1467/V1470. The PCR fragment was cloned into pCR2.1-TOPO (Invitrogen) to yield pCR2.1-\textit{CAN2}, and then sequenced for confirmation. The plasmid was digested with EcoRI, and the \textit{CAN2} full-length fragment was biolistically transformed into the \textit{can2} mutant, and transformants were selected on YPD medium in ambient air. Since the \textit{can2} mutant did not grow in ambient air, clones recovered under these conditions were potential \textit{CAN2} reconstituted strains. These transformants were patched on YPD-nourseothricin plates. Inability to grow on this medium was considered as an indication of \textit{can2}+\textit{CAN2} reconstituted strains with \textit{CAN2} integration in the native locus, resulting in the removal of the \textit{can2}::\textit{NAT} allele. These reconstituted strains were further confirmed for single \textit{CAN2} homologous integration event by Southern blot. One of these clones termed as \textit{can2}+\textit{CAN2} was used for further investigations.

\textbf{Analysis of nutritional requirements of \textit{can2} mutant}

Cultures grown overnight in YPD broth at 30°C with 5% CO$_2$ were washed with sterile water, inoculated at OD$_{600} = 0.1$ in YNB containing various sugars, and incubated in ambient air with shaking for 1 week. To determine amino acid and fatty acid requirements, 5μl of serial dilutions of yeast suspension from original stock of 10$^7$/ml were spotted on an appropriate medium supplemented with various amino acids or fatty acids. Cultures were incubated for 2-5 days at 30°C in ambient air (0.036% CO$_2$) or in 5% CO$_2$.
Mating assays

V8 medium, buffered either with 100 mM MOPS for pH 7.0 or with sodium citrate for pH 5.0 and filament agar (pH 5.0) was used for mating and monokaryotic fruiting assays [20]. Cultures grown overnight in YPD broth at 30°C with high CO₂ (5%) were washed twice with sterile distilled water, and were re-suspended in water at a concentration of 5 × 10⁷ cells/ml. An equal number of cells of the opposite mating type cells was mixed, and 5μl of the mixture inoculated on buffered V8 medium, and incubated at 30°C with or without CO₂ for up to 8 weeks. For monokaryotic fruiting, 5-10μl of individually washed cells (5 × 10⁷/ml) were inoculated on filament agar and buffered V8 agar media, and incubated with or without CO₂ for 8 weeks. Images of hyphal growth were captured with an Olympus AX70 microscope equipped with a digital camera as described previously [27].

Assays for virulence factor expression and stress sensitivity

The C. gattii WT, can1, can2 single mutants, can1can2 double mutant, and can2+CAN2 reconstituted strains were incubated for 16–18 hours in YPD broth at 30°C with 5% CO₂. Cells were washed with sterile distilled water, counted, and adjusted to 10⁸/ml. Five microliters of yeast suspension were spotted on DME agar, on Christensen’s agar, and on egg yolk agar and incubated for 24–72 hours at 30°C with 5% CO₂ for respective assessments of capsule, urease, and phospholipase production. For determination of stress sensitivity, yeast cells grown as described above were serially diluted (10³–10⁷), and spotted on YPD medium containing redox cycling agents menadione (3μg/ml), paraquat (1μM), sodium nitrite (0–10 mM), and sodium chloride (1–1.8 M), and incubated at 30°C with 5% CO₂.

Virulence assays

The pathogenic potentials of the C. gattii WT, can1, can2 single mutants, can1can2 double mutant, and can2+CAN2-reconstituted strains were assessed in a mouse model of pulmonary and systemic cryptococcosis [26, 29]. BALB/c mice (6–8 weeks) were procured from Charles River Laboratories, Inc., and procedures for safe and pain-free handling of animals were followed as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC), Wadsworth Center, New York State Department of Health, Albany, NY, USA. Cultures grown overnight in YPD broth at 30°C with 5% CO₂ were washed, and then re-suspended in sterile phosphate buffered saline (PBS), pH 7.4, at a concentration of 1 × 10⁷/ml. Group of five mice were injected intravenously with 10⁶ CFU of each strain. The animals were given food and water ad libitum, and were observed twice daily for any sign of distress. Mice that appeared moribund or in pain were sacrificed using CO₂ inhalation and cervical dislocation as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC), Wadsworth Center, New York State Department of Health, Albany, NY, USA.
Survival data were analyzed by Kaplan-Meyer survival curve using the SAS software (SAS Institute Inc., Cary, NC, USA).

To determine the pathogenic potential of test strains in pulmonary infection, we inoculated a group of three mice with 10^5 CFU of each strain in a volume of 30μl via nasal inhalation as previously described [26]. Animal care procedures were as per approved IACUC protocol. Animals were sacrificed after 14 days of infection, lungs and brains were removed aseptically, homogenized, serially diluted, and plated on YPD agar, and incubated at 30℃ with 5% CO₂ for CFU enumeration.

For histopathology, the left lung lobe was dissected and immersion-fixed in formalin; it was embedded and processed into paraffin blocks, sectioned at 4μm and stained with mucicarmine (Richard-Allan Scientific, Kalamazoo, MI).

**Results**

**CAN2 but not CAN1 is a major β-CA required for C. gattii growth at ambient air**

We identified two CA encoding genes, CAN1 and CAN2 in the C. gattii genome database for related strain R265 (www.broad.mit.edu/annotation/genome/cryptococcus_neoformans_b/Blast.html). The pair-wise comparison revealed 58% and 42% identity at nucleotide and amino acid levels. Both deduced Can1p and Can2p sequences exhibited β-CA signature motif comprising one histidine, two cysteins, and one aspartate residue critical for zinc-binding and enzyme activity. Comparison of deduced amino acid sequences of C. gattii Can1p and Can2p with that of C. neoformans Can1p and Can2p revealed them to be 89% and 97% identical, respectively, indicating that the two genes are highly conserved in C. neoformans and C. gattii [20].

To assess the role of β-CA in C. gattii biology, we created can1, and can2 single knockout mutants and a can1can2 double knockout mutant through homologous integration (see method and Figure S1). The can1 mutant did not exhibit any growth defects in either ambient air (0.036% CO₂) or in a high-CO₂ (5%) environment. The can2 mutant in contrast, exhibited a severe growth defect in ambient air, but not in a high-CO₂ environment. The can1can2 double knockout mutants exhibited a growth phenotype similar to that of can2 single mutant. The severe growth defect of the can2 mutant in ambient air was rescued by re-introduction of wild-type CAN2 allele (Fig. 1a). These results indicated that CAN2 but not CAN1 is essential for C. gattii growth in ambient air. Prolonged incubation of the can2 mutant in ambient air was irreversibly lethal; majority of the cells could not be rescued by a shift to a high-CO₂ environment (Fig. 1b).

**CAN2 is critical for fatty acid biosynthesis but not required for adenyl cyclase (CAC1) gene expression**

We reasoned that the inability of the can2 mutant to grow in air could be due to limiting amounts of bicarbonate, a critical substrate required for the synthesis of
several cellular carboxylases important in metabolism [31]. Bicarbonate is also a critical substrate for CAC1 gene activation, and that in turn leads to the synthesis of cAMP, a ubiquitous second messenger that regulates a large variety of essential physiological processes [21, 22]. Interestingly, addition of exogenous cAMP (2–10 mM) or sodium bicarbonate (1–10 mM) either singly or in combination, failed to complement the growth defect of the can2 mutant in ambient air. Similarly, addition of various cellular metabolites and carbon sources, including citrate, succinate, oxalaacetate, malate, α-ketoglutarate failed to complement the
growth defect of the \textit{can2} mutant (data not shown). In contrast to the report published for \textit{C. neoformans}, the growth defect of the \textit{can2} mutant was barely rescued by addition of exogenous fatty acids, 0.1 mM and 1 mM palmitate (Fig. 2a), indicating that \textit{CAN2} is essential for fatty-acid biosynthetic processes in ambient air in \textit{C. gattii}. We observed a clear zone surrounding the colonies of \textit{C. gattii} WT and \textit{can2+CAN2} reconstituted strains (2 mM and 5 mM palmitate) (Fig. 2a). This might be due to the fact that WT and reconstitute strains were able to utilize fatty acids from media resulting in clear zone surrounding the growth.

To explore the link between \textit{CAN2} and \textit{CAC1}, RNA was extracted from WT, \textit{can2} mutant, and \textit{can2+CAN2} reconstitute strain grown for 3 days in ambient air in the presence of 1mM sodium palmitate or in a high-CO\textsubscript{2} environment. We found that the \textit{can2} mutant remains viable (100%) but do not multiply in the presence of 1 mM sodium palmitate in ambient air for up to 4 days (data not shown). Semi-quantitative RT-PCR revealed that \textit{CAC1} transcript was expressed with or without CO\textsubscript{2} in both \textit{can2} mutant and in the WT strain and also \textit{CAC1} expression appeared to be marginally induced without CO\textsubscript{2}, which was consistent for WT, \textit{can2} mutant and \textit{can2+CAN2} reconstituted strains (Fig. 2b). These results indicated that \textit{CAC1} expression is independent of \textit{CAN2}, in other words, \textit{CAN2} is not required for \textit{CAC1} expression. Also, semi-quantitative RT-PCR analysis of \textit{CAN2} transcript in \textit{C. gattii} WT revealed similar expression pattern in both ambient air or in high CO\textsubscript{2} environment. These results indicated that \textit{CAN2} gene expression is not regulated by CO\textsubscript{2} (Fig. 2c).

\textbf{CO\textsubscript{2} is a powerful inducer of monokaryotic hyphae development in \textit{C. gattii}}

Mating is an important process by which \textit{Cryptococcus} generates filaments and spores that might be important in its ecological fitness. It is clear that \textit{C. gattii} associates with various plant species in nature [1]. However, it is not clear how this fungus survives and propagates on plant substrates. Since most of the plants utilize CO\textsubscript{2} for photosynthesis, and they possess a CO\textsubscript{2} concentration mechanism through RubisCO, an enzyme specifically found in chloroplasts of bundle sheath cells [18], we asked whether high CO\textsubscript{2} induces mating and hyphae development in \textit{C. gattii}. The \textit{C. gattii} WT, \textit{can1} and \textit{can2} single mutants, \textit{can1can2} double mutant, and \textit{can2+CAN2} reconstitute strains were inoculated on filament agar and V8 agar for monokaryotic and sexual mating. The inoculated plates were incubated in ambient air or in high CO\textsubscript{2}. To our surprise, we found that \textit{C. gattii} WT strain undergoes hyphae development as part of monokaryotic fruiting more vigorously in high CO\textsubscript{2} than in ambient air (Fig. 3a). Filaments on the edges of \textit{C. gattii} WT growth appeared as early as 1-week post-incubation under high CO\textsubscript{2}, compared to 4-weeks post-incubation under low CO\textsubscript{2}. The \textit{can2} but not \textit{can1} mutation caused further enhancement of filamentation as judged by long and dense filaments on the colony edges (Fig. 3a; lower panel). Light microscopic mounts of these filamentous projections from the WT as well from the \textit{can2} mutant revealed hyphae and blastospores but not basidiospores. The \textit{can2} mutant
hyphae harbored several blastospores, whereas the WT and can2+CAN2 reconstituted strains harbored few blastospores (Fig. 3b; lower panel). The topology of hyphae harboring blastospores was consistent with our earlier report where these structures were analyzed by scanning electron microscope [27]. The monokaryotic filamentation was also observed on V8 agar at pH 7.0 with or without CO2 but not at pH 5.0. However, filamentation was not as robust as on filament agar (data not shown).

In contrast, high CO2 completely suppressed sexual mating (α-α) in C. gattii WT, can2 mutant, and can2+CAN2 reconstituted strains, as no filamentation on the edges of the colonies was observed even after 8 weeks of incubation on V8 agar medium adjusted to either pH 5 or 7 (Fig. 4a). C. gattii WT and can2+CAN2 reconstituted strains showed robust sexual mating under low CO2 in V8 agar medium adjusted to pH 7.0 (Fig. 4b), but not to pH 5.0 (data not shown). Hyphae cells produced during sexual mating contained two nuclei (single arrow)
that were linked by fused clamp (double arrow) connections (Fig. 4c & 4d). It should be pointed out here that we used only unilateral crossing in which WT MATa strain (NIH 198) was used as the opposite mating partner in the sexual mating assay. Overall, these results indicated that high CO2 is a powerful inducer of monokaryotic hyphae differentiation but not in sexual mating providing important distinction in these two developmental programs in C. gattii.

Figure 3. CO2 is a powerful inducer of monokaryotic hyphae development in C. gattii. C. gattii strains were individually cultured on filament agar and hyphae development was assessed macroscopically at 8 weeks-post incubation. (a) Upper panel- Few filamentous projections (arrow) seen at the edge of the colonies of WT and can2+CAN2 reconstituted strains. No growth of can2 mutant in ambient air (low CO2). Lower panel-Robust filaments (arrow) in the presence of high CO2 with dense and long hyphal extension in can2 mutant. (b) Upper panel - Light microscopic analyses of hyphae development (magnification, × 100) in WT, can2, and can2+CAN2 strains in the presence of high CO2. Lower panel - Filamentous growth on the edge of the colony were carefully removed, mounted on lactic acid cotton blue and gently pressed and photographed (magnification, × 200). Filaments bearing blastospores (arrows) seen in all the strains except that can2 mutant revealed more blastospores compared to the WT and can2+CAN2 reconstituted strains.

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β-CA activity is not required for the expression of *C. gattii* virulence repertoire

Since β-CA activity was found to be essential for *C. gattii* growth in ambient air, we asked whether this enzyme is required for *C. gattii* virulence factor expression, and furthermore, for disease development in mammalian hosts. The *can1* and *can2* single mutants, as well as *can1can2* double mutant strains expressed major virulence factors (melanin, capsule, phospholipase, urease) at levels comparable to those for the WT strain, in a high-CO$_2$ environment. Similarly, mutants did not exhibit any altered sensitivity to oxidative, osmotic or nitrosative stress (Figure 4).
These results indicated that β-CA activity is neither required for general stress response nor for the expression of virulence traits, at least when CO₂ is in abundance. Furthermore, β-CA activity is not essential for C. gattii to induce disease in mammalian host. Mice infected intravenously with the can1 or can2 single mutant, or can1can2 double mutant strains manifested severe disease similarly to mice infected with the WT strain (Fig. 5a & 5b).

Given the importance of gaseous exchange in the lungs, with the high oxygen content in the terminal alveoli, as well the lungs' vigorous defense mechanisms against pathogens, we probed if can2 mutant is able to colonize the lungs as efficiently as the WT strain. The organ load experiment revealed that the fungal burden imposed by the can2 mutant was almost as high as the burden imposed by the WT strain (Fig. 6a). Also, the can2 mutant was able to produce capsule in the lungs as large as those produced by the WT strain (Fig. 6b). Furthermore, histopathological examinations of lungs infected with can2 mutant or the WT strain revealed similar tissue responses, including severe and diffuse interstitial pneumonia, and the presence of numerous organisms in the alveoli and airways (Fig. 6c). Altogether, these results confirmed that CAN2 deletion has no influence on C. gattii virulence traits and pathogenesis, in agreement with previous findings for C. neoformans and Candida albicans [20, 21].

Discussion

The present study revealed that high CO₂ strongly induced monokaryotic hyphae development in C. gattii while it completely repressed sexual (η-α) hyphae development, indicating an important distinction in environmental responses by these two developmental programs. Considering the fact that C. gattii grows on plants known to concentrate CO₂ through RuBisCO [32], the observed association between high CO₂ and morphological transition in C. gattii indicates an ecological adaptation for survival and propagation in nature.

Nitrogen starvation, water deprivation and high temperature have been linked to monokaryotic fruiting in C. neoformans [16, 17]. Also, darkness is an additional factor associated with hyphae production and fruiting structures in C. neoformans [33]. We have now added high CO₂ (5%) to this list as it strongly induced hyphae development in C. gattii; filamentation was discernable as early as 1-week post incubation in high CO₂ compared to its appearance at 4 weeks in a low-CO₂ (ambient air) environment. Recently, CO₂ has also been shown to be powerful inducer of filamentation in C. albicans that requires CAC1 but bypasses Ras [21]. CAC1 activation requires both bicarbonate and G proteins in C. albicans as well as in C. neoformans [21, 22, 34]. Interestingly, we did not find any link between CAN2 and CAC1 as can2 mutant produced equivalent amount of CAC1 transcript as the WT strain. Additionally, CAC1 transcript was induced more in ambient air than in high CO₂ while opposite was true for hyphae development where high CO₂ served as powerful inducer. These results indicate that C. gattii CAC1 may not be directly involved in CO₂-induced monokaryotic hyphae development as
opposed to its critical role assessed in sexual mating [34]. These results support the hypothesis that there are probably different signaling pathways in the development of hyphal projection, a prerequisite for spore formation in monokaryotic fruiting and sexual mating. The search of C. gattii database for a related strain R265 (http://www.broad.mit.edu) revealed single copy of CAC1 gene as reported earlier for C. neoformans [34]. Interestingly, we found that can2
mutant undergoes robust monokaryotic filamentation with blastospore formation indicating that either CAN2 serves as a repressor, or certain threshold levels of CO2−HCO3− interconversion is critical in this developmental pathway.

We also found that CAN2, but not CAN1, was essential for C. gattii growth under ambient air (0.035% CO2). In this regard, C. gattii is similar to its closely related species C. neoformans where CAN2 was major β-CA for growth under ambient air [20–22]. The precise mechanism for observed growth defects of C. gattii can2 mutant in ambient air is not clear at present but defective fatty acid
biosynthesis might be partially responsible, consistent with earlier report for C. neoformans [20]. Since CAN2 was dispensable for survival, proliferation, and lethality during intravenous and intranasal infection, its role in C. gattii pathogenesis appeared to be redundant.

Although very little is known about morphological forms of C. gattii in nature, a hyphal phase appears to be an integral part of C. gattii biology. The recent outbreak of C. gattii on Vancouver Island revealed that the fungus inhabits several tree species (Douglas fir, alder, maple, and Garry oak) [1, 4, 9]. The Vancouver Island air samples contain particles of 1–2 μm in diameter, a size consistent with spores [1]. Also, all of the isolates from this outbreak belonged to MATa mating type, further bearing out the predominant mode of reproduction possibly through monokaryotic fruiting. Additionally, the endemic nature of C. gattii in Australia, majority of Australian isolates being sterile, and their well-known association with Eucalyptus trees strongly suggest that the monokaryotic fruiting might be the driving force for the survival and propagation of C. gattii in nature [35, 36]. Although, mixed populations of MATa and MATα strains of C. gattii have been identified colonizing hollows in Eucalyptus trees in Australia [37–40], no meiotic recombination has been detected in isolates recovered from these hollows; thus monokaryotic fruiting could still be the main mode of propagation of C. gattii in nature.

In summary, we have demonstrated that high CO2 conditions induced robust development of monokaryotic hyphae and spores in C. gattii. Conversely, high CO2 completely repressed hyphae development in sexual mating. Both CAN1 and CAN2 were dispensable for CO2 induced morphogenetic transitions and expression of pathogenic traits. Further investigations are warranted to dissect CO2-mediated signaling pathways to determine relevant sensor(s) required for monokaryotic fruiting.

**Supporting Information**

Figure S1. **Characterization of can1, and can2 single knockout mutants, can1can2 double knockout mutant, and can2+CAN2 reconstituted strains.** (a-b) Diagnostic PCR and Southern hybridization analysis for can1 mutants: (a) Primers (V1609/v1610) designed from the CAN1 flanking NAT gene amplified 1.7-kb PCR product from the genomic DNA of C. gattii WT and 3.0-kb amplicon from the genomic DNA of can1-1 and can1–2 mutants obtained through two independent transformation events. (b) Genomic DNA was digested with Sac I (cuts once within CAN1 gene) and probed with 612-bp PCR product amplified from CAN1 ORF. The C. gattii WT produced 1.4-kb band, while both can1-1 and can1–2 mutants produced 3.3-kb bands. (c-e) Diagnostic PCR, RT-PCR, and Southern hybridization analyses of can2 mutant and can2+CAN2 reconstituted strains. (c) Primers (V1496/V1497) designed from the CAN2 flanking NAT gene amplified 1.4-kb PCR product from the genomic DNA of C. gattii WT and can2+CAN2 reconstituted strains while same primer set produced 2.9-kb PCR.
product from the genomic DNA of can2 mutant. (d) Total RNA was isolated, reverse transcribed to cDNA and amplified with primers (V1600/V1532) directed against CAN2 or primers (V548/V549) directed against SOD1. RT-PCR products were fractionated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. C. gattii WT and can2+CAN2 reconstituted strains yielded 515-bp CAN2 transcript while can2 mutant did not. SOD1 transcript served as a loading control. (e) Genomic DNA from C. gattii WT, can2 mutant, and can2+CAN2 reconstituted strains were cut with Hind III (non-cutter within CAN2 gene), and probed with 372-bp PCR product amplified from the CAN2 gene. The C. gattii WT and can2+CAN2 reconstituted strains produced 3.0-kb band while can2 mutant produced 4.5-kb band. (f-g) Diagnostic PCR and Southern hybridization analyses of can1can2 double knockout strains: For creation of can1can2 double knockout strain, CAN2 gene was disrupted in can1 mutant using can2:HYG allele. (f) Primers (V1496/V1497) yielded 1.4-kb amplicon from the genomic DNA of C. gattii WT as shown in figure C while same primer pair yielded 3.2-kb amplicon from the genomic DNA of can1can2 double knockout strains. (g) Genomic DNA from C. gattii WT, can1can2-1, and can1-can2-2 double knockout mutants were cut with Hind III and probed with CAN2 PCR product. The C. gattii WT produced 3.0-kb band, while both can1can2 double knockout mutants produced 4.9-kb bands.

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Figure S2. β-CA activity is dispensable for virulence factor production and for various stresses in C. gattii. WT and various can mutant strains were grown overnight at 30˚C in 5% CO2, washed, and adjusted to OD600 = 1.0. The 10-fold serial dilutions were prepared and 4μl of each dilution was spotted on YPD alone, YPD containing NaNO2 (nitrosative), NaCl (osmotic), menadione and paraquat (oxidative) and incubated at 30˚C for 72 h. Also assessed were the production of melanin (Niger seed agar), urease (Christensen agar), phospholipase (egg-yolk agar) and capsule (DME agar). Mutant strains neither exhibited any altered sensitivity to stress nor were defective in the production of major virulence factors.

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Author Contributions

Conceived and designed the experiments: SC VC. Performed the experiments: PR SC. Analyzed the data: PR SC VC. Contributed reagents/materials/analysis tools:
PR SC VC. Wrote the paper: SC VC. Acquisition of data and interpretation of data: PR SC VC. Revised the manuscript critically for important intellectual content: SC VC. Final approval of the version to be published: PR SC VC.

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