A capsule-associated gene of *Cryptococcus neoformans*, CAP64, is involved in pH homeostasis

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**Abstract**

The CAP64 gene is known to be involved in capsule formation in the basidiomycete yeast *Cryptococcus neoformans*. A null mutant of CAP64, Δcap64, lacks a capsule around the cell wall and its acidic organelles are not stained with quinacrine. In order to clarify whether the Cap64 protein indeed maintains vacuole or vesicle acidification, so that the vesicle containing the capsule polysaccharide or DBB substrate are transported to the cell membrane side, the relationship between CAP64 and intracellular transport genes and between CAP64 and enzyme-secretion activity were analysed. Laccase activity was higher in the Δcap64 strain than in the wild-type strain, and the transcriptional levels of SAV1 and VPH1 were also higher in the Δcap64 strain than in the wild-type strain. The intracellular localization of the Cap64 protein was analysed by overexpressing an mCherry-tagged Cap64 and observing its fluorescence. The Cap64 protein was accumulated within cells in a patch-like manner. The quinacrine-stained cells were observed to analyse the acidified cell compartments; quinacrine was found to be accumulated in a patch-like manner, with the patches overlapping the fluorescence of CAP64-mCherry fusion protein. Quinacrine was thus accumulated in a patch-like fashion in the cells, and the mCherry-tagged Cap64 protein position was consistent with the position of quinacrine accumulation in cells. These results suggest that CAP64 might be involved in intracellular acidification and vesicle secretion via exocytosis.

**INTRODUCTION**

Diazonium Blue B (DBB), 3,3′-dimethoxy-[1,1′-biphenyl]–4,4′-bis tetrachlorozincate, has been used to distinguish basidiomycetous yeasts from ascomycetes yeasts [1, 2]. Ascomycetous yeast colonies are not stained by DBB, but basidiomycetous ones are stained to red or purple. However, the molecular targets for DBB staining are still unknown. Identifying the targets would provide clues as to how their ancestors have evolved into the present basidiomycetes or ascomycetes. *Cryptococcus neoformans* is a model basidiomycetous yeast for molecular biological study, and was used to clarify DBB staining mechanisms in this study.

During our study of the mechanism of DBB staining, we found that only the cell edge of the CAP64 mutant (Δcap64) strain was not fluorescent when we performed a DBB staining test using CAP gene-deletion mutants [3]. We also determined that the PMT2 gene coding protein o-mannosyl transferase is necessary for the DBB staining in *C. neoformans* [4]. The PMT2 deletion mutant is negative by the DBB colony staining test but has a thick capsule around the cell wall [4]. These results suggested that the molecular targets for the DBB staining reaction might not be related to the capsule formations, and CAP64 might play an additional role in the DBB staining.

Quinacrine diffuses across membranes and accumulates in intracellular acidic compartments, and it has therefore been used as a marker for yeast vacuoles and investigations into the morphology of yeast vacuoles or vesicles [5–7]. We investigated the characteristics and phenotype of the Δcap64 strain, and found that intracellular quinacrine accumulation was not observed in Δcap64, but was clearly seen in the wild-type strain [3]. This result suggests that CAP64 regulates the acidification of vacuoles or vesicles.

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Acidification of vesicular or vacuolar compartments plays an important role in many intracellular processes, such as protein secretion, glycosylation of proteins in the Golgi, osmotic and pH stability, and autophagic degradation [7–9]. A common vacuolar proton pump has been identified in Golgi-derived and vacuolar membranes in Saccharomyces yeast [9]. This vacuolar (H^+)-ATPase proton pump contains about 13 subunits, including a regulatory subunit that is encoded by the VPH1 gene [10]. In C. neoformans, the VPH1 gene was isolated by screening for mutants defective in laccase activity, and its deletion mutant showed defects in capsule formation and urease expression [8]. On the other hand, it was shown that post-translational modifications of virulence factor expression involving polysaccharides depend on vesicular acidification [11].

The capsule of C. neoformans consists mainly of glucuronoxylomannan (GXM) which comprises more than 90% of the capsule’s polysaccharide mass, and galactoxylomannan (GalXM) [12]. The polysaccharide comprises an α-1,3-linked mannose backbone which is O-acetylated, and substituted with glucuronic acid and xylose residues [13]. GXM synthesis is thought to occur in Golgi-related structures. GXM traffic to the cell surface involves transport by secretion of polysaccharide-containing vesicles, which cross the cell wall, releasing their contents into the extracellular space. The released polysaccharide is then connected to the cell wall or incorporated into the growing capsule [14].

In Saccharomyces yeast cells, one of the small GTPases regulating the exocytosis process is known as Sec4p [15]. In C. neoformans, a SAV1-encoding Sec4p homologue was cloned and this gene disruption resulted in a decrease in acid phosphatase secretion, and an accumulation of post-Golgi exocytotic vesicles in the cytoplasm [16]. The observation of these vesicles connecting with antibodies against GXM indicated that the GXM transport occurs via secretory vesicles derived from the Golgi apparatus.

In this study, in order to clarify the relation between CAP64 and both VPH1 and SAV1, the expression levels of VPH1 and SAV1 in the Δcap64 cells were investigated and were compared with the levels in wild-type. Then, the Cap64p localization pattern was also compared with the quinacrine staining pattern.

### METHODS

#### Strains and growth conditions

The strains used in this study are summarized in Table 1.

| Strains   | Genotype               | Source                      |
|-----------|------------------------|-----------------------------|
| KN3501a   | MATa                   | Nielsen et al. [33]         |
| KN3501b   | MATa                   | Nielsen et al. [33]         |
| TYCC77    | MATa, cap64::ADE2      | Moyrand et al. [32]         |
| KGU 10016 | MATa, cap64::ADE2, CAP64::Hyg | Imanishi et al. [3]        |
| KGU 10048 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10049 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10050 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10051 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10052 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10053 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10054 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10055 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10056 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10057 | MATa, cap64::ADE2, CAP64::mCherry::Hyg | This study                 |
| KGU 10058 | MATa, cap64::ADE2, mCherry-CAP64::Hyg | This study                 |
| KGU 10059 | MATa, cap64::ADE2, mCherry-CAP64::Hyg | This study                 |
| KGU 10060 | MATa, cap64::ADE2, mCherry-CAP64::Hyg | This study                 |
| KGU 10061 | MATa, cap64::ADE2, mCherry-CAP64::Hyg | This study                 |

Yeast cells were grown in potato dextrose agar (PDA) [24 g l\(^{-1}\) potato dextrose broth (Difco), 15 g l\(^{-1}\) agar] or YPDA (1% yeast extract, 2% polypeptone, 2% glucose, 2% agar) for a couple of days at 25 °C. For the induction of laccase production, asparagine medium [1 g l\(^{-1}\) l-asparagine, 0.5 g l\(^{-1}\) MgSO\(_4\) · 7H\(_2\)O, 3 g l\(^{-1}\) KH\(_2\)PO\(_4\), 1 g l\(^{-1}\) thiamine, 1 g l\(^{-1}\) biotin, 1 mM L-DOPA (Nacalai), pH 5.6] was used [17]. Escherichia coli DH5α was the host strain for the recovery of a plasmid and was grown in LB medium [10 g l\(^{-1}\) polypeptide, 5 g l\(^{-1}\) bacto yeast extract (Difco), 10 g l\(^{-1}\) NaCl, pH 6.8] containing 25 μg ml\(^{-1}\) kanamycin for 12 h at 37 °C at 150 r.p.m.

#### Construction of an mCherry-tagging Cap64 overexpression strain

To study the intracellular localization of Cap64p in C. neoformans cells, a cassette coding for an mCherry-tagged Cap64p, designated the CAP64-mCherry cassette, was driven by the GDP promoter and was introduced into the Δcap64 strain. The cassette was constructed by overlap PCR using the oligos listed in Table 2 by the method described by Davidson et al. [18]. In order to insert an mCherry tag on the C-terminal side of Cap64p, an mCherry DNA fragment was PCR-amplified with the primers mcherry-F and mcherry-R.
using the plasmid pmCherry DNA as a PCR template. The vector containing the CAP64 gene was PCR-amplified with the primers cap64-linker-F2 and cap64-linker-R2 using pKIS615. Those PCR products were combined and cloned using NEBuilder HiFi DNA Assembly Master Mix (New England Bio Labs), and pYI601 was constructed (Fig. 1). Its insert DNA was sequenced and confirmed to encode the designed protein. In order to insert an mCherry tag on

| Primer name                                                                 | Sequence (5′–3′)                                      |
|----------------------------------------------------------------------------|------------------------------------------------------|
| **CAP64 primers for the qRT-PCR analysis**                                 |                                                      |
| CAP64_F                                                                     | CTGATCAAACCGATCTGTCATTTCT                             |
| CAP64_R                                                                     | GATCAGGTCTCAAAAGGATGCTTCT                             |
| **LAC1 primers for the qRT-PCR analysis**                                  |                                                      |
| RT-lac1.F2                                                                  | AACCGTACTCTGCAATTATCACACA                             |
| RT-lac1.r2                                                                  | AACCATTCCATTACCAGGATACCT                              |
| **SAV1 primers for the qRT-PCR analysis**                                  |                                                      |
| SAV1_RT_F                                                                   | ATCTGTAGCTGATGAGAAGGA                                 |
| SAV1_RT_R                                                                   | AATCGCTTTGCTTGTAGACATT                                |
| **VPH primers for the qRT-PCR analysis**                                   |                                                      |
| VPH_RT_F                                                                    | TGCCTCGATAAGTCCATTGAAATT                              |
| VPH_RT_R                                                                    | ATTACATTTCCATTCTTGTGTGCA                              |
| **ACT1 primers for the qRT-PCR analysis**                                  |                                                      |
| RT-ACT1.UP                                                                  | ATGGAAAGAAGAGTCCGCCG                                  |
| RT-ACT1.LP                                                                  | TAGAAGGGAAGACGTCG                                      |
| **mCherry primers for the qRT-PCR analysis**                               |                                                      |
| mcherry_RT_R                                                                | CGAGTTCATCTCAAAAGGTGAAGCT                             |
| mcherry_RT.F                                                                | TACATGAACTGAGGGAGACAAGGA                              |

### Table 2. Primers used for PCR in this study

| Primer name                                                                 | Sequence (5′–3′)                                      |
|----------------------------------------------------------------------------|------------------------------------------------------|
| **Ter- cap64**                                                             | TACTGTAAACCCCATACATAGGTOCCCAAGACTACAGT                 |
| **Link- mcherry-R**                                                        | GGCTTCCTGAGCATCTTGTACAGCTGTCATCGE                     |
| **cap64-mcherry**                                                          | GACGAGCTGTAAGATGCTCGAGAAAGCCAGGT                     |
| **link- mcherry-F**                                                        | TTAGCATAATACAAATGGTGAGCAAGGGGAGGA                    |
| **CAP64-vector**                                                           | AGCTTTTGAGACCTAGTATAGGGGTCTACAGTAGCTG               |
| **mche-vector**                                                            | GACCGTTGCTCACATTGTATATTGTAGCAAGTATA                  |
| **Primers for construction of the cassette coding CAP64 tagged with mCherry at the N-terminus of CAP64 (pYI602)** |                                                      |
| mcherry-F                                                                  | TGTAGCTTTTGGGACCATGAGGAAGCCAGGAG                   |
| mcherry-R                                                                  | CTACGTGTAACCCCATACATTAGGTOCCCAAGACTACAGT              |
| **cap64-linker-F2**                                                        | GACACATAGCCATCGCAGAGGCCAGGT                        |
| **cap64-linker-R2**                                                        | GCACGTGTAAGATGAGGGGTCTACAGTAGCTGA                    |
| pro-cap64                                                                  | TTAGCATAATACAAATGGTGAGGACAAGGGGAGGA                |
| **Primers for amplification of the cassette**                             |                                                      |
| M13 forward primer                                                         | CGCGAGGGTTTCCCCACTACGAC                               |
| M13 reverse primer                                                         | AGCGGATAACATCTTCACACAG                               |
the N-terminal side of Cap64p, a DNA fragment of CAP64 was PCR-amplified with the primers cap64-mcherry and ter-cap64 using gDNA of the wild-type strain as template DNA, and the mCherry DNA fragment was PCR amplified with the primers link-mcherry-F and link-mcherry-R using the plasmid pmCherry DNA as a PCR template. The vector was PCR-amplified with the primers CAP64-vector and mche-vector using pKIS612 (Shimizu, unpublished) DNA digested by the restriction enzyme HindIII. These PCR products were combined and cloned into pKIS612 using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs), and pYI602 was constructed (Fig. 1). Its insert DNA was sequenced and confirmed to encode the designed protein. A plasmid carrying the CAP64-mCherry cassette was used as a template, and the DNA fragment of the cassette was amplified by PCR with the M13 forward primer and M13 reverse primer. The PCR product was purified using an EZ-10 Spin Column PCR Products Purification Kit (Bio Basic, Ontario, Canada) and introduced into cryptococcal cells as described previously [19].

Genomic DNA was isolated from transformants and was confirmed to contain the DNA fragment of the CAP64-mCherry cassette by PCR amplification with the ter-cap64 and link-mcherry-R primers. The PCR product was detected by agarose gel electrophoresis and sequenced. For DNA extraction, yeast cells were harvested from PDA cultures for DNA extraction according to the benzyl chloride method described by Zhu et al., with minor modifications [20]. PCR was performed using the KAPA Taq EXtra HS ReadyMix PCR Kit (Nippon Genetics, Tokyo) according to the manufacturer’s instructions. PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics) according to the manufacturer’s manual and directly sequenced on an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA). Sequences were analysed using GENETYX ver.11 software (Genetyx).

**Fluorescence microscopy**

Quinacrine staining of vacuoles or vesicles was performed by the method described by Corbacho et al. (2012) with modifications [21]. Cells were collected by centrifugation and 5×10⁶ cells were resuspended in 1ml of YPD buffer containing 50mM phosphate (pH 7.6) and 100mM quinacrine dihydrochloride hydrate (Tokyo Chemical Industry Co.). Cells were incubated for 15 min at 25°C, and harvested cells were resuspended in 100µl of 50mM phosphate buffer (pH 7.6) and examined with a Leica optical microscope DM 2500 equipped with a fluorescence source (450/490 nm excitation, 510 nm dichroic mirror, 515 nm-emission). In order to observe the intercellular Cap64p localization, the mCherry-tagged Cap64 overexpression strain was inoculated into YPD medium and cultured for 20h at 25°C, and then the cells were examined with a Leica optical microscope DM 2500 equipped with a fluorescence source (515/560 nm excitation, 580 nm dichroic mirror, 590 nm-emission). Data analyses were performed using ImageJ [22].

For DAPI staining, cells were grown in YPD liquid medium for 20 h and collected by centrifugation. Cells were washed with sterilized distilled water and incubated in 95% ethanol on ice for 1 h. Fixed cells were collected by centrifugation and suspended in PBS. Cells were collected by centrifugation, suspended in PEMS (100 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, 1.2 M sorbitol ) containing 1µg ml⁻¹ DAPI (Merck, Darmstadt, Germany) and incubated at 30°C for 30 min. After the incubation, cells were examined with a Leica optical microscope DM 2500 (Leica Microsystems).
equipped with a fluorescence source (360/40 nm excitation, 400 nm dichroic mirror, 470/40 nm emission).

**Capsule observation**

Yeast strains were grown according to the methods described previously [3]. To observe the capsule, a drop of Indica ink was added to the cell suspension on the slide grass, and the samples were observed using a Leica optical microscope DM 2500 (Leica Microsystems).

**Laccase activity**

Strains were grown for 12 h in YPD at 25 °C with shaking at 150 r.p.m. After cultivation, cells were collected by centrifugation, washed twice in 50 mM PBS (pH 7.0), counted by hemocytometry, and adjusted to achieve an inoculum of 1×10⁷ cells ml⁻¹. Cells were then incubated in asparagine medium for 24 h at 30 °C. After incubation, the supernatants were obtained after removal of the cells via centrifugation. The amount of pigment produced in the supernatants was determined by a spectrophotometer (U-3310; Hitachi, Tokyo) at a 475 nm wavelength. Assays were repeated three times.

**qRT-PCR**

Total RNA extraction was performed using Trizol reagent (Life Technologies, Carlsbad, CA) and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) following the provided instructions. The cDNA was used as a template in the qRT-PCR analysis using THUNDERBIRD qPCR MixSYBR (Toyobo) according to the manufacturer’s recommendations. Step One Plus Real-Time PCR (Thermo Fisher Scientific) was used to detect and quantify the PCR products. The PCR was conducted using the following protocol: incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each set of PCR included a triplicate of each target gene. The data were normalized to the expression levels of the housekeeping gene ACT1 (encoding actin) in each set of PCRs. The sequences of the primers for the qRT-PCR analysis are listed in Table 2.

**RESULTS**

**Deletion of CAP64 inhibits the separation of daughter cells from mother cells**

The CAP64 gene has been shown to be involved in capsule formation in *C. neoformans*, but its deletion mutant was negative for DDB staining, with irregular vacuole morphology [3]. CAP64 thus seems to play an additional role other than capsule formation, and we therefore re-observed the cell morphology in detail. At first, the cell morphology of the Δcap64 strain was compared to that of the wild-type or CAP64 complement strain. Cells were grown in a YPD liquid medium, and the cell morphology of the log phase (cells grown for 6 h) or of the stationary phase (cells grown for 24 h) was observed under a phase contrast microscope. The cell morphology of the Δcap64 strain at log phase revealed that several cells were connected and many daughter cells could not separate from their mother cells, although the cell morphology of the wild-type or CAP64 complement strain showed that only a few cells were connected and the cell division proceeded normally (Fig. 2a). For the purpose of statistical analysis, the cells were classified into four groups: a single cell group (A), a two-cell connected group (B), a three-cell connected group (C), and a group of four or more connected cells (D). The number of cells in each group was counted and the ratio to the total number of cells observed in each culture was expressed as a percentage (b).

![Fig. 2. Cell morphology comparison among the Δcap64 strain, the wild-type strain KN3501α, and the CAP64 complement strain KGU 10016. Cells were grown in YPD medium for 6 or 24 h and observed microscopically (a). Cells were classified into a single cell group (a), a two-cell connected group (b), a three-cell connected group (c), and a group of four or more connected cells (d). The number of cells in each group was counted and the ratio to the total number of cells observed in each culture was expressed as a percentage (b).](image-url)
inhibition is only partially released at the stationary phase. When the wild-type strain was grown in a YPD liquid medium, the amount of \textit{CAP64} mRNA was analysed by qRT-PCR analysis. The amount of \textit{CAP64} transcript at the stationary phase was 0.65-fold the amount in the log phase (Fig. 3a). The results of \textit{CAP64} transcript analysis and the observation of the separation of daughter cells from mother cells indicated that gene deletion of \textit{CAP64} inhibits the separation of daughter cells in log phase. But when growth enters the stationary phase, other factors might be related to cell separation.

**Correlation between \textit{CAP64} and laccase**

The enzyme laccase is well known as one of the virulence factors of \textit{C. neoformans} and is produced and released by the vesicle secretion system [23–25]. If \textit{CAP64} is required for vesicle acidification, it might affect laccase secretion as well. Cells of \textit{Δcap64}, the wild-type, and the \textit{CAP64} complement strains were incubated in asparagine medium to induce laccase production and the amount of pigment from each culture supernatant was determined. Fig. 4a shows the observation of pigmentation of the wild-type, \textit{Δcap64}, and complement strains after incubation for 24 h in asparagine medium. The colour of the \textit{Δcap64} culture supernatant became darker than the other two culture supernatants, suggesting that the \textit{Δcap64} cells produced more pigment. The absorbance of the \textit{Δcap64} culture supernatant was 0.420, which was 2.1-fold the absorbance of the wild-type culture after 24 h incubation in asparagine medium (Fig. 4b). Cells of the wild-type, \textit{Δcap64} and the \textit{CAP64} complement strains were incubated in asparagine medium for 3 or 6 h, and the amount of \textit{LAC1} transcript was analysed by qRT-PCR (Fig. 4c). The amounts of \textit{LAC1} transcript in the \textit{Δcap64} cultures with 3 h and 6 h incubation were 1.85 or 3.00-fold those in the 3 h and 6 h wild-type cultures. The amounts of \textit{LAC1} transcript in the \textit{CAP64} complement cultures with 3 h or 6 h incubation were 1.43 or 1.22-fold the amounts in the corresponding wild-type cultures. The amount of \textit{LAC1} transcript in the \textit{Δcap64} culture tended to increase, but there was no significant difference in the \textit{LAC1} transcript between the \textit{Δcap64} strain and the \textit{CAP64} complement strain.

**Effect of deletion of \textit{CAP64} on \textit{VHP1} or \textit{SAV1} transcription**

Quinacrine accumulation is associated with the vesicular (H+)\textsuperscript{+}-ATPase protein pump gene \textit{VPH1}, and thus deletion of the \textit{CAP64} gene was expected to have some effect on the transcription of \textit{VPH1}. We have already showed that the intracellular quinacrine accumulation was not observed in \textit{Δcap64}, but was clearly seen in the wild-type strain when cells were grown in YPD liquid medium [3]. The amounts of \textit{VPH1} transcript in the wild-type, \textit{Δcap64}, and \textit{CAP64} complement strain cultures were compared. Cells of the wild-type, \textit{Δcap64}, or \textit{CAP64} complement strains were grown in YPD liquid medium for 6 h or 20 h, respectively, and the amount of \textit{VPH1} transcript was analysed by qRT-PCR analysis. No signal was detected under this culture condition, although three independent experiments were performed. Next, cells of the wild-type, \textit{Δcap64}, or \textit{CAP64} complement strains were incubated in asparagine medium for 6 h, and the amount of \textit{VPH1} transcript was analysed by qRT-PCR analysis. This is because the amount of \textit{LAC1} transcript in the culture with 6 h incubation was higher than that in the culture with 3 h incubation in the \textit{Δcap64} strain (Fig. 4c). Cells of the wild-type or \textit{Δcap64} strain were cultured in asparagine medium for 6 h,
and the amount of $VPH1$ transcript in the $\Delta cap64$ strain was compared to that in the wild-type strain. The $VPH1$ transcript of the $\Delta cap64$ strain was 1.8 times that of the wild-type strain (Fig. 5a). The amount of $VPH1$ transcript in the cap64 strain tended to be higher compared to that of the wild-type, but there was no significant difference between the $\Delta cap64$ strain and the $CAP64$ complement strain ($p$ value=0.0503, Fig. 5a).

Next, the amount of the $SAV1$ gene transcript, which is involved in vesicle exocytosis, was measured. The wild-type strain or $\Delta cap64$ strain was cultured in YPD liquid medium for 6 or 24 h, and the $SAV1$ transcript levels were analysed by qRT-PCR. The amount of $SAV1$ transcript was represented as the ratio of the amount of transcript in each culture to the amount of $SAV1$ transcript in the 6 h culture of the wild-type strain. The amount of $SAV1$ transcript in the 6 h culture of the $\Delta cap64$ strain was 4.0-fold the amount in the 6 h culture of the wild-type. The amounts of $SAV1$ transcript in the 24 h culture of the wild-type strain and $\Delta cap64$ strain were 2.3-fold and 2.2-fold the amounts in the 6 h culture of the wild-type strain, and there was not a significant difference in the amount of $SAV1$ transcript between the 24 h culture of the wild-type strain and the 24 h culture of the $\Delta cap64$ strain. The $SAV1$ transcript levels in the cultures performed in asparagine medium were also analysed. The $SAV1$ transcript levels of the wild-type strain and $\Delta cap64$ strain, which were incubated for 6 h in asparagine medium, were not significantly different (Fig. 5c).

**Intracellular localization of Cap64**

Because deletion of the $CAP64$ gene eradicated the quinacrine accumulation and the $VPH1$ gene coding for the vacuolar ($H^+$)-ATPase proton pump was not transcribed in the YPD culture, the Cap64 protein may be localized intracellularly and may be associated with acidic organelles which are stained with quinacrine. In order to examine this hypothesis, $CAP64$-mCherry overexpression strains were constructed. The $CAP64$-mCherry cassette was constructed and introduced into the $\Delta cap64$ strain. Two types of plasmids were constructed, in which mCherry was placed at the N- or C- terminals of $CAP64$ (Fig. 1). From these plasmids, the $CAP64$-mCherry cassette was amplified by PCR and transformed into cells of $\Delta cap64$. When the $\Delta cap64$ strain was transformed with the $CAP64$-mCherry cassette obtained by PCR amplification using pYI601 as a template, ten transformants on YPD plates containing hygromycin B were obtained. Genomic DNA was isolated from each of these ten transformants and was confirmed to contain the DNA fragment of the $CAP64$-mCherry cassette by PCR amplification with the pro-cap64 and mcherry-R primers. However, these transformants were unable to produce a capsule (data not shown). Then, the $\Delta cap64$ strain was transformed with the $CAP64$-mCherry cassette obtained by PCR amplification using pYI602 as a template, and nine transformants on YPD plates containing hygromycin B were obtained. Among them, five transformants could produce a capsule (Fig. 6). The integration of the transformation cassette was confirmed by appropriate PCR followed by the sequence analysis. The amount of $CAP64$ transcript of these four transformants was measured for the culture in YPD medium and was four-fold the amount of $CAP64$ transcript in the wild-type strain. Therefore, these transformants were selected as
CAP64-mCherry overexpression strains to analyse Cap64p subcellular localization.

The CAP64-mCherry overexpression strains were cultured in YPD liquid medium for 20h, and the cells were collected and stained with quinacrine or DAPI, and the fluorescence of mCherry, quinacrine or DAPI was observed (Fig. 7). The mCherry fluorescence was clearly accumulated in cells in a patch-like manner, and the sites of quinacrine accumulation coincided with the location of the mCherry fluorescence patches (Fig. 7a−c). The fluorescent pattern of the mCherry was different from that of the DAPI staining (Fig. 7e), although cells stained with DAPI were fixed with ethanol, but it was determined that Cap64p did not accumulate in the nucleus. This observation suggests that Cap64p accumulates in vesicles during the cell growth stages.

DISCUSSION

In C. neoformans, the CAP64 gene is well recognized to be involved in capsule synthesis because a null mutant, Δcap64, lacks a capsule [12–14, 26]. On the other hand, we reported that cells of the Δcap64 strains stained negative for DBB staining [3]. Therefore, CAP64 appears to have a different role other than capsule synthesis, and the phenotype of the Δcap64 strain was reanalysed. As the result of morphological observation, the Δcap64 strain was found not only to have abnormal quinacrine staining morphology, but also to have a morphology in which daughter cells could not be separated from parent cells. In Saccharomyces cerevisiae, this phenomenon of daughter cells being inseparable from parent cells is known to arise partly as the result of a failure in the extracellular secretion of mother/daughter separation enzymes (MDS proteins), which act to separate daughter cells from parent cells [27]. If CAP64 is associated with organelle acidification and intracellular trafficking, CAP64 deletion should affect these phenomena, leading to abnormalities in the expression of genes related to intracellular transport or the vesicle secretory system. When we compared the transcription levels of VPH1 and SAV1 among the wild-type, Δcap64, and CAP64 complement strains, the amount of VPH1 or SAV1 transcript in the Δcap64 strain was increased compared to that in the wild-type or CAP64 complement strain. Quinacrine diffuses across membranes and accumulates in intracellular acidic compartments, but the amounts of VPH1 transcripts in the wild-type, Δcap64, and CAP64 complement strains after YPD medium cultivation were undetectable, although quinacrine accumulation was observed in cells of the wild-type and CAP64 complement strains (data not shown). This result suggests that the VPH1 transcript might be restricted by the components of the YPD medium, and there might be other genes associated with organelle acidification in YPD culture.

On the other hand, when cells were cultured in asparagine medium, the amount of VPH1 transcript in the Δcap64 strain was higher than that in the wild-type strain, suggesting that VPH1 is involved in vesicle acidification of the laccase secretion system. Ericsson et al. analysed the Δvph1 mutant of the C. neoformans H99 strain and found that disruption of VPH1 resulted in defects in capsule formation and laccase and urease expression, but they did not find the VPH1 disruption effect in cells under relatively stress-free environmental conditions, such as YPD, neutral pH, and 30°C [8]. This result is consistent...

Fig. 5. The amounts of VPH1 or SAV1 transcript in the wild-type and Δcap64 strain cultures were compared. The amount of VPH1 transcript was examined after culturing in asparagine medium for 6h (a), and the amount of SAV1 transcript was examined after culturing in YPD medium for 6 or 24h (b), and after culturing in asparagine medium for 6h (c). Student’s t-test was used to compare them. Experiments were carried out in triplicate. Values are reported as the mean±SD. *Statistically significant by Student’s t-test (P<0.05).
with our VPH1 transcript analysis. The yeast may contain paralogs that cause acidification of vesicles, which are required for growth under stress-free conditions. We also revealed that CAP64 was highly transcribed during C. neoformans growth in YPD medium and that Cap64p was associated with vesicle acidification at the growth phase. But CAP64 disruption seemed not to affect the activity of laccase or urease [16].

In S. cerevisiae, Vph1p or Stv1p is included in the V-ATPase complex as the ‘a’ subunit, and the Stv1 protein (similar to Vph1p) is a paralog of Vph1p [28–31]. Vph1p is localized to the vacuoles, whereas Stv1p is present in the Golgi apparatus or other compartments, which are endosome compartments, and acts as a V-ATPase [29–31]. Interestingly, it is known that intracellular glucose depletion is involved in the dissociation of the V-ATPase complex [30]. In C. neoformans, the presence of an STV1 gene was not found in a search of cryptococcal genomic databases [8]. When the CAP64-mCherry overexpressing strain was observed, Cap64p was accumulated in a patch-like manner and a quinacrine accumulation site was observed at the same location, although the cells were grown in YPD medium and Vph1p was not expressed. In addition, the patch size was smaller than the vacuole, which appeared to be the size of a vesicle. Cap64p seems to have a function similar to Stv1p, but there is no homology to a subunit of the vacuolar (H+) -ATPase complex from diverse organisms including S. cerevisiae, and therefore, C. neoformans might express an Stv1-protein paralog and this paralog that is localized to the vesicles and acts as a V-ATPase. It is thus necessary to investigate whether Cap64p is contained in vesicles and whether Cap64p is a component of the V-ATPase.

When the wild-type strain and Δcap64 strain were grown in YPD medium, the amount of SA V1 transcript of the Δcap64 strain was 4.0-fold that of the wild-type strain, indicating that the SA V1 was highly expressed in the Δcap64 strain. But when these strains were incubated in asparagine medium, there was no detectable difference in the SA V1 expression level between the wild-type strain and the Δcap64 strain (Fig. 5), although the laccase activity in the Δcap64 strain was high (Fig. 4). This is because SA V1 was not directly involved in laccase secretion, suggesting that there may be genes that perform the same function as SA V1.

Fig. 8 shows a model of vesicle or vacuole acidification. In YPD culture, CAP64 activates the paralog of the Stv1 protein, forming acidic vesicles. The acidic vesicles contain some proteins secreted extracellularly which are necessary for the separation of mother and daughter cells. VPH1 is not transcribed, but some acidified vesicles become vacuoles during the stationary phase.
of cell growth. When CAP64 is disrupted, the vesicles are not acidified and vesicle migration is not regulated, leading to the high SAV1 transcription. On the other hand, when cells are incubated in asparagine medium, VPH1 is highly transcribed and the vacuoles are acidified. Because VPH1 expression is up-regulated due to CAP64 disruption, CAP64 may suppress VPH1 expression. Some acidified vacuoles might change to vesicles from which extracellular proteins such as laccase are secreted. Disruption of CAP64 does not affect SAV1 expression but activates laccase secretion. These findings suggest that the Sav1p homologue may be present and may be associated with laccase secretion [16].

The DBB colony staining method is one of the methods used to distinguish basidiomycetous yeasts from ascomycetes [1, 2]. If CAP64 is involved in the acidification of intracellular vesicles, it seems unlikely that CAP64 plays a direct role in the ability of DBB colony staining to distinguish basidiomycetous yeasts from ascomycetous yeasts. However, many homologous genes of the Cas3p/Cap64p family have been isolated from basidiomycetes, and isolation of those genes is limited to the phylum Basidiomycota [32]. Basidiomycetous yeasts may have a genus-specific secretory system, and CAP64 may be involved in it. On the other hand, we determined that the PMT2 gene, which encodes o-mannosyl transferase, is also related with the DBB staining in C. neoformans [4]. The PMT2 deletion mutant is negative by the DBB colony staining test but has a thick capsule around the cell wall [4]. Therefore, the DBB stained substrates may be o-mannosylated by Pmt2 at the ER and subsequently transferred to the Golgi apparatus and vesicles, and sorted to the cell membrane or cell wall. In a previous paper [3], it was shown that DBB stains the cell wall or cell membrane of the wild-type strain, so that the DBB-stained substrate is transported to the cell wall or cell membrane. In the Δcap64 strain, the colonies are slightly stained by DBB compared to those of ascomycetous yeasts, but the cell wall and the cell membrane are not stained by DBB. This result suggests that the DBB-stained substrate is synthesized in the Δcap64 strain, but it is not transported to the cell wall or cell membrane properly. It was also shown that in the Δcap64 strain, quinacrine cannot accumulate in vacuoles as
in the wild-type strain (Fig. 7f–g) [3]. In the present study, the location of the mCherry-tagged Cap64 protein was consistent with the location of intracellular quinacrine accumulation, as shown in Fig. 7. Since the vesicles are not sorted by Δcap64, it is considered that the DBB substrate could not be delivered to the cell wall or the cell membrane. If there are some proteins that complex with Cap64p and accumulate in vesicles, and these complexes maintain vesicle acidification, then these complex proteins should be detected and characterized, and their relationship with PMT2 and CAP64 should be analysed.

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Conflicts of interest
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