Original Article

Genetic and Phenotypic analyses of Calcineurin A subunit in *Arthroderma vanbreuseghemii*

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Abstract

Calcineurin is a serine/threonine protein phosphatase that consists of catalytic (calcineurin A) and regulatory (calcineurin B) subunits. The conserved protein plays important roles in various biological processes. Drug combination of fluconazole and the calcineurin inhibitor (FK506) showed synergistic effects against dermatophytes. In the current study, we identified the calcineurin A homologous gene (*TmcanA*) in the dermatophyte *Arthroderma vanbreuseghemii* (anamorph: *Trichophyton mentagrophytes*). Knockdown mutants were produced from *A. vanbreuseghemii*, resulting in a defection in growth properties in accordance with dose of the suppressing reagent. The *TmcanA* gene restored the ability of calcineurin A-deficient *Cryptococcus neoformans* strain to grow at elevated temperatures. Repression of *TmcanA* at 37°C resulted in severely stunted growth, suggesting that this protein plays a role in tolerance to elevated temperatures. In addition, TMCANA showed an interaction with high osmolarity glycerol (HOG) signalling pathway by governing the secretion of a secondary metabolite. Moreover, expression of the hydrophobin A gene (*TmHF*) decreased significantly under the *TmcanA*-repressive condition, suggesting that TMCANA is involved in its regulation. In conclusion, calcineurin A is a multifunctional gene that is involved in the regulation of several biological processes and therefore is worth being considered as a drug target for treatment of dermatophytoses.

Key words: dermatophytes, calcineurin A, Hydrophobin A, copper repressible promoter, secondary metabolites.
Introduction

The filamentous fungus *Arthroderma vanbreuseghemii* (anamorph: *Trichophyton mentagrophytes*) is one of the dermatophytic species that invade the keratinised tissues of human and animals. They secrete a set of proteases belonging to two gene families that degrade keratinous materials and, therefore, extract nutritional elements for their own survival.\(^1\)\(^2\) Gaining a comprehensive understanding of their biological processes and the mechanisms they employ to invade their hosts has yet to be achieved.

Next-generation technologies prompt efforts to sequence whole genomes of dermatophytic species that are of medical significance.\(^3\)\(^–\)\(^5\) Meanwhile, our research group has been working on establishing molecular tools for dermatophytes, which would enable advanced level of research.\(^6\)\(^–\)\(^9\) They include the development of the conditional promoter P\(_{CTR4}\) as an alternative reverse genetic approach to explore functions/phenotypes of essential genes or of genes that cannot be knocked out.

Calcineurin is a serine/threonine protein phosphatase (calcineurin A) and regulatory (calcineurin B) subunits.\(^10\) Activation of this heterodimer protein occurs following the binding of Ca\(^{2+}\)/calmodulin to calcineurin A at the calmodulin-binding domain, displacing the auto-inhibitory domain.\(^10\) The binding of Ca\(^{2+}\) to calcineurin B also plays a role in this activation. Calcineurin is conserved from yeasts to human; however, it displays unique roles in different cell types, for example, modulating immune responses, impacting muscle development, cell death, morphogenesis, stress resistance, and the ability to grow at elevated temperatures.\(^11\)\(^–\)\(^15\)

An *ex vivo* study showed that targeting the calcineurin pathway at the level of the peptidyl-prolyl isomerase FKBP12 by tacrolimus (FK506) had synergetic therapeutic effects against the dermatophyte *T. mentagrophytes*, in clearing conidia and preventing skin’s damages.\(^16\) For this reason, calcineurin might be a candidate for research aimed at finding novel antifungal agents against dermatophytes.

In the current study, we isolated the calcineurin A gene, designated *TmcanA*, from *A. vanbreuseghemii*, produced knockdown mutants based on the copper responsive promoter P\(_{CTR4}\), and studied the characteristics associated with this gene. The results showed that TMCANA preserves some functions that are conserved among other fungi and is involved in the regulation of phenotypes that have not been reported yet.

Material and methods

Strains and media

The strains used in this study are listed in Table 1. They were incubated at 28°C unless otherwise stated. Conidiation of *A. vanbreuseghemii* strains was induced on solid MOPS-buffered RPMI 1640 medium (Nissui, Tokyo, Japan).

Putative calcineurin A knockdown mutants were selected on Sabouraud glucose agar (SDA) supplemented with 300 μg/ml of hygromycin B, 25 μg/ml of chloramphenicol, 200 μg/ml of cefotaxime, and 20 μg/ml of bathocuproine disulfonate (BCS) as a copper-specific chelator (Dojin, Kumamoto, Japan). For the complementation test, yeast extract-peptone-dextrose (YPD) agar rich medium supplemented with 100 μg/ml of nourseothricin and 200 μg/ml of cefotaxime was used.

The *Agrobacterium tumefaciens* strain EHA105 used in the *A. tumefaciens*-mediated transformation (ATMT) was maintained as described in previous research.\(^6\)

*TmcanA* gene identification

DNA sequences of calcineurin catalytic A subunit from *Aspergillus fumigatus*, *A. nidulans* and *Coccidioides immitis* were compared to isolate a homologous sequence in *A. vanbreuseghemii*. A pair of mix primers, Calci-FA and Calci-RA, was designed from highly conserved domains to amplify an internal fragment using total DNA from *A. vanbreuseghemii* TIMM2789. Fragment 1 from the 5′ end was extended using Calci-FC mix and Calci-RC primers. The 3′ end of fragment 1 was amplified by rapid amplification of the cDNA ends (RACE)-PCR using primers, Calci-FB1 and Calci-FB2 (for nested polymerase chain reaction [PCR]), which were designed based on the nucleotide sequence of fragment 1. Aliquots of 1 μg of total RNA were reverse transcribed according to the 3′ RACE method with Super Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The nucleotide sequence of fragment 2 was determined by analysis of the previous amplicons. Specific primers, Calci-F1 and Calci-R1, were designed to amplify the open reading frame (ORF) of *TmcanA*, while Calci-FC1 and Calci-RC1 (and their nested primers Calci-FC2 and Calci-RC2) were employed to extend the ORF of TIMM2789 in upstream direction. Sequencing of the novel fragments was carried out using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA). Table 2 lists the nucleotide sequences of the primers used in this study.

To estimate the number of copies of the *TmcanA* gene in *A. vanbreuseghemii*, Southern hybridization was...
Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype / description</th>
<th>Reference</th>
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<tr>
<td><em>Arthroderma vanbreuseghemii</em></td>
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<td>TIMM 2789</td>
<td>Wild-type, anamorph: <em>Trichophyton mentagrophytes</em></td>
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<tr>
<td>TmL28</td>
<td>tmlig4Δ::nptII</td>
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<td>DRC A1</td>
<td>tmlig4Δ::nptII, tmcanAΔ::phph::Pctr4</td>
<td>This study</td>
</tr>
<tr>
<td>DRC A2</td>
<td>tmlig4Δ::nptII, tmcanAΔ::phph::Pctr4</td>
<td>This study</td>
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<td>DRC A2</td>
<td>tmlig4Δ::nptII, tmcanAΔ::phph::Pctr4</td>
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<td><em>Cryptococcus neoformans</em></td>
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<td>H99</td>
<td>Wild-type</td>
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<td>AO4</td>
<td>cna1Δ::ADE2</td>
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<td>AO4Con3</td>
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<td><em>Escherichia coli</em></td>
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<td>DH5α</td>
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<td>Nippon gene</td>
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<td>(rK−, mK+), deoR, supE44, λ−, thi-1, gyrA96, relA1</td>
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</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>containing Ti helper plasmid</td>
<td>18</td>
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</table>

performed. Total DNA from TIMM2789 was digested with five types of restriction enzymes, *Bam*HI, *Xho*I, *Hin*III, *Pst*I, and *Sal*I. An internal fragment of the coding region of the calcineurin A gene was then amplified using Calci-FB2 and Calci-RC primers and used as a hybridization probe.

Construction of transformation vectors

Complementation construct

The calcineurin A complementation cassette was constructed to harbor the cDNA of the *TmcanA* gene under control of the promoter *Pact* and the terminator *Trp1*. The three fragments were merged by overlap extension PCR using three pairs of primers, cDNA-F(O.E.PCR) and cDNA-R(O.E.PCR), *Pactin*-FA/*Bam*HI and *Pactin*-R(O.E.PCR), and *Trp1*-F(O.E.PCR) and *Trp1*-R/*Sac*I, respectively (Fig. 1). The construct was subcloned into the binary vector pAg1NAT at the *Bam*HI and *Sac*I restriction sites.

Knockdown vector

The knockdown vector pAg1H-*TmcanA*/D was engineered from the binary vector pAg1-*hph* at the *XbaI* and *Sma*I restriction sites. A 1.8 kb of upstream region of the *TmcanA* locus (nucleotide position: -2031 to -264) was amplified using two primers, *Nru*I-*TmcanA*-pro-F and *Bgl*II-*TmcanA*-pro-R, and finally subcloned into the previous binary vector containing the conditional expression cassette (Fig. 2).

Transformation

*A. vanbreuseghemii* was transformed by ATMT method as described in previous research. Transformants were selected on RPMI 1640 agar supplemented with 300 μg/ml of hygromycin B.

*Cryptococcus neoformans* was also transformed by ATMT as described in previous research with minor modifications. The co-cultivation was performed for 48 h on *Agrobacterium* induction medium. Following co-cultivation, the membranes were washed with sterilized H2O and aliquots were spread on YPD plates supplemented with 100 μg/ml of nourseothricin and 100 μg/ml of cefotaxime.

Molecular biological analysis

Total DNA from each *A. vanbreuseghemii* strain was extracted from growing mycelia as described by Girardin and Latge. Subsequently, DNA was digested with appropriate restriction endonucleases, fractionated on 0.8% (w/v) agarose gels, blotted onto Hybond N+ membranes (GE Healthcare Limited, UK) and hybridized using the ECL Direct Nucleic Acid Labeling and Detection system (GE Healthcare Limited).
Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5‘-3’)</th>
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<td>Calci-FA</td>
<td>GGCICARTAYTAYGATTTATGGA</td>
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<td>Calci-RA</td>
<td>CATRTCIGTDATYTYYTTCICCC</td>
</tr>
<tr>
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<tr>
<td>Calci-RC</td>
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<td>Calci-FB1</td>
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<td>Calci-FB2</td>
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<td>Calci-F1</td>
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<td>Calci-RC1</td>
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<tr>
<td>Calci-FC2</td>
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<td>Calci-RC2</td>
<td>GAACCCTTCAGACACGGC</td>
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<td>cDNA-F(O.E.PCR)</td>
<td>TAAACTCGCCCAACATGTCTATGGGAAGGAAACTGG</td>
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<tr>
<td>cDNA-R(O.E.PCR)</td>
<td>CTTAATCGCCCAACATGTCATGGAAGGAAACTGG</td>
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<td>Pactin-FA/BamHI</td>
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<td>TrpR/SacI</td>
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<td>Pactin-R(O.E.PCR)</td>
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<td>Trp1-F(O.E.PCR)</td>
<td>GCCGGGAGCTGCAAGAGATGTAGAAACTAGC</td>
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<tr>
<td>Trp1-R/SacI</td>
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<td>Xba- PCTR4</td>
<td>AGCCTGCAAGTCTCGAGGTAGAAGTTGTCAG</td>
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<td>PCTR4-TmcanA-R</td>
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<td>Sma-TmcanA-term-R</td>
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<td>Nru-TmcanA-pro-F</td>
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<td>BglII-TmcanA-pro-R</td>
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<td>DRCA-RT-R</td>
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<td>TmHF-F1</td>
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<tr>
<td>TmHF-R1</td>
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<tr>
<td>KpnI/TmCalci(F2)RA</td>
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<tr>
<td>TmCalci/GW4F</td>
<td>CCATCGAATGTTATCGTCAG</td>
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</tbody>
</table>

I: (deoxyuridine); R: (A,G), Y: (C,T), D: (A,G,T).

Phenotype restoration

Each C. neoformans strain was cultured onto YPD agar supplemented with 100 μg/ml of nourseothricin at 28°C for three days. Subsequently, they were spotted onto YPD agar and incubated at 28, 37, 38, and 39°C for four days. The strains were also grown on YPD agar supplemented with 1 μg/ml of FK506 (Funakosi, Japan) and incubated at 37°C.

Phenotypic analyses

Growth under TmcanA-suppressive conditions

Growth under TmcanA-suppressive conditions, conidia of each A. vanbreuseghemii strains (2 × 10³ cell) were spotted onto RPMI1640 agar supplemented with 1, 5, 10, 50, 100, and 200 μM of CuSO₄. Cultures were incubated at 28°C for up to five days.

Growth under stress conditions

Growth properties of the calcineurin A knockdown mutant (DRCA1) in response to stress factors were studied under TmcanA-repressive, that is, medium with 5 μM of CuSO₄, and expressive conditions. Conidia (2 × 10³ cell) were spotted onto RPMI 1640 agar with pH adjusted to 5, 7, 8 or a high-salt condition, 0.5 or 1 M of NaCl (pH 7), at 28°C or 37°C for five days.

Scanning electron microscopy (SEM)

Conidia (3 × 10³ cell) of each TmL28 and DRCA1 were cultured in RPMI 1640 broth in Petri dishes with-
Figure 1. Construction of calcineurin A complementation vector. For details review the material and methods section.

Figure 2. Construction of the knockdown vector. (a) The restriction map of the knockdown vector pAg1H-TmcanA/D. RB, right border; LB, left border; Hyb. probe, hybridisation probe. (b) Southern blotting analysis of total DNA samples from transformants. Fifteen microgram of total DNA from each A. vanbreuseghemii strains were digested with SpeI and fractionated by electrophoresis on 0.8% (w/v) agarose gels. TmL28 was used as negative control. The hybridisation probe was constructed by amplifying around 800 bp of the TmcanA locus by PCR using the two primers, KpnI/TmCalc(F2)RA and TmCalc/GW4F. 1, 2, 3, DRCA1–3.

out shaking at 28°C for two days under the TmcanA-suppressive and expressive conditions. Mycelia were fixed with 2% glutaraldehyde in 0.1 M of cacodylate buffer (pH 7.2) at 4°C for 5 h. Following three time of washing with 0.1 M of cacodylate buffer (pH: 7.2), mycelia were post-fixed with 1% osmium tetroxide in 0.1 M of cacodylate buffer (pH: 7.4) at 4°C for 24 h and dehydrated through a graded series of acetone, which was finally replaced with t-butyl alcohol. The samples were finally freeze-dried, coated with osmium tetroxide using an osmium
plasma coater (OPC60A; Filgen Inc., Aichi, Japan), and observed by high-resolution field-emission type SEM (JSM-7500F; JEOL, Tokyo, Japan) at an acceleration voltage of 1 kV.

Identification of TmHF sequences

A homologue of hypA from A. benhamiae (NCBI accession number: XP_003014413.1) was isolated from A. vanbreuseghemii using a pair of primers, TmHF-F1 and TmHF-R1. A total of 420 bp, encoding 139 amino acids, was isolated and confirmed by sequencing.

Real-time PCR

To study the timecourse of the relative expression levels of calcineurin A in DRCA1, each A. vanbreuseghemii strain was grown in RPMI 1640 broth at 28°C for 72 h and CuSO₄ was added at a final concentration of 5 μM. Mycelium was collected at 0, 4, and 12 h after supplementation of CuSO₄.

Whereas the relative expression of hydrophobin A (TmHF) in DRCA1 was estimated by growing A. vanbreuseghemii strains under the TmcanA-suppressive, that is, 5 μM of CuSO₄, and expressive conditions at 28°C. Mycelia were collected from 72 h culture 12 h after applying copper. Total RNA was extracted using a RNeasy plant mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Invitrogen, Carlsbad, CA). Reverse transcription PCR was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using Fast SYBR Green PCR Master Mix on an ABI PRISM 7500 Fast Real-Time PCR system (Applied Biosystems) under the standard conditions recommended by the manufacturer. Relative expression was calculated using the 2⁻ΔΔCt method and was relatively normalised to the house keeping gene 18S rRNA.

Nucleotide accession number

The nucleotide sequence data of TmcanA and TmHF have been deposited in the GenBank under the accession numbers AB366433 and KR108211, respectively.

Results

Identification of the A. vanbreuseghemii TmcanA homologue

At the time of isolating the homologue of the catalytic A subunit of the calcineurin gene in A. vanbreuseghemii, no whole genome sequences of dermatophytic species had yet been released. Therefore, to isolate the calcineurin A homologue, sets of primers were designed based on comparison of three fungal species. The identification revealed a homologous sequence with a 1662 bp ORF interposed with four introns. Southern hybridization analysis indicated the presence of a single copy of the TmcanA locus throughout the chromosomes of A. vanbreuseghemii (data not shown). The identified ORF encodes a putative product of 554 amino acids with 73%, 86%, 87%, and 99% identities with calcineurin A of each C. neoformans (H99), A. fumigatus, A. nidulans, and Trichophyton rubrum, respectively.

Phenotype restoration

To ensure that the isolated TmcanA gene encodes the highly conserved calcineurin A subunit in A. vanbreuseghemii, cDNA of TmcanA was introduced into C. neoformans strain AO4 (a generous gift from Dr. Joseph Heitman of Duke University) that is deficient in calcineurin A subunit encoding gene (CNA1). The resultant transformants were confirmed by PCR using two primers, Paccinin/FA/BamHI/ and TrpR/SacI (data not shown). A comparative analysis of C. neoformans strains revealed that the wild-type, cna1Δ, the calcineurin A revertant (cna1Δ::TmcanA::NAT) and the negative control (cna1Δ::NAT) were able to grow on YPD incubated at 28°C for four days (Fig. 3). However, when they were incubated at 37°C, both cna1Δ and cna1Δ::NAT strains were unable to grow unlike the wild-type and calcineurin A revertant strains. To exclude the role of suppressor mutations in restoring the ability to grow at elevated temperatures, the four strains were inoculated on YPD supplemented with 1 μg/ml of FK506 and incubated at 37°C. Both the wild-type and revertant strains displayed sensitivity to the calcineurin inhibitor (Fig. 3). Therefore, the cDNA of TmcanA restored the phenotype of the cna1Δ to its wild-type so that it could grow on elevated temperatures. Accordingly, the TmcanA gene functions similarly to CNA1 in its resistance to high temperatures. Moreover, only the wild-type grew at 38°C and none of the C. neoformans strains grew at 39°C (data not shown).

Production of TmcanA knockdown mutants

Regardless of the many attempts to produce TmcanA null mutants by homologous recombination, none were successful, compelling us to consider alternative ways to study functions and potential roles of calcineurin A in dermatophytes. In a previous study, we demonstrated that the copper-responsive promoter PCTR4 from T. rubrum is tightly functional in A. vanbreuseghemii.⁸ The calcineurin
A down-regulation cassette was constructed by placing the ORF of the TmcanA gene under the control of \( P_{CTR4} \) (Fig. 2). The cassette was introduced into TIMM2789 by means of ATMT. The resultant transformants underwent molecular analyses by direct PCR and Southern blotting (Fig. 2). Three of the putative down-regulated mutants, DRCA1–3, were chosen randomly for downstream analyses.

The growth of DRCA strains on media supplemented with serial concentrations of copper showed that growth properties were dose-dependent: the higher the copper concentration, the weaker the growth (Fig. 4). However, the copper concentration of 5 \( \mu \)M proved to be the lowest concentration that ensured the minimum growth for allowing further analyses (Fig. 4). In addition, growth of the three DRCA strains was identical under the TmcanA-repressive and expressive conditions, implying that they are stable. Therefore, DRCA1 was chosen to represent all three strains in the downstream experiments.

**Kinetics of TmcanA down-regulation**

The kinetics of calcineurin A subunit repression in DRCA1 and TmL28 strains were estimated at three time points—0, 4, and 12 h after applying CuSO\(_4\). Mycelia were collected and RNA was extracted. A pair of primers, DRCA-RT-F and DRCA-RT-R, was used to amplify a total of 160 pb of cDNA from the TmcanA. The relative expression of TmcanA was normalised to 18S rRNA using two primers, 18S-1-F and 18S-1-R.25 As shown in Figure 5, the relative expression levels of TmcanA in DRCA1 under the TmcanA-repressive condition were 94% and 97% lower than their levels under the expressive condition at the time point 4 h and 12 h, respectively. On the other hand, the expression of TmcanA in the TmL28 strain did not change significantly after the broth culture was treated with copper.

**Phenotypic analyses of DRCA1 under stress conditions**

The responses of the TmcanA down-regulated mutant (DRCA1) to various stress conditions were evaluated in comparison with its parental strain, TmL28. As shown in Figure 6, the colony sizes of DRCA1 were smaller under the TmcanA-repressive condition than the expressive condition and smaller than the colony sizes of TmL28 under both conditions. Moreover, TmcanA-repression in the DRCA1 strain displayed a preference for acidic media, that is, pH 5 at 28°C rather than neutral or alkaline media. However, growth at 37°C resulted in severe deficiency in the ability of the fungi to generate filaments during the suppression of TmcanA. In addition, the DRCA1 colony became opaque under the TmcanA-repressive condition at elevated temperature and pH 7.
In addition, both DRCA1 and TmL28 were stunted under the repressive and expressive conditions in media with high osmotic pressure. However, the colonies of each A. vanbreuseghemii strain were yellow in colour when incubated at 28°C with or without copper, except for DRCA1 under the TmcanA-repressive condition.

### Microscopic observations

While no morphological changes were observed in TmL28 after applying copper (data not shown), direct microscopy and SEM observations of A. vanbreuseghemii strains revealed highly branched and stunted hyphal branches during the TmcanA-repression of DRCA1 unlike the copper-free condition (Fig. 7). Moreover, during down-regulation of TmcanA in DRCA1, hyphal walls were irregularly shaped and hyphal threads were thicker than hyphae under the expressive condition.

### Calcineurin is involved in hydrophobicity

The repression of TmcanA led to stunted growth, suggesting that calcineurin A plays a role in cell wall related proteins. One of the candidate proteins was hydrophobin A. A pair of primers, TmHF-RTF and TmHF-RTR, was used to amplify a total of 134 pb of TmHF cDNA, and normalisation was done to 18S rRNA using two primers, 18S-1-F and 18S-1-R. The relative expression of TmHF in DRCA1 after 12 h of TmcanA repression was significantly decreased (P < 0.05) in comparison with CuSO4-free medium (Fig. 8). However, the expression of hydrophobin in the control strain, TmL28, did not alter when CuSO4 was added, rolling out the effect of copper on the expression of hydrophobin A.

### Discussion

Calcineurin is a serine/threonine protein phosphatase composed of catalytic (calcineurin A) and regulatory (calcineurin B) subunits. Homologues of this conserved protein have been proven to play important roles in various biological processes. A previous study showed that a combination of fluconazole and FK506 that targets the calcineurin pathway could result in synergistic therapeutic effects against T. mentagrophytes that clear conidia and prevent skin damage. Accordingly, we hypothesised that studying the phenotypic characteristic associated with this

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**Figure 4.** Growth impairment in copper-containing media. Conidia of each A. vanbreuseghemii strains (2 × 10^3 cell) were spotted onto RPMI1640 agar with or without CuSO_4_. Control, copper-free media; DRCA1–3, calcineurin A down-regulated mutants; TIMM2789, wild-type; TmL28, parent strain of DRCA. Growth was observed after five days of incubation at 28°C. This Figure is reproduced in color in the online version of Medical Mycology.
Figure 5. The timecourse of the transcriptional repression of \( TmcanA \). Strains DRCA1 and TmL28 were grown in RPMI 1640 broth for three days, and then 5 \( \mu \text{M} \) of \( \text{CuSO}_4 \) was added. Samples were collected at 0, 4, and 12 h after supplementation of copper. The expression levels of \( TmcanA \) at each time point are indicated as relative fold changes compared to the level at time zero. Error bars indicate the standard error for triplicate measurements from a representative experiment.

Figure 6. The phenotypic characteristics of DRCA1 and TmL28 under stress conditions. Conidia (2 \( \times \) 10^3 cell) of each strain were grown at 28\(^\circ\)C or 37\(^\circ\)C for five days onto RPMI 1640 solid media under \( TmcanA \)-repressive, i.e., with 5 \( \mu \text{M} \) of \( \text{CuSO}_4 \), or expressive conditions. (a) pH 7; (b) pH 8; (c) pH 5; (d) pH 7 with 1M NaCl. Bar = 5 mm. This Figure is reproduced in color in the online version of Medical Mycology.

protein would be of great importance in finding novel antifungal agents against dermatophytes.

A previous report showed that calcineurin governs the growth of \( C. \text{neoformans} \) at 37\(^\circ\)C, mammalian body temperature.\(^\text{15}\) The complementation test in this study revealed that \( TmcanA \) could restore the original phenotype of \( C. \text{neoformans} \) that is deficient in calcineurin A, implying that it harbours similar domains required for growth at elevated
temperatures. On the other hand, the down-regulation of \( \text{TmcanA} \) at 37°C led to severe inhibition in the ability of \( \text{A. vanbreuseghemii} \) to produce filaments, suggesting that TM-CANA also plays a role in the resistance to elevated temperatures, unlike \( \text{A. fumigatus} \), which is not temperature-dependent. However, down-regulation of the \( \text{TmcanA} \) shows almost identical morphological effects of hyphal morphogenesis, including hyphal tip growth, branching and cell wall thickness. The inability of the \( \text{TmcanA} \)-revertant \( \text{C. neoformans} \) strains to grow at temperatures higher than 37°C can be explained by the fact that \( \text{TmcanA} \) is a heterogeneous gene derived from the dermatophytic \( \text{A. vanbreuseghemii} \) which is naturally adapted to growing at the temperature of mammalian skin (~35°C), unlike the systemic mycoses-causing \( \text{C. neoformans} \) that grows in more elevated temperatures. In addition, the deduced amino acids from \( \text{TmcanA} \) share only 73% similarity with the \( \text{CNA1} \) from \( \text{C. neoformans} \). Accordingly, being able to restore the phenotype at 37°C does not necessarily mean that it will function as efficiently as the homogenous \( \text{CNA1} \). Moreover, the revertant strain was as sensitive as its wild-type against the calcineurin inhibitor, suggesting that restoration of the phenotype at 37°C was the direct result of heterogeneous complementation rather than suppressor mutations.

In the current study, trials to produce \( \text{TmcanA} \) null mutants were unsuccessful. The ability to generate a null mutant by homologous recombination is dependent on several factors such as locus, genes, and length of homologous sequences. In this study, although we failed to produce a knockout mutant, we were able to generate down-regulated mutants of \( \text{TmcanA} \), from the same locus, with almost same length of the flanking homologous sequences (around 2kb). This implies that the position of the locus on the chromosomes and length of homologous DNA sequences had no role in failing homologous recombination in this case. Accordingly, \( \text{TmcanA} \) might be involved in an essential biological process that disrupting of its deduced protein could lead to lethal effects or growth arrest in certain stages. This hypothesis is supported by the fact that growth inhibition of \( \text{A. vanbreuseghemii} \) was dose-dependent under \( \text{TmcanA} \)-repressive conditions. It is also noteworthy that high concentrations of CuSO\(_4\) resulted in severe growth inhibition (data not shown), given the fact that there must be a certain level of leakage from the promoter P\(_{\text{CTR4}}\), regardless of its tight regulation. Moreover, a previous report

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**Figure 7.** Microscopic observation by SEM. DRCA1 and TmL28 were grown on RPMI 1640 or medium supplemented with 5 \( \mu \text{M} \) CuSO\(_4\) at 28°C for two days. Specimens were prepared as described in the material and methods section. (a) and (b) are DRCA1 under the \( \text{TmcanA} \)-repressive condition, (c) and (d) are DRCA1 under the expressive condition, i.e., without CuSO\(_4\). Bars = 1 \( \mu \text{m} \).
The expression level of TmHF during the repression of calcineurin A. DRCA1 and TmL28 were grown in RPMI 1640 broth in duplicate for three days at 28°C. Only one set of cultures was supplemented with CuSO₄ (final concentration of 5 μM), and total RNA was collected after 12 h. The expression levels of TmHF under the TmcanA-repressive condition are indicated as relative fold changes compared to the expressive condition for same strain. Error bars indicate standard error for four independent experiments.

Figure 8. The expression level of TmHF during the repression of calcineurin A. DRCA1 and TmL28 were grown in RPMI 1640 broth in duplicate for three days at 28°C. Only one set of cultures was supplemented with CuSO₄ (final concentration of 5 μM), and total RNA was collected after 12 h. The expression levels of TmHF under the TmcanA-repressive condition are indicated as relative fold changes compared to the expressive condition for same strain. Error bars indicate standard error for four independent experiments.

Microorganisms adapt to environmental stimuli to ensure their survival and propagation. Previous study showed that the production of secondary metabolites, including pigments, is affected by the high osmolarity glycerol (HOG) signalling pathway. Moreover, Delgado-Jarana et al. proposed the existence of cross-talk between the calcineurin and HOG pathways in the fungus Trichoderma harzianum. In the current study, A. vanbreuseghemii strains produced yellow pigments in hyperosmotic environment, except for DRCA1, under the TmcanA-repressive condition (Fig. 6). Therefore, the down-regulation of calcineurin A in this fungus prevents the production of one of its secondary metabolites, suggesting a preliminary evidence that there may be a link between the two pathways. However, the TmcanA knockdown mutant did not produce a similar level of yellow pigmentation under the expressive condition, which might have been due to traces of copper in the medium, causing slight repression of TmcanA and, thus, lower pigment production.

The recognition of pathogens by the host immune system is an important element for disease progression. A previous study showed that the ability of the cellular immune defence system to recognize the dermatophyte A. benhamiae was significantly affected by the existence of the rodlet layer formed by the hydrophobin HypA. Therefore, hydrophobin is a crucial factor for the invasion of dermatophytes. The current study demonstrated that calcineurin A is involved in the regulation of hydrophobin A and, therefore, in recognition by the host immune system. As shown in Figure 8, the expression level of TmHF mRNA significantly reduced under the TmcanA-repressive condition; thus, when the expression level of TmcanA is down-regulated, the expression level of TmHF reduces accordingly.

In conclusion, calcineurin A of the dermatophytic species A. vanbreuseghemii is a multifunctional protein that is involved in many biological processes. It is involved in hyphal morphogenesis and stress resistance. In addition, there are signs that it participates in the regulation of secondary metabolites, that is, pigments, produced under osmotic pressure. Moreover, calcineurin A is involved in regulation of hydrophobin A, which forms the rodlet surface layer, one of elements recognized by the host immune system. Future studies on transcriptome level may reveal whether TMCANA is involved in essential biological processes and, therefore, whether it influences the failure to produce the TmcanA null mutant in A. vanbreuseghemii.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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