

# Development of a Tightly Regulatable Copper-Mediated Gene Switch System in Dermatophytes

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Targeted gene deletion is now available for molecular genetic research of dermatophytes, and the physiological roles of several genes have been elucidated. However, this method cannot be applied to essential genes, which can be potential drug targets. To overcome this limitation, we have developed a conditional gene knockdown system using a copper-responsive promoter. The promoter sequence of the copper transporter gene *CTR4* ( $P_{CTR4}$ ) and that of the copper efflux pump gene *CRP1* ( $P_{CRP1}$ ) derived from *Trichophyton rubrum* were examined for their response to copper in *Arthroderma vanbreusegheimii*.  $P_{CTR4}$  was demonstrated to repress expression of a reporter gene in the presence of copper, while the activity of  $P_{CRP1}$  was induced by addition of copper. Importantly,  $P_{CTR4}$  regulated the gene expression more tightly. Furthermore, when  $P_{CTR4}$  was applied to regulate the expression of the endogenous genes *ERG1* and *TRP5*, their conditional mutants exhibited decreased growth activity under the repressive conditions. These results suggest that the  $P_{CTR4}$ -based gene regulation system represents a powerful tool for identification and characterization of a broad range of genes, including essential genes, in dermatophytes.

Dermatophytes are a group of filamentous fungi that cause superficial infection (so-called ringworm) in humans and animals. Due to their ability to utilize keratin, they colonize keratinized tissues, such as the skin, nails, and hair. Unlike deep-seated mycoses, e.g., aspergillosis and candidiasis, dermatophytosis is rarely fatal but severely decreases the quality of life of patients. More than 20% of the world's population is estimated to be infected with dermatophytes (12). Therefore, management of dermatophyte infection is a global concern, because currently available antifungal agents do not provide satisfactory results in many cases (11).

Basic tools for molecular genetic studies of dermatophytes have been developed in the last few years, including improved genetic transformation methods (1, 2, 31, 32, 33). In addition, genome sequence databases of seven dermatophyte species became available from the Broad Institute ([http://www.broadinstitute.org/annotation/genome/dermatophyte\\_comparative](http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative)). Considering genetic relatedness among dermatophytes (16), their genome sequence databases are useful for identification of homologs in strains of other dermatophytic species, including our experimental strain of *Arthroderma vanbreusegheimii*, one of the teleomorphs of the *Trichophyton mentagrophytes* complex. These technical advances have made it possible to conduct the functional analyses of several nonessential genes by efficient targeted gene deletion (6, 7, 10, 30).

Conditional gene knockdown methods are often used for identification and evaluation of essential genes, but no such methods have been reported for use in dermatophytes. Conditional gene knockdown systems have been established for some fungi, including the *Aspergillus nidulans* *alcA* promoter system (25), the nitrogen-regulated promoter system (*pNiiA*) in *Aspergillus fumigatus* (14), and the *Escherichia coli* tetracycline (Tet) resistance operon-based system in *A. fumigatus*, *Candida albicans*, and *Candida glabrata* (19, 24, 29). In the dimorphic fungus *Histoplasma capsulatum*, the promoter region of the copper efflux pump *CRP1* was shown to function as a copper-inducible promoter (8). Conversely, expression of the copper transporter *CTR3* was re-

pressed in the presence of copper. In *Cryptococcus neoformans*, the promoter of the copper transporter *CTR4* (a homolog of *H. capsulatum* *CTR3*) was demonstrated to be an excellent copper-repressive promoter and to be suitable for validation of essential genes (4, 15, 22).

The present study was performed to establish a conditional gene knockdown system for use in dermatophytes. For this purpose, we focused on copper-responsive promoters for the following reasons: (i) *Histoplasma* spp. are phylogenetically related to dermatophytes; (ii) copper-based systems are simple and do not require exogenous *trans*-acting factors such as tTA or rtTA in the Tet system; and (iii) copper-based systems are thought to be widely applicable, including in animal models, unlike other systems regulated by nutritional sources, such as carbon or nitrogen.

In this study, two copper-responsive promoters,  $P_{CTR4}$  and  $P_{CRP1}$ , derived from *Trichophyton rubrum* were isolated. The *lacZ* reporter gene was used to evaluate their applicability as conditional promoters in *A. vanbreusegheimii*. The results of a  $\beta$ -galactosidase assay suggested that both  $P_{CTR4}$  and  $P_{CRP1}$  were copper-responsive promoters. Furthermore,  $P_{CTR4}$  was shown to function as a copper-repressive promoter, allowing tight regulation of essential genes. Here, we report a conditional gene knockdown system in dermatophytes. We expect this  $P_{CTR4}$ -based conditional gene knockdown system to facilitate analysis of essential genes and thus advance molecular genetic research in dermatophytes.

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TABLE 1 Fungal and bacterial strains used in this study

Organism and strain	Description or genotype	Source or reference
<i>A. vanbreuseghemii</i>		
TIMM2789	Wild type	2
TmL28	$\Delta TmLIG4::nptII$	2
PCTR4lacZ	$\Delta TmLIG4::nptII \Delta Tmku80::Pch::hph::TtrpC::P_{CTR4}::lacZ::TtrpC$	This study
PCR1lacZ	$\Delta TmLIG4::nptII \Delta Tmku80::Pch::hph::TtrpC::P_{CRP1}::lacZ::TtrpC$	This study
PLESSlacZ	$\Delta TmLIG4::nptII \Delta Tmku80::Pch::hph::TtrpC::lacZ::TtrpC$	This study
PCTR4ERG1	$\Delta TmLIG4::nptII Pch::hph::TtrpC::P_{CTR4}::ERG1$	This study
PCTR4TRP5	$\Delta TmLIG4::nptII Pch::hph::TtrpC::P_{CTR4}::TRP5$	This study
<i>A. tumefaciens</i> EHA105	Carrying Ti plasmid pEHA105	13

## MATERIALS AND METHODS

**Strains and culture media.** The strains used in this study are listed in Table 1. *A. vanbreuseghemii* TmL28 was used as a host strain for transformation experiments throughout this study. The strain lacks its homolog of human DNA ligase 4 and is deficient in the nonhomologous end-joining pathway, which enables homologous recombination to occur efficiently (2). Conidial formation of TmL28 was induced at 28°C using modified solid 1/10 Sabouraud dextrose agar (SDA) (0.1% [wt/vol] Bacto peptone, 0.2% [wt/vol] glucose, 0.1% [wt/vol]  $KH_2PO_4$ , and 0.1% [wt/vol]  $MgSO_4 \cdot 7H_2O$ ) (28). After transformation, test strains were maintained on solid MOPS ([*N*-morpholino]propanesulfonic acid)-buffered RPMI 1640 medium (RPMI 1640A; 0.165 M 3-[*N*-morpholino]propanesulfonic acid, pH 7.0, 10.4 g/liter RPMI 1640). To test the effects of copper on the phenotypes of test strains, copper sulfate ( $CuSO_4$ ) was added at 0.1 to 100  $\mu M$ . To deplete copper ions from media completely, bathocuproine disulfonate (BCS) (Dojin, Kumamoto, Japan), a copper ion-specific chelator, was added at 10  $\mu M$ .

*Agrobacterium tumefaciens* strain EHA105 (13) for *A. tumefaciens*-mediated transformation (ATMT) was maintained as described previously (33).

**Southern hybridization analyses.** The growing mycelia from each dermatophyte strain were collected after incubation on SDA for 3 days at 28°C. The mycelia were frozen and ground under liquid nitrogen with a Multi-Beads shocker (Yasui Kikai, Osaka, Japan) at 1,800 rpm for 10 s, and this step was repeated 10 times. Total DNA was extracted as described previously (9). For Southern blotting, the total DNA samples were digested with an appropriate restriction enzyme, separated by electrophoresis on 0.8% (wt/vol) agarose gels, and transferred onto Hybond  $N^+$  membranes (GE Healthcare Limited, Buckinghamshire, United Kingdom). Southern blotting hybridization was performed using the ECL direct nucleic acid labeling and detection system (GE Healthcare Limited) according to the manufacturer's instructions.

**Preparation of  $P_{CTR4}$  and  $P_{CRP1}$  from *T. rubrum* total DNA.** To identify a *Trichophyton rubrum* homolog of *C. neoformans*  $CTR4$  (GenBank accession number [AAW43046](#)), the *T. rubrum* CBS118892 genome sequence database was searched using the local BLASTp (protein-protein BLAST) provided by the Broad Institute. A region of approximately 1.3 kb upstream of the start codon of the identified gene TERG\_01401 was amplified from *T. rubrum* total DNA by PCR with a pair of primers, PCTR4-F/BamHI and PCTR4-R (Table 2). The obtained fragment was subcloned into pUC118, sequenced, and used as a putative repressive promoter ( $P_{CTR4}$ ). Similarly, approximately 1.3 kb of upstream sequence of TERG\_07477, a homolog of *H. capsulatum*  $CRP1$  (GenBank accession number [EGC43700](#)), was amplified by PCR with two primers, PCR1-F/BamHI and PCR1-R, subcloned into pUC118, sequenced, and used as a putative inducible promoter ( $P_{CRP1}$ ).

**Construction of transformation vectors.** To generate *lacZ* reporter strains, binary vectors pAgPCTR4lacZ and pAgPCR1lacZ were constructed (Fig. 1A). For pAgPCTR4lacZ,  $P_{CTR4}$ , *E. coli lacZ* (18), and the *A. nidulans trpC* terminator (*TtrpC*) (27) were amplified separately by PCR with primers PCTR4-F/BamHI and lacZ/PCTR4-R, PCTR4/lacZ-F and

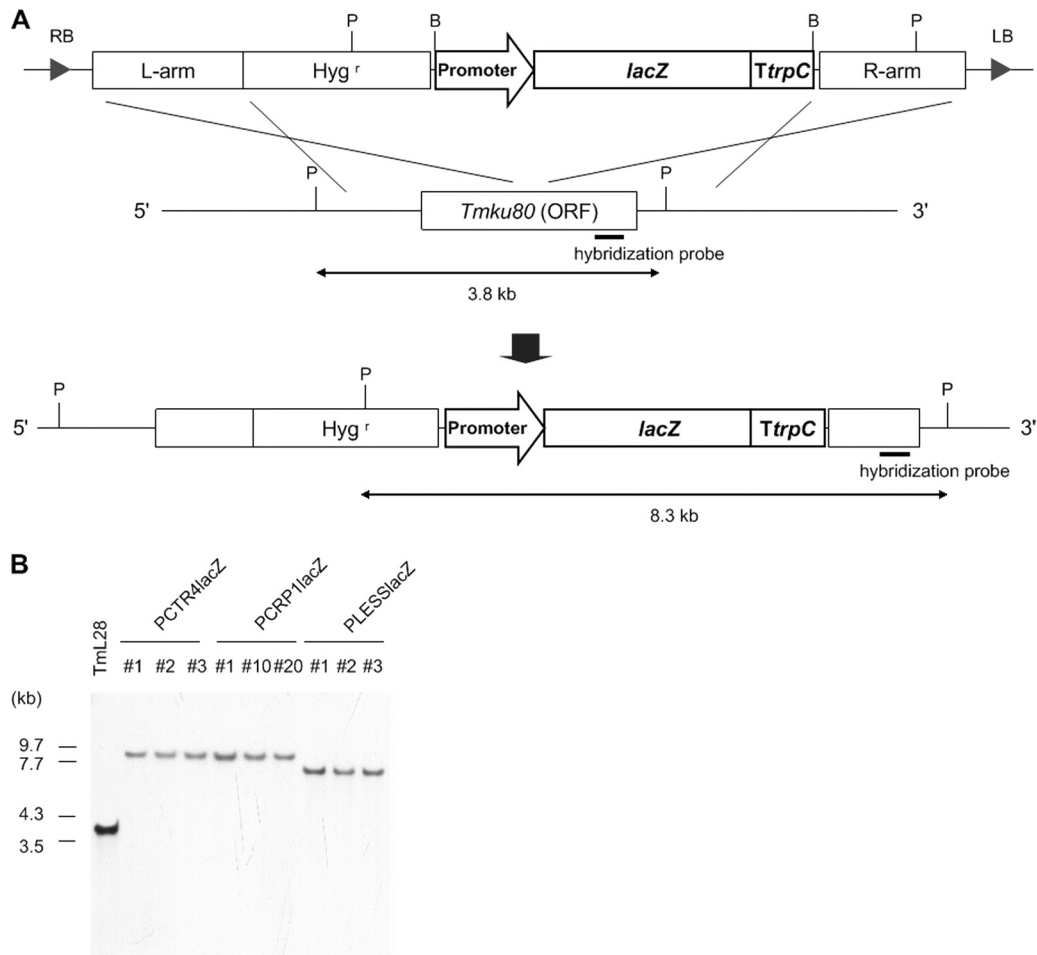
*TtrpC/lacZ*-R, and lacZ/*TtrpC*-F and *TtrpC*-R/BamHI, respectively (Table 2). These three fragments were gel purified and fused by overlap PCR (5) with the primer pair PCTR4-F/BamHI and *TtrpC*-R/BamHI. The resulting reporter cassette was subcloned into pUC118 and sequenced. The cassette was then excised from the plasmid by BamHI digestion and ligated into the binary vector pAg1-*Tmku80/T* (31) at the BamHI site to give pAgPCTR4lacZ. pAgPCR1lacZ was constructed through the same procedure, except using  $P_{CRP1}$  instead of  $P_{CTR4}$ . pAgPLESSlacZ, which carried a promoterless *lacZ* reporter cassette, was also constructed to generate a negative-control strain.

Transformation vectors for production of conditional knockdown strains were constructed as follows. First, the nucleotide sequences of *A. vanbreuseghemii* *ERG1* and *TRP5* loci were determined by chromosome walking. For pAgPCTR4ERG1, a binary vector for construction of the  $P_{CTR4}$ -*ERG1* conditional mutant, approximately 2 kb of sequence beginning from the start codon of *ERG1* (R arm) was amplified by PCR from *A. vanbreuseghemii* total DNA with the primer pair comprising PCTR4/ERG1R-F and ERG1R-R/SacI.  $P_{CTR4}$  and the R arm of *ERG1* were then fused by overlap PCR with a pair of primers, PCTR4-F/KpnI and ERG1R-R/SacI. The resulting fragment was digested with KpnI/SacI and ligated into the corresponding site of the binary vector pAg1-*hph* (33). In the second step, an approximately 2-kb region upstream of the start codon of *ERG1* (L arm) was amplified by PCR from *A. vanbreuseghemii* total DNA with the two primers ERG1L-F/SpeI and ERG1L-R/SpeI. The resulting fragment was digested with SpeI and inserted into the SpeI-digested vec-

TABLE 2 Primers used in this study

Primer	Sequence (5'-3') <sup>a</sup>
PCTR4-F/BamHI	ATGGATCCTCGTAGGTACGCC
PCTR4-R	ATGAATCTTCGCAGGCTTTGTAC
PCR1-F/BamHI	ATGGATCCGGAAGCTGTTCTTCCATATC
PCR1-R	ATGAATTCGGTGATAGACGGTTCAGTG
lacZ/PCTR4-R	AGCCGTTTCAATTCGCAGGCTTTGTACTTTG
PCTR4/lacZ-F	AGCCTGCGAAATGAACGGCTCCGGAGCTTG
<i>TtrpC/lacZ</i> -R	GAGACCCGGGTTTTTGTACACCAGACCAAC
lacZ/ <i>TtrpC</i> -F	GTGTCAAAAACCCGGGTCTCGAGCAGCTAG
<i>TtrpC</i> -R/BamHI	TCCGGATCCAAAGAAGGATTACCTCTAAAC
PCTR4-F/KpnI	GCATGGTACCTCGTAGGTACGCCCTTCCGG
ERG1R/PCTR4-R	ACCATTGTAGTTCGCAGGCTTTGTACTTTG
PCTR4/ERG1R-F	GCCCTGCGAATCACTAATGGTTGTAGAGGCTC
ERG1R-R/SacI	GTTTGAGCTCATTATCTGGCCACCTTTGTT
ERG1L-F/SpeI	TCCAAGTACTGGAATTAGATAAATCTGGCC
ERG1L-R/SpeI	CCATACTAGTAAGTGTGCGAAGGGGATAAA
TRP5R-F	CAATGGAGCAAATAAAGGAGAC
TRP5R-R	TGAATCCAAATTAGGGCCTCTG
TRP5L-F	GAAATCATTGGGAAGTATTC
TRP5L-R	TGAATGGTGTGTATATGTTGTGTGTCG

<sup>a</sup> Restriction recognition sites are underlined.



**FIG 1** Construction of *lacZ* reporter strains. The *lacZ* reporter cassette was integrated into the *Tmku80* locus by ATMT and confirmed by Southern blotting. (A) Schematic representation of homologous recombination. Restriction sites and sizes of expected fragments on Southern blotting are shown. “Promoter” represents either  $P_{CTR4}$  or  $P_{CRPI}$ . B, BamHI; P, PstI; Hyg<sup>r</sup>, hygromycin B resistance cassette; L and R arms, homologous sequences for recombination at the *Tmku80* locus; LB and RB, left and right borders, respectively. (B) Total DNA from each strain was digested with PstI and separated by gel electrophoresis. Southern blotting hybridization was performed as described in Materials and Methods. A partial fragment of *Tmku80* 393 bp in length was used as a hybridization probe.

tor to give pAgPCTR4ERG1. pAgPCTR4TRP5, a binary vector for construction of the  $P_{CTR4}$ -*TRP5* conditional mutant, was also constructed according to a similar procedure. The R arm and L arm for *TRP5* were amplified by PCR with the primer pairs comprising TRP5R-F/TRP5R-R and TRP5L-F/TRP5L-R, respectively, and then assembled into a binary vector.

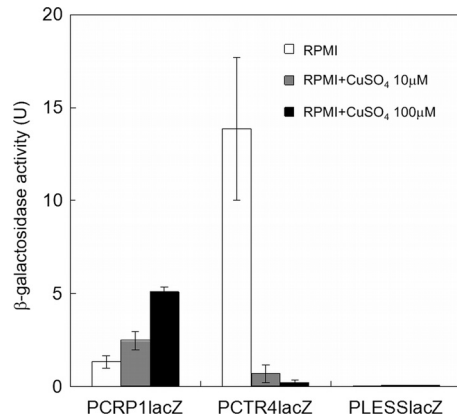
**Transformation of *A. vanbreuseghemii*.** *A. vanbreuseghemii* was transformed by ATMT as described previously (33). After cocultivation, transformants were selected on SDA or RPMI 1640 containing 100 to 300  $\mu$ g/ml hygromycin B. The desired transformants were confirmed by Southern blotting.

**$\beta$ -Galactosidase assays.** Quantitative  $\beta$ -galactosidase assays using cell extracts of reporter strains were performed as follows. Reporter strains were grown at 28°C for 5 days in RPMI 1640 broth in the presence or absence of copper. Mycelia were harvested by filtration using a cell strainer with 40  $\mu$ M nylon mesh (Becton, Dickinson and Company, Sparks, MD) and stored at -80°C. Frozen mycelia were ground under liquid nitrogen with a Multi-Beads shocker at 2,200 rpm for 15 s, and this step was repeated 10 times. Mycelial powders were suspended in 300  $\mu$ l of extraction buffer (100 mM sodium phosphate, pH 7.0, 10 mM  $\beta$ -mercaptoethanol, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 10 mM EDTA) and mixed well. The extracts were centrifuged for 10 min at 20,000  $\times$  g to

remove cell debris, and the supernatants were collected as crude protein extracts. Protein concentration was measured with a Pierce protein assay kit (660 nm; Thermo Scientific, Rockford, IL), using bovine serum albumin (BSA) as a standard.

An enzyme reaction was performed in 96-well microplates. Aliquots of cell extracts (20  $\mu$ l, corresponding to approximately 1  $\mu$ g of protein) were incubated with 150  $\mu$ l of 1-mg/ml o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) in Z buffer (100 mM sodium phosphate, pH 7.0, 50 mM  $\beta$ -mercaptoethanol, 10 mM KCl, 1 mM MgSO<sub>4</sub>) at 30°C until a yellow color developed (usually up to 30 min). Optical density at 405 nm (OD<sub>405</sub>) was then immediately measured with a Multiskan FC microplate reader (Thermo Scientific). Units were defined as numbers of OD<sub>405</sub> units/min of reaction/mg of protein.

**Real-time RT-PCR.** Strain PCTR4*lacZ* was grown in RPMI 1640 broth at 28°C for 5 days, and then CuSO<sub>4</sub> was added to give a final concentration of 50  $\mu$ M and culture was continued for a further 24 h. Mycelia were harvested by filtration and ground into powder as described above. Total RNA was extracted using an RNeasy plant minikit (Qiagen, Hilden, Germany) and was treated with DNase I (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) and stored at -30°C until use. Real-time reverse transcription-PCR (RT-PCR) was performed using Fast



**FIG 2** Copper-dependent responses of  $P_{CTR4}$  and  $P_{CRP1}$ . Strains PCTR4lacZ, PCR1lacZ, and PLESSlacZ were grown for 5 days in RPMI 1640 broth supplemented with 0, 10, or 100  $\mu\text{M}$   $\text{CuSO}_4$ . The mean  $\beta$ -galactosidase activity is shown in units (U) on the y axis. Error bars indicate standard deviations for three clones.

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. A dissociation curve of the PCR-amplified products was plotted to confirm the absence of nonspecific products and primer dimers. Primers were designed using Primer Express software (Applied Biosystems). The relative expression level of *lacZ* after addition of  $\text{CuSO}_4$  was calculated using the standard curve method and was normalized relative to the 18S rRNA level.

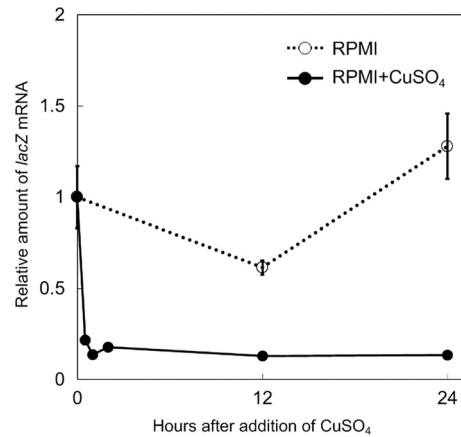
**Phenotypic analysis of conditional mutants.** Aliquots of 30  $\mu\text{l}$  of conidial suspensions (containing  $3 \times 10^4$  conidia) of each conditional mutant were spotted onto solid medium supplemented with 0 to 50  $\mu\text{M}$   $\text{CuSO}_4$  or 10  $\mu\text{M}$  BCS and grown at 28°C for 4 days. RPMI 1640A for PCTR4ERG1 and yeast nitrogen base without copper (YNB; ForMedium, Norfolk, United Kingdom) supplemented with 1% glucose and amino acids for PCTR4TRP5 were used as the basal medium.

**SEM.** TmL28 and PCTR4ERG1 were grown at 28°C for 2 days as described above, except with a shorter duration of culture. Agar blocks containing single colonies were excised and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 24 h. After a thorough washing with the buffer, the samples were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 20 h, dehydrated through a graded series of acetone, and transferred into isoamyl acetate. The samples were dried in a critical-point drying chamber (HCP-1; Hitachi, Tokyo, Japan) using liquid carbon dioxide, coated with osmium tetroxide using an osmium plasma coater (OPC60A; Filgen, Inc., Aichi, Japan), and observed by high-resolution field emission-type scanning electron microscopy (SEM) (JSM-7500F; JEOL, Tokyo, Japan) at an acceleration voltage of 1 kV.

**Nucleotide sequence accession numbers.** The newly determined sequences of *ERG1* and *TRP5* loci from *A. vanbreuseghemii* were deposited in the GenBank database under accession numbers [AB690298](#) and [AB690299](#), respectively.

## RESULTS

**Isolation of copper-responsive promoters from the *T. rubrum* genome.** When we started this study, the whole-genome sequence of *A. vanbreuseghemii* was not available. Instead, as dermatophytes share closely related genetic backgrounds, we searched the genome sequence database of *T. rubrum* CBS118892 for a homolog of *C. neoformans* *CTR4* or *H. capsulatum* *CRP1*. The results of protein-protein BLAST yielded one candidate for each gene in the *T. rubrum* genome. The gene TERG\_01401 showed 36% identity to *C. neoformans* *CTR4*, and the gene TERG\_07477 showed 56%

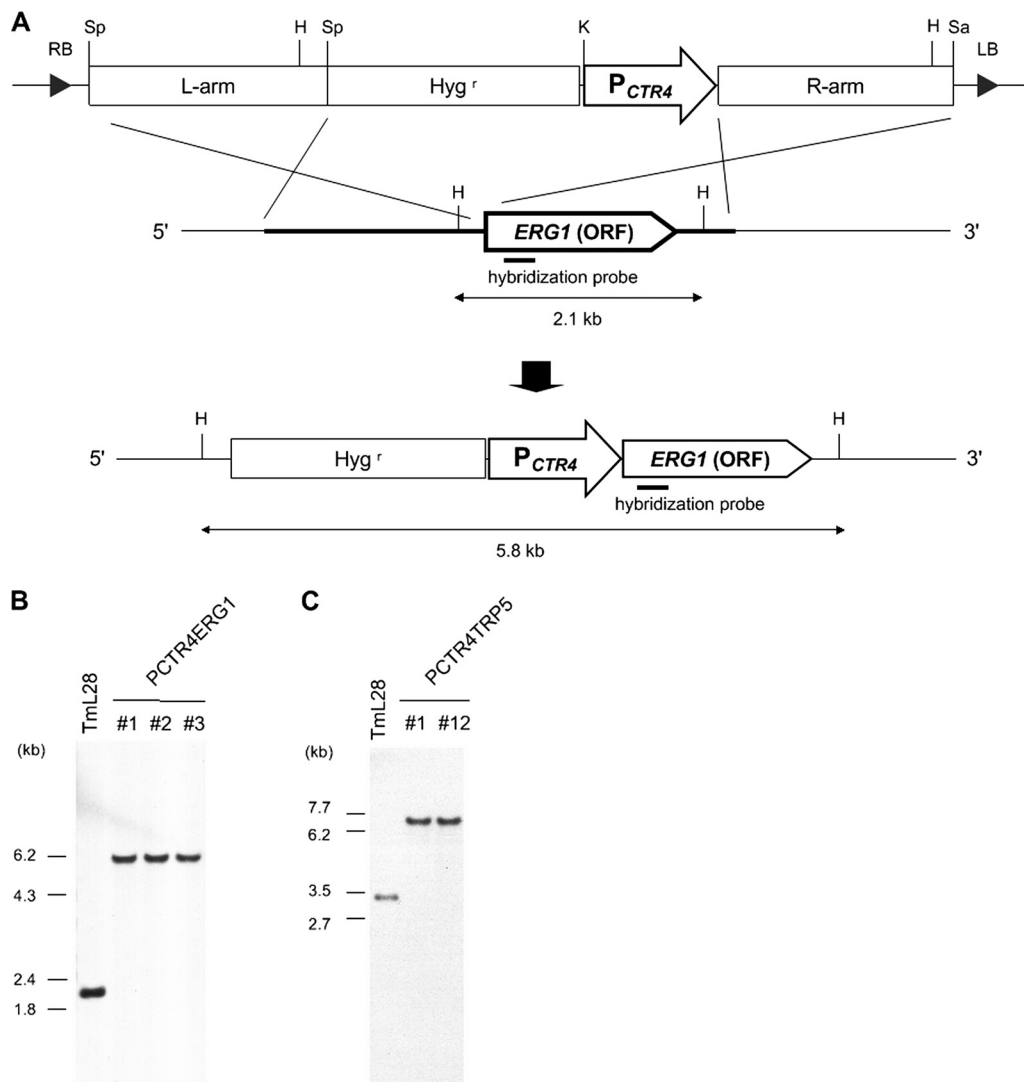


**FIG 3** Time course of transcriptional repression by  $P_{CTR4}$ . Strain PCTR4lacZ 1 was grown in RPMI 1640 broth (inducing conditions) for 5 days, and then 50  $\mu\text{M}$   $\text{CuSO}_4$  was added. Samples were collected at 0.5, 1, 2, 12, and 24 h after addition of copper. Real-time RT-PCR was performed as described in Materials and Methods. The expression levels of *lacZ* at each time point are indicated as relative fold changes compared to the level at time zero (addition of  $\text{CuSO}_4$ ). Error bars indicate standard deviations for triplicate measurements from a representative experiment.

identity to *H. capsulatum* *CRP1*. An upstream sequence of approximately 1.3 kb of each homologous gene was amplified from *T. rubrum* total DNA by PCR, and these sequences were designated  $P_{CTR4}$  and  $P_{CRP1}$ .

To confirm that the  $P_{CTR4}$  and  $P_{CRP1}$  fragments can function as copper-repressive or -inducible promoters in *A. vanbreuseghemii*, their copper dose-responses were examined with *A. vanbreuseghemii* transformants harboring a  $\beta$ -galactosidase (*lacZ*) reporter cassette. Three types of reporter cassette were constructed and introduced into TmL28 by ATMT (Fig. 1A), yielding PCTR4lacZ, PCR1lacZ, and PLESSlacZ (Table 1). Southern blotting confirmed that reporter cassettes were successfully integrated into the *Tmku80* locus (Fig. 1B). Confirmed clones of PCTR4lacZ (strains #1, #2, and #3), PCR1lacZ (#1, #10, and #20), and PLESSlacZ (#1) were further analyzed by a quantitative  $\beta$ -galactosidase assay.

These reporter strains were grown for 5 days in RPMI 1640 broth in the presence or absence of copper and assayed for  $\beta$ -galactosidase activity. As shown in Fig. 2, both  $P_{CTR4}$  and  $P_{CRP1}$  were shown to function as copper-responsive promoters in *A. vanbreuseghemii*. PCTR4lacZ exhibited marked repression of  $\beta$ -galactosidase activity when cultured in the presence of  $\geq 10$   $\mu\text{M}$   $\text{CuSO}_4$ . Maximum repression was observed at 100  $\mu\text{M}$ , where the activity was repressed approximately 70-fold compared with that in the absence of copper. In contrast,  $\beta$ -galactosidase activity of PCR1lacZ was induced in a copper dose-dependent manner. The activity in the presence of 100  $\mu\text{M}$   $\text{CuSO}_4$  was approximately 4-fold higher than that in the absence of copper. Note that higher leakage activity was observed for PCR1lacZ, i.e., enzyme activity of PCR1lacZ in the absence of  $\text{CuSO}_4$  was higher than that of PCTR4lacZ in the presence of 100  $\mu\text{M}$   $\text{CuSO}_4$ . PLESSlacZ showed a very low level of  $\beta$ -galactosidase activity, suggesting that *A. vanbreuseghemii* does not have intrinsic  $\beta$ -galactosidase activity. None of the tested strains showed apparent growth impairment even in the presence of 100  $\mu\text{M}$   $\text{CuSO}_4$  (data not shown). These results indicated that  $P_{CTR4}$  would be more suitable for tight reg-



**FIG 4** Construction of conditional mutants of endogenous *ERG1* or *TRP5*.  $P_{CTR4}$  was inserted directly upstream of target genes by ATMT and confirmed by Southern blotting. (A) Schematic representation of homologous recombination at the *ERG1* locus. Restriction sites and sizes of expected fragments on Southern blotting are shown. Sp, SpeI; K, KpnI; Sa, SacI; H, HindIII;  $Hyg^r$ , hygromycin B resistance cassette; L arm, 5' homologous sequence 2 kb upstream of the start codon of *ERG1*; R arm, 2-kb 3' homologous sequence beginning at the start codon of *ERG1*; ORF, open reading frame. Parts of host DNA which are homologous to the L and R arms are shown as a thick line. (B) Total DNA from each strain was digested with HindIII and separated by gel electrophoresis. Southern blotting hybridization was performed as described in Materials and Methods. A partial fragment of *ERG1* (length, 415 bp) or (C) *TRP5* (length, 414 bp) was used as a hybridization probe.

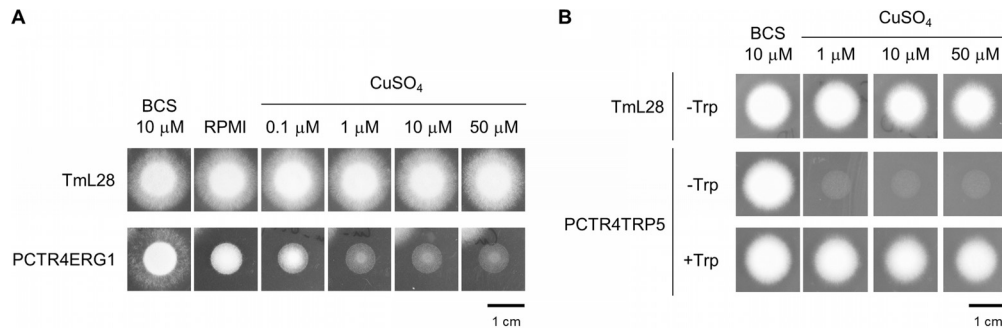
ulation of gene expression. Therefore, we focused on  $P_{CTR4}$  in the subsequent studies.

**Time course study of the  $P_{CTR4}$ -*lacZ* reporter strain.** To characterize  $P_{CTR4}$  in detail, the time course of the copper response of  $P_{CTR4}lacZ$  was evaluated by real-time RT-PCR.  $P_{CTR4}lacZ$  was grown for 5 days in RPMI 1640 broth in the absence of copper (inducing conditions), after which 50  $\mu$ M  $CuSO_4$  was added. The levels of *lacZ* mRNA were quantified at 0.5, 1, 2, 12, and 24 h after application of  $CuSO_4$ . As shown in Fig. 3, the *lacZ* gene expression level rapidly decreased to approximately 20% of the initial level within 0.5 h after administration of  $CuSO_4$ . The minimal level of expression (approximately 10% of the initial level) was observed at 12 h and lasted for 24 h after addition of  $CuSO_4$ .

**Regulation of endogenous *ERG1* and *TRP5* genes by  $P_{CTR4}$ .** From the above-described experimental results,  $P_{CTR4}$  was ex-

pected to be a potential tight conditional promoter. Here, we applied  $P_{CTR4}$  to regulate the expression of specific endogenous genes in *A. vanbreuseghemii*. For this purpose, *ERG1* and *TRP5* were chosen as target genes. *ERG1*, which encodes squalene epoxidase involved in the ergosterol biosynthesis pathway, is a target molecule of antifungal agents such as terbinafine (26) and was expected to be essential for cell growth. *TRP5* encodes tryptophan synthase in the tryptophan biosynthesis pathway, and its down-regulation caused tryptophan auxotrophy in *A. fumigatus* (14). Two binary vectors for conditional regulation of the expression of these genes were constructed and introduced into TmL28 by ATMT (Fig. 4A), yielding  $P_{CTR4}ERG1$  and  $P_{CTR4}TRP5$  (Table 1). Southern blotting confirmed that  $P_{CTR4}$  was correctly inserted directly upstream of the target genes (Fig. 4B and C).

Strain  $P_{CTR4}ERG1$  was grown at 28°C for 4 days on RPMI



**FIG 5** Growth phenotypes of conditional mutants. Conidia were spotted onto solid medium supplemented with the indicated concentrations of  $\text{CuSO}_4$  or  $10 \mu\text{M}$  BCS and grown at  $28^\circ\text{C}$  for 4 days. Two or three clones for each mutant were tested, and the results for one representative clone are shown. (A) Strain PCTR4ERG1 1 was grown on RPMI 1640A. (B) Strain PCTR4TRP5 12 was grown on YNB-based solid medium with (+Trp) or without (–Trp) tryptophan.

1640A in the presence or absence of copper. As shown in Fig. 5A, growth of PCTR4ERG1 was significantly repressed on RPMI 1640A supplemented with  $\geq 0.1 \mu\text{M}$   $\text{CuSO}_4$ . Growth was also mildly suppressed on RPMI 1640A without any supplementations of copper, implying the existence of a trace amount of copper in the agar, as RPMI 1640 is a synthetic copper-free medium. However, addition of a copper ion-specific chelator, BCS, restored the growth of PCTR4ERG1 to the level of the host strain TmL28. These macroscopic observations were supported by SEM analysis. Short, irregularly shaped hyphae were observed in PCTR4ERG1 grown in the presence of  $10 \mu\text{M}$   $\text{CuSO}_4$  (Fig. 6). Swelling and bending were seen frequently over the whole length of the hyphae. On the other hand, long elongated hyphae with smooth surfaces were observed in PCTR4ERG1 grown in the presence of  $10 \mu\text{M}$  BCS as well as in TmL28 under both conditions (Fig. 6).

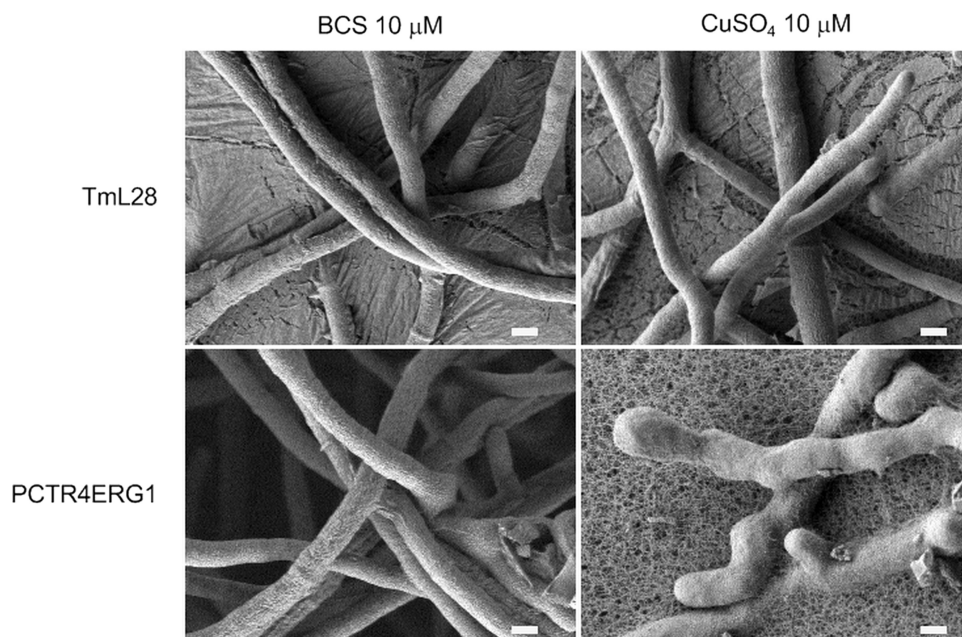
Clear phenotypic differences were also observed in strain PCTR4TRP5. After incubation at  $28^\circ\text{C}$  for 4 days, PCTR4TRP5 grew independent of tryptophan on solid YNB (without trypto-

phan [–Trp]) under inducing conditions, supplemented with  $10 \mu\text{M}$  BCS. On the other hand, growth was markedly repressed on solid YNB (–Trp) medium in the presence of  $\geq 1 \mu\text{M}$   $\text{CuSO}_4$  (Fig. 5B). This auxotrophy was restored by adding tryptophan to the growth medium.

These results suggested that in both conditional mutants, the transcriptional activity of  $P_{CTR4}$  under inducible conditions was sufficiently high to support their growth and that residual activity under repressive conditions was sufficiently low to impair growth.

## DISCUSSION

There has been steady development of molecular tools for genetic studies of dermatophytic species, including selectable markers (1, 32), transformation methods (2, 31, 33), and genome sequence databases. However, conditional knockdown systems, which are indispensable for proving that a gene is essential, have yet to be developed because no conditional promoters have been reported. In the present study, we demonstrated that  $P_{CTR4}$  and  $P_{CRP1}$  could



**FIG 6** Microscopic analysis by SEM. PCTR4ERG1 1 and TmL28 were grown on RPMI 1640A supplemented with  $10 \mu\text{M}$   $\text{CuSO}_4$  or  $10 \mu\text{M}$  BCS at  $28^\circ\text{C}$  for 2 days. Agar blocks containing single colonies were excised, and SEM samples were prepared as described in Materials and Methods. Bars =  $1 \mu\text{m}$ .

function effectively as copper-responsive conditional promoters in *A. vanbreuseghemii*. Furthermore, analyses of conditional mutants of endogenous genes suggested that the  $P_{CTR4}$ -based conditional knockdown system can be useful for identification of essential genes.

In previous studies, promoters of *CTR4* and *CRP1* genes were shown to be copper responsive in *C. neoformans* (22) and *H. capsulatum* (8), respectively. To evaluate the applicability of these promoters in *A. vanbreuseghemii*, cognate promoters of *CTR4* and *CRP1* homologs in *T. rubrum* were isolated. The criteria for choosing *T. rubrum*-derived promoters were (i) the availability of the full genome sequence of *T. rubrum*, (ii) its genetic relatedness to *A. vanbreuseghemii*, and (iii) avoidance of the introduction of endogenous sequences with knockdown cassettes, therefore increasing homologous recombination efficiency. The isolated promoter sequences,  $P_{CRP1}$  and  $P_{CTR4}$  were tested for their responses to copper by a  $\beta$ -galactosidase reporter assay. As shown in Fig. 2,  $P_{CRP1}$  showed inducible characteristics in accordance with copper concentration, while  $P_{CTR4}$  was repressive, consistent with previous reports (8, 22). Despite the phylogenetic relatedness of the two genera *Histoplasma* and *Trichophyton*, the levels of induction of  $P_{CRP1}$  were considerably lower than those reported to occur in *H. capsulatum*, which reached as high as 125-fold (8), whereas only 4-fold induction was observed in the present study (Fig. 2). Similarly, fold repression of  $P_{CTR4}$  was not as high as that reported to occur in *C. neoformans* (22). On the other hand, residual activity under repressive conditions was lower in  $P_{CTR4}$  than in  $P_{CRP1}$ . Therefore,  $P_{CTR4}$  can regulate gene expression more tightly than  $P_{CRP1}$  in *A. vanbreuseghemii*, suggesting that  $P_{CTR4}$  is more reliable for genetic studies.

Real-time RT-PCR was used to study the kinetics of  $P_{CTR4}$  under repressive conditions. The expression level of *lacZ* decreased within 30 min after administration of copper (Fig. 3). This rapid response gives the  $P_{CTR4}$  knockdown system an advantage over other systems regulated by nutrient sources, which require several hours. Monteiro and De Lucas reported that in *A. fumigatus*, ammonium regulation of the *niiA* promoter requires 36 h to significantly decrease *triA* expression level, while the *alcA* promoter repressed *triA* expression after 18 h (17). Thus, the tightness and promptness of the response vary depending on the promoter. Furthermore, transcriptional repression by  $P_{CTR4}$  lasted for at least 24 h after addition of copper (Fig. 3), which may be due to excessive copper ions in liquid medium provided by high and essentially unaltered levels of copper during the testing period.

The ability of  $P_{CTR4}$  to cause phenotypic changes in *A. vanbreuseghemii* through its tight regulation of the endogenous genes *ERG1* and *TRP5* was confirmed. As shown in Fig. 5, the levels of  $P_{CTR4}$ -*ERG1* and  $P_{CTR4}$ -*TRP5* growth were markedly reduced due to repression of *ERG1* and *TRP5*, as the growth of their parental strain, Tml28, was unaffected in the presence of copper. Scanning electron microscopy of  $P_{CTR4}$ -*ERG1* showed short hyphae with swelling (Fig. 6) similar to the ultrastructural changes caused by terbinafine (20). When growing hyphae of *T. mentagrophytes* were treated with 2 ng/ml terbinafine, swollen hyphal tips were observed. Furthermore, severely damaged hyphae were observed microscopically with a higher terbinafine concentration of 20 ng/ml (20). Terbinafine has been shown to have fungicidal activity against dermatophytes (3, 23). However, no fungicidal effects caused by the repression of *ERG1* expression were observed even when  $P_{CTR4}$ -*ERG1* was grown under high (50  $\mu$ M or higher)

concentrations of copper (Fig. 5A and data not shown). One possible reason is that transcriptional repression by  $P_{CTR4}$  was not as stringent as enzymatic inhibition by terbinafine, and thus, leakage of  $P_{CTR4}$  promoter activity might allow  $P_{CTR4}$ -*ERG1* to grow slowly. Another explanation is that the fungicidal effect and hyphal disruption were due to off-target effects of terbinafine. Further studies are therefore required for clarification.

The  $P_{CTR4}$ -based system can potentially participate in elucidation of dermatophytosis mechanisms under various conditions and particularly *in vivo* studies, due to its highly sensitive responses to copper (at a level as low as 0.1  $\mu$ M  $\text{CuSO}_4$ ) (Fig. 5A). In addition, the parental strain of the host cells used in our knockdown system, *A. vanbreuseghemii* TIMM2789, is a zoophilic strain of *T. mentagrophytes* complex, and this strain has been used in a guinea pig model of tinea pedis (21, 28). Therefore, we hopefully expect that functional analyses of genes can be performed *in vivo* by infecting animals and treating them with copper-containing agents.

The applicability of any conditional knockdown system is highly correlated with the expression level of its promoter, whether it is sufficiently high to support growth of a mutant under inducible conditions or low enough to cause phenotypic changes under repressive conditions. Furthermore, the expression level of a conditional promoter may vary from one locus to another. The successful application of the  $P_{CTR4}$ -based system to *ERG1* and *TRP5* loci suggested that this  $P_{CTR4}$ -based conditional knockdown system can be applied to most genes. Several other genes are currently being tested in our laboratory.

In conclusion, the two copper-responsive promoters  $P_{CTR4}$  and  $P_{CRP1}$  are the first to be reported for dermatophytes. The applicability and tightness of  $P_{CTR4}$  in *A. vanbreuseghemii* were confirmed for two loci, *ERG1* and *TRP5*. Therefore, the  $P_{CTR4}$ -based conditional knockdown system represents a potentially powerful tool for identification of essential genes. This system will contribute to further progress in molecular genetic research in dermatophytes.

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