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Brief Report

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Terbinafine resistance conferred by multiple copies of the salicylate 1-monooxygenase gene in *Trichophyton rubrum*

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Abstract

Resistance to antifungals is a leading concern in the treatment of human mycoses. We demonstrate that the *salA* gene, encoding salicylate 1-monooxygenase, is involved in resistance of the dermatophyte *Trichophyton rubrum* to terbinafine, one of the most effective antifungal drugs against dermatophytes. A strain with multiple copies of *salA* was constructed and exhibited elevated expression of *salA* and increased terbinafine resistance. This reflects a mechanism not yet reported in a pathogenic fungus.

Key words: salicylate 1-monooxygenase, terbinafine, dermatophyte, naphthalene, *T. rubrum*.

The dermatophyte *Trichophyton rubrum* is one of the most common causes of skin and nail mycoses in humans. Infection is often chronic and recurrent following conventional treatments, particularly in immunocompromised patients or when associated with additional risk factors, such as predisposing health or environmental conditions.[1]

Terbinafine (TRB) is an antifungal that is highly active against dermatophytes. This drug belongs to the chemical class of allylamines and inhibits squalene epoxidase, a key enzyme in the ergosterol biosynthetic pathway. This inhibition leads to accumulation of squalene, depletion of ergosterol, and fungal growth inhibition.[2–4] Mutations in or overexpression of the squalene epoxidase gene have been found to be associated with TRB resistance in fungi, such as *Aspergillus fumigatus, Aspergillus nidulans*, and *T. rubrum*.[5–9] In *A. fumigatus*, plasmidial extra copies of the squalene epoxidase gene conferred resistance to TRB. After successive culture passages without TRB, plasmid cure was detected and associated with loss of TRB resistance.[7] An additional form of TRB resistance involving the *salA* gene, which codes for a salicylate 1-monooxygenase, was suggested in *A. nidulans*.[10] The role of salicylate 1-monooxygenase, an enzyme in the naphthalene degradation pathway, has been well-characterized in *Pseudomonas*.[11,12] In this work, we evaluated the involvement of *salA* in the resistance and response of *T. rubrum* to TRB.

A strain of *T. rubrum* carrying multiple *salA* copies was constructed. The *T. rubrum salA* gene is identified with accession number TERG_03218 in the NCBI database (https://www.ncbi.nlm.nih.gov/gene/?term=terg_03218). Briefly, a 2247-bp fragment containing the *salA* gene and its promoter region from wild-type (WT) *T. rubrum* strain CBS118892 (Centraalbureau voor Schimmelcultures [CBS]) was amplified by polymerase chain reaction

(PCR) using salA_F (5′-GCGGCTGGGATATTATTGC-3′) and salA_R (5′-CTGGACATGTTCCAACAATACCT-3′) primers. This fragment was cloned into the pGEM-T vector (Promega), generating the p*salA* plasmid, which was subsequently transformed into protoplasts of the same strain, as previously described.[13] The transformed strain, HLS01, was selected on Sabouraud medium plates containing 0.1 μg/ml of TRB following incubation for 21 days at 28 °C.[10,13] As a control, protoplasts were transformed with the empty pGEM-T vector (lacking the *salA* gene), plated on the same medium containing TRB (0.1 μg/ml), and incubated for the same period, but we did not get any colony. Subsequently, the minimal inhibitory concentration (MIC) of TRB was determined, according to the CLSI M38-A2 protocol.[14] The MIC was determined to be 0.0244 μg/ml TRB for the *T. rubrum* WT strain and 0.0976 μg/ml TRB for the HLS01 strain. Therefore, compared to the WT strain, HLS01 exhibited increased resistance to TRB. However, HLS01 cultured in the absence of TRB presented a gradual loss of resistance to TRB, and after six serial passages on culture medium without TRB, HLS01 displayed irrelevant growth in the presence to TRB, suggesting that the HLS01 strain had been cured of psalA. The MIC value of the cured HLS01 strain was 0.0244 μg/ml TRB.

DNA was extracted from each strain, as previously described,[13] cleaved with *Hind*III and *Stu*I, and probed for the presence of the *salA* gene with a 507-bp fragment of *salA* obtained by PCR using salApr_F (5′-GTGCCGGAACTACCTACGTG-3′) and salA_R primers (Fig. 1A). The DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) was used for labeling and detection. Southern blot analysis confirmed the presence of a single copy of *salA* in the WT and cured HLS01 strains and the presence of at least an extra copy of *salA* in HLS01, indicating that the p*salA* was lost in the cured HLS01 strain (Fig. 1B). A phenotypic assay for assessment of TRB susceptibility in a solid medium was performed for these three strains. The initial growth of the cured HLS01 strain in the presence of TRB remained restricted to the inoculum point and did not progress in the following 21 days, whereas HLS01 was able to grow (Fig. 2A).

The transcription profile of the *salA* gene was analyzed after exposing *T. rubrum* strains to TRB, as previously described.[15,16] Briefly, approximately 10^6 conidia were inoculated into 100 ml of Sabouraud broth and incubated at 28 °C and 100 rpm for 96 h. Mycelia were transferred to RPMI 1640 medium (Sigma) with or without 0.2 μg/ml TRB and incubated for 24 h at 28 °C and 100 rpm. The experiments were performed in triplicate. RNA isolation, gene expression, and statistical analyses were performed as previously described.[17]

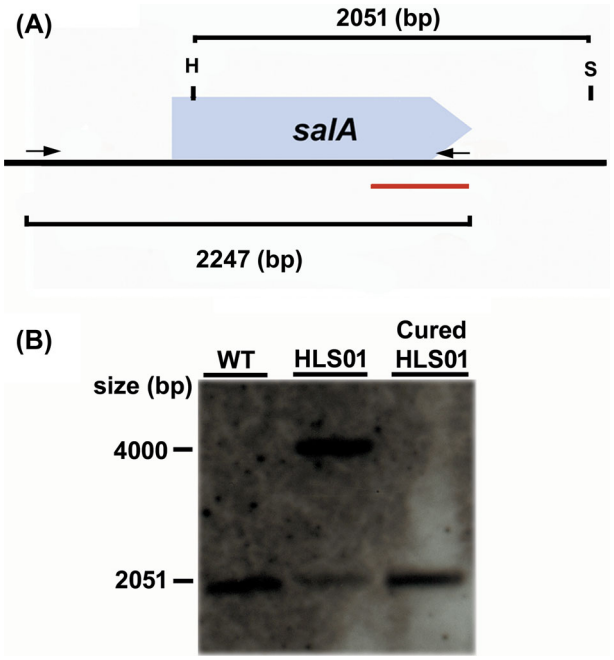

Figure 1. Generation of a *Trichophyton rubrum* strain carrying multiple *salA* copies. **(A)** Schematic representation of the *salA* gene (box) in the genome of *T. rubrum* WT. The arrows represent the position of the forward and reverse primers. Restriction sites for *Hind*III and *Stu*I are represented by H and S, respectively. Bar represents the *sal*A fragment used as a probe. **(B)** Southern blot analysis of the DNA from WT, HLS01, and cured HLS01 strains digested with *Hind*III and *Stu*I and probed with a *salA* fragment. This Figure is reproduced in color in the online version of *Medical Mycology*.

For quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *salA*, the primers qPCRsalA_F (5′-GTGCCGGAACTACCTACGTG-3′) and qPCRsalA_R (5′-GACACACCTTGTCCCGAAGT-3′) were used. The endogenous gene *rpb2* was used as a standard, as previously described.[18]

Gene expression analysis revealed that *salA* was transcriptionally upregulated in *T. rubrum* in response to TRB. Moreover, expression of *salA* was higher in HLS01 than that in WT even in the absence of TRB, which is consistent with a *salA* multicopy effect. Following p*salA* cure, *salA* expression in cured HLS01 strain was lower than in HLS01 and was similar to that in the WT strain, compatible with the loss of extra copies of *salA* (Fig. 2B).

These results indicate the involvement of *salA* in the resistance and response of *T. rubrum* to TRB. Resistance to TRB mediated by multiple *salA* copies was also observed in another dermatophyte, *Trichophyton interdigitale*, transformed with the *salA* gene of *A. nidulans* (data not shown), suggesting a general phenomenon among fungi.

The involvement of salicylate 1-monooxygenase in TRB resistance was first reported in *A. nidulans*.[10] Salicylate 1-monooxygenase is an enzyme that participates in the naphthalene degradation pathway, converting the intermediate

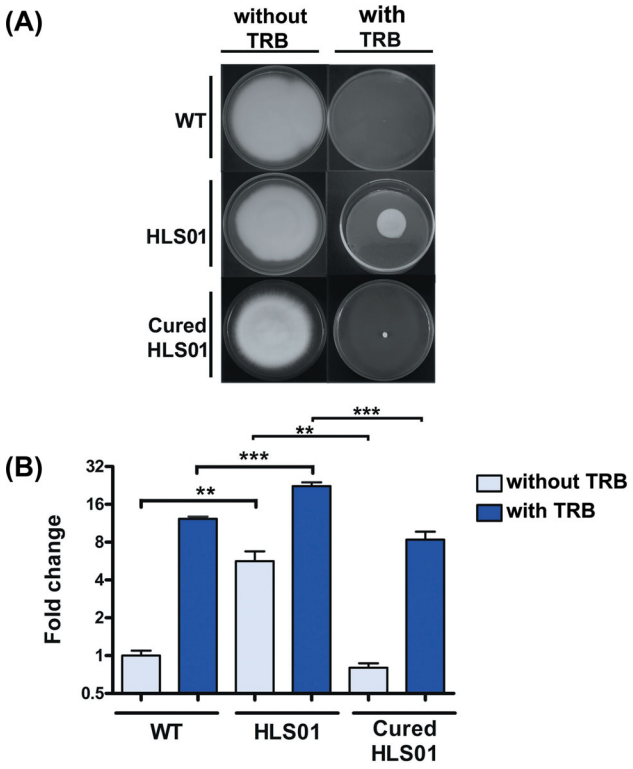

Figure 2. Involvement of *sal*A gene in resistance of *Trichophyton rubrum* to terbinafine (TRB). **(A)** Phenotypic TRB susceptibility assay. Mycelia of *T. rubrum* wild-type (WT), HLS01 (carrying plasmid p*salA*, harboring the salicylate 1-monooxygenase gene), and cured HLS01 strains were inoculated onto the center of Sabouraud agar plates in the absence or presence of 0.1 μg/ml TRB, and incubated at 28 °C for 21 days. **(B)** Transcription profile of *salA* gene of *T. rubrum* in response to terbinafine (TRB). Expression of *salA* was determined by qRT-PCR after mycelia incubation of WT, HLS01, and cured HLS01 strains in the absence of TRB or presence of 0.2 μg/ml TRB for 24 h. Statistical significance was determined by ANOVA followed by Tukey's ad hoc test and is indicated by asterisks (** $P < .01$; *** $P < .001$). This Figure is reproduced in color in the online version of *Medical Mycology*.

metabolite salicylic acid into catechol.[11] The antifungal drug TRB contains a naphthalene nucleus in its molecular structure. Thus, the mechanism of resistance to TRB described here possibly occurs via degradation of the naphthalene portion of TRB, mediated by the action of salicylate 1-monooxygenase. Recently, the role of fungal salicylate 1-monooxygenase in salicylic acid degradation was functionally demonstrated in the endophytic fungus *Epichloë festucae* and in the phytopathogen *Ustilago maydis*.[19,20]

In conclusion, our results show that the *salA* gene plays a role in the resistance and response of *T. rubrum* to TRB. This mechanism has not been previously reported in dermatophytes. Our findings therefore provide insight into the molecular strategies used by fungal pathogens to cope with exposure to TRB, a drug commonly used in the treatment of dermatophytoses.

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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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