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The bZIP Ap1 transcription factor is a negative regulator of virulence attributes of the anthropophilic dermatophyte *Trichophyton rubrum*

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ABSTRACT

Trichophyton rubrum is a fungus that causes chronic skin and nail infections in healthy individuals and immunocompromised patients. During infection, *T. rubrum* invades host cutaneous tissues by adapting to the acidic pH and the innate immune response of the host. Several genes are upregulated during the growth of *T. rubrum* in substrates found in human tissue, including the *ap1* gene, which codes for the transcription factor Ap1. Here, we generated a null mutant strain by deleting the *T. rubrum ap1* gene and performed a functional analysis of this gene. Our results showed that the Δ*ap1*mutant increased its growth in nail fragments and co-cultures with keratinocytes compared to the wild type. Furthermore, the mutant displayed hyperpigmentation, thickening of the conidia cell wall, increased conidia susceptibility to calcofluor-white compared to the wild type, and loss of control of the keratinolytic activity. Although the *ap1* gene was upregulated during exposure to the antifungal drugs amphotericin B, nystatin, and terbinafine, its deletion did not alter the fungal susceptibility to these drugs, revealing the role of the *ap1* gene in the physiological response to the stress caused by these drugs, but not in their resistance. Moreover, *ap1* was also involved in the oxidative stress response caused by menadione, but not paraquat or hydrogen peroxide. These findings indicate that the *ap1* gene plays a role in the negative control of virulence-related attributes and may contribute to the chronicity of nail infection caused by *T. rubrum*.

1. Introduction

Cutaneous mycoses are the most frequent fungal infections affecting healthy and immunocompromised patients worldwide. The *Candida* genus and filamentous fungi such as dermatophytes may cause these infections (Hube et al., 2015). Dermatophytes are keratinophilic fungi that degrade keratin, providing their nutrients. *Trichophyton rubrum,* an anthropophilic dermatophyte, is the most frequently isolated species from human nails and skin lesions, also causing subcutaneous and deep infection in both healthy and immunocompetent individuals (Petrucelli et al., 2020; Sang et al., 2021; Zhu et al., 2021).

During infection, dermatophytes must sense the host tissue to scavenge nutrients and evade immune response mechanisms, aiming for their complete installation. This sensing triggers several signaling pathways targeting the activation of a diverse range of genes, allowing fungal installation, growth, and maintenance of dermatophytes in the host tissue (Martinez-Rossi et al., 2021, 2017; Martins et al., 2020). We previously showed the upregulation of some genes during *T. rubrum* growth on keratin medium and *ex vivo* infection of human skin and nails. Among them, the *ap1* gene showed high transcript levels during *ex vivo* infection and keratin growth, suggesting its role in virulence and thus in the pathogenic process (Peres et al., 2016).

This gene encodes the Ap1 transcription factor (TF), belonging to the bZIP family that comprises several leucine zipper proteins, and regulates genes that participate in various cellular processes. (Simaan et al., 2019). In yeasts under oxidative stress, Ap1 translocates into the nucleus, promoting the expression of the enzymes catalase and superoxide dismutase (Rodrigues-Pousada et al., 2019, 2004). In the pathogenic mold *Aspergillus fumigatus*, Ap1 has been implicated in oxidative stress; however, its deletion did not affect virulence in a murine infection model (Qiao et al., 2008). In *Magnaporthe grisea*, the *ap1* gene was highly expressed in the conidia and during invasive hyphal growth. Moreover, conidia production, pigmentation, production of peroxidases, and infection of plant leaves were decreased in an Ap1 mutant strain of this

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Table 1
Primers used in this study, for gene replacement and expression (qPCR).

Gene name - description	Primer ID	Sequence (5'- 3') (*)
	Amplification of the 5'flanking sequence:	
ap1 - AP1 bZIP transcription factor (TERG_ 02940)	ap15'UTRF (P1)	GTGGTAAGCCATCGTTCTGG
	ap15'UTRR (P2)	ACTGGCCGTCGTTTTACTGTCGTCTGGATTGAAGCTG
	Amplification of the 3'flanking sequence:	
	ap13'UTRF (P3)	GTCATAGCTGTTTCCTGGTCGACAACCTCTGCACTCA
	ap13'UTRR (P4)	CAGCAACAACAAGCTCCATC
	Fusion PCR:	
	ap15'UTRnestF (P5)	TGTGAACCGGTGCTACTGTC
	ap13'UTRnestR (P6)	TCAAAATCGACCACCTCCAC
	qPCR:	
	ap1F (P7)	GAAACAGCTTTTCCCCAACA
	ap1R (P8)	TCATATGAGCCCCAGGTAGG
rpb2 – RNA polymerase 2 (TERG_05742)	rpb2F	TGCAGGAGCTGGTGGAAGA
	rpb2R	GCTGGGAGGTACTGTTTGATCAA
tub-beta tubulin (TERG_07904)	tubF (**)	CGGTATGATGGCCACTTTCT
	tubR	CTGACCTGGGAAACGAAGAC
rlp38 - ribosomal protein L38	rlp38F	TCAAGGACTTCCTGCTCACA
	rlp38R	AAAGGTATCTGCTGCATCGAA
il-6 – interleukin-6	il-6F	ACTCACCTCTTCAGAACGAATTG
	il-6R	CCATCTTTGGAAGGTTCAGGTTG
il-8 – interleukin-8	il-8F	ACTGAGAGTGATTGAGAGTGGAC
	il-8R	AACCCTCTGCACCCAGTTTTC
tnfa – tumor necrosis factor alpha	tnfF	GAGGCCAAGCCCTGGTATG
	tnfR	CGGGCCGATTGATCTCAGC
il-1b – interleukin-1beta	il-1bF	TTCGACACATGGGATAACGAGG
	il-1bR	TTTTTGCTGTGAGTCCCGGAG

(*) Underlined sequences represent the complementary regions with the M13 universal primers, used to amplify the *hph* gene.
(**) tub primers were used as a control to confirm the knockout by PCR.

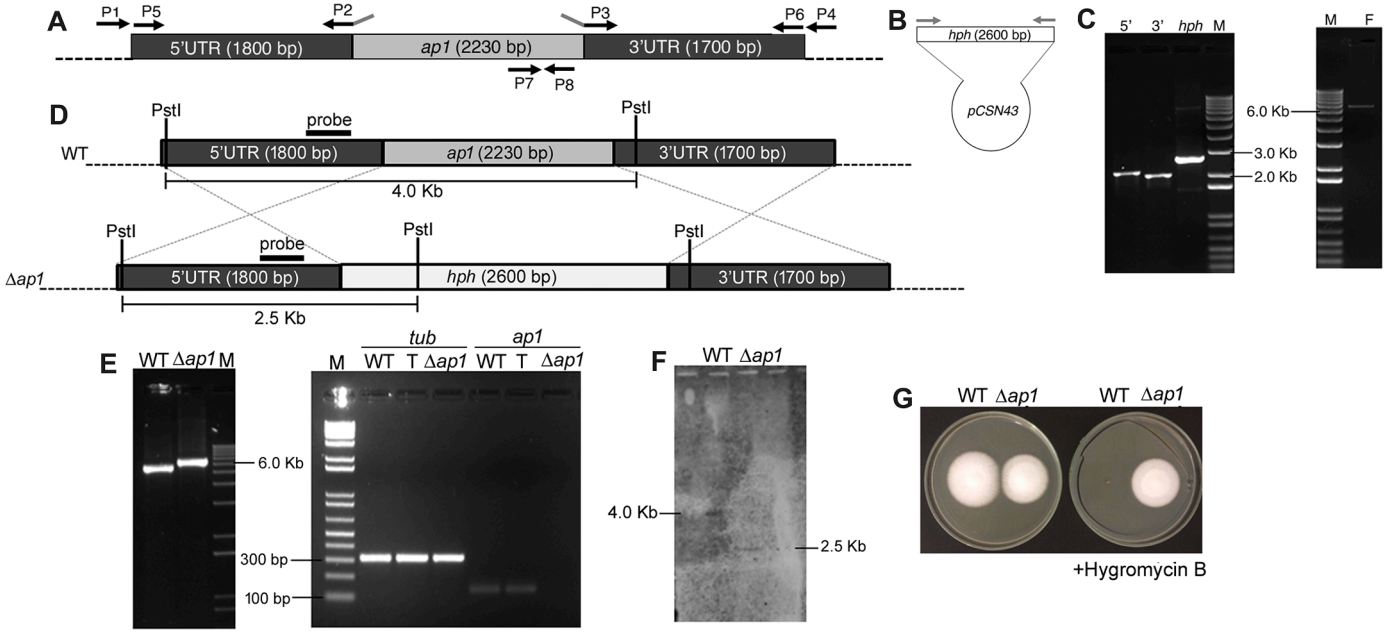

Fig. 1. Generation of *T. rubrum* Δ*ap1* strain. (A) Schematic representation of the WT locus shows the primers used to generate the knockout cassette and validate the gene replacement by PCR. (B) pCSN43 plasmid containing the hygromycin B resistance gene *hph* (gray arrows represent the M13 universal primers annealing sites). (C) Agarose gel electrophoresis of the fusion PCR used to obtain the knockout cassette, showing the PCR products of the 5' and 3' flanking regions of the *ap1* gene, the *hph* reporter gene (left panel), and the fusion fragment [F] 5':*hph*:3' (right panel). (D) Schematic representation of the wildtype (WT) and mutant (Δ*ap1*) loci after homologous recombination. (E) PCR amplification of the *ap1* loci of the WT and the knockout mutant strains, using primers (P1 and P4) annealing outside the knockout cassette (left panel), and the primers (P7 and P8) that amplify an internal fragment of the *ap1* gene (right panel). Primers for the tubulin gene (*tub*), were used as a control (right panel). T: A hygromycin-resistant transformant but with the *ap1* gene intact. (F) Southern blot of the WT and Δ*ap1* strains, the genomic DNA of both strains was digested with PstI (probe is shown in d). (G) WT and Δ*ap1* strains growth on Sabouraud agar (SDA) in the absence or presence of 450 μg/ml hygromycin B.

fungus. (Guo et al., 2011). In *Talaromyces marneffei*, the *ap1* gene is essential for cell development, oxidative and nitrosative stress, and virulence (Dankai et al., 2016).

The high prevalence of *T. rubrum* in human cutaneous infections worldwide and its anthropophilic nature prompted the question of whether the role of the *ap1* gene in cutaneous disease-related pathogens might correlate with that described for systemic mycosis agents and plant pathogens. This knowledge is crucial for understanding the

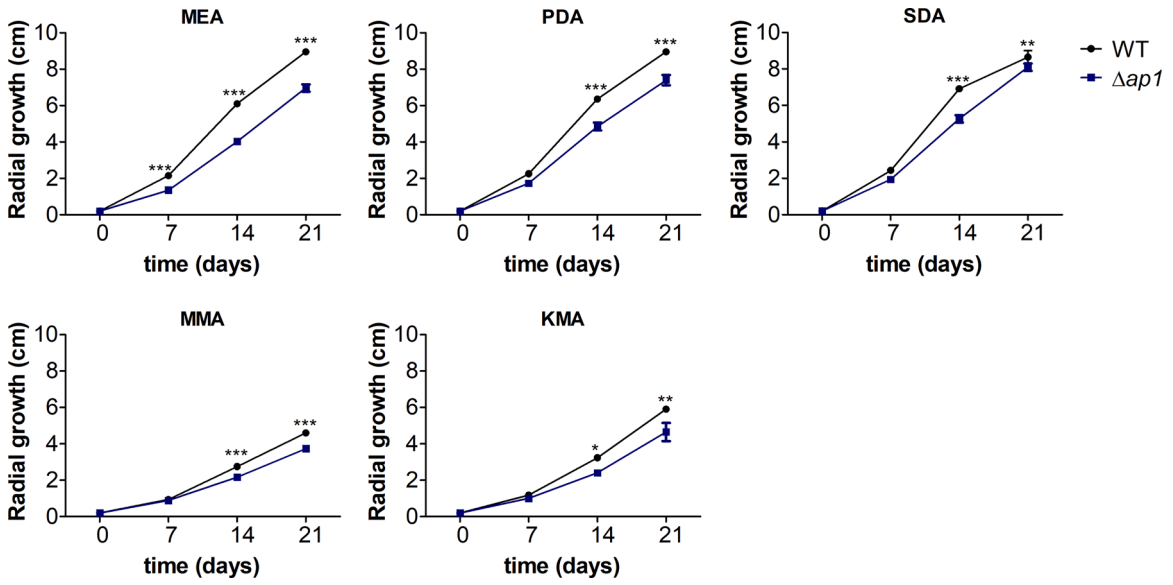

Fig. 2. *ap1* gene deletion alters *T. rubrum* growth on different agar media. *T. rubrum* strains were grown on agar plates, and the radial growth was measured in the indicated time points. Values are the average from three experiments and respective standard deviations. Statistical analysis was performed using ANOVA followed by Tukey's ad hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). MEA: Malt Extract Agar; PDA: Potato Dextrose Agar; SDA: Sabouraud Dextrose Agar; MMA: Minimal Medium Agar; KMA: Keratin Medium Agar.

pathogenic process of dermatophytosis to develop novel therapeutic and prophylactic strategies. Indeed, variations in the role of *ap1* among several fungi, especially regarding virulence, drug susceptibility and response, and osmotic and oxidative stress, have been reported (Simaan et al., 2019). This work describes, for the first time, the role of the *ap1* gene in a pathogenic filamentous fungus that causes cutaneous infections.

Here, we generated a *T. rubrum* strain with a deletion of the *ap1* gene to investigate its functional role under several environmental conditions.

2. Materials and Methods

2.1. Strain and growth conditions

T. rubrum strain CBS 119892 (WT) was kindly donated by the CBS-KNAW Fungal Collection (Westerdijk Fungal Biodiversity Institute). *T. rubrum* culture was carried out in Malt Extract Agar (MEA, 2 % malt extract (w/v), 2% glucose (w/v), 0.1% peptone (w/v) – Sigma Aldrich) at 28 °C. Conidia were recovered by scraping the mycelium from 15-day MEA plates flooded with sterilized 0.9% NaCl, followed by vortexing and filtration through glass wool. After centrifugation, the microconidia concentration was estimated by counting on the Neubauer chamber. Other media used in this study were: Sabouraud Dextrose Agar (SDA - 2% dextrose (w/v), 1% peptone (w/v), pH 5.7), Minimal Medium (MM) pH 5.0 (Cove, 1966), Potato Dextrose Agar (PDA - 0.4% potato extract (w/v) (Sigma Aldrich), 2% dextrose (w/v), pH 5.7), and Keratin Medium (KM - 2.5 g/L keratin powder from hooves and horns - MP Biomedicals, pH 5.5). The solid medium contained 2% agar (w/v).

2.2. Culture conditions for ap1 gene expression analysis

To evaluate *ap1* gene expression in response to sub-lethal concentrations of antifungal drugs, cell wall, and membrane stress compounds, 1×10^6 conidia from the WT *T. rubrum* strain were cultured in 100 ml liquid Sabouraud for 96 h at 28 °C (Jacob et al., 2012; Persinoti et al., 2014). After germination, 1.75 μg/ml acriflavine (ACR), 0.07 μg/ml amphotericin B (AMB), 0.2 μg/ml nystatin (NYT), or 0.2 μg/ml terbinafine (TRB) was added to the medium and incubated at 28 °C for 3 h or 24 h, under agitation. Concentrations represent 70% of minimal inhibitory concentration (MIC) values of each drug for this *T. rubrum* strain in the Sabouraud broth medium, which were established before the expression assays by serial microdilution analysis. To evaluate stress response, 0.5 M NaCl, 0.01% sodium dodecyl sulfate (SDS), 200 μg/ml calcofluor-white (CFW, Sigma Aldrich), 200 μg/ml Congo red (CR, Sigma Aldrich), or 1.6 mM H_2O_2 (Sigma Aldrich) was added to the medium and incubated for 1 h or 3 h at 28 °C. The resulting mycelia were recovered by filtration and stored at -80°C until RNA extraction.

2.3. Total RNA extraction and qPCR analysis

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific), following the manufacturer's instructions. Then, 1 μg of total RNA was treated with RNase-free DNase I (Sigma Aldrich) and converted to cDNA using the High-Capacity cDNA synthesis kit (ThermoFisher Scientific) according to the manufacturer's protocols. qPCR was performed with 50 ng cDNA, 1X SYBR Green master mix (Thermo Fisher Scientific), and 300 nM of each primer using the StepOne Real-Time PCR System (ThermoFisher Scientific), with standard parameters. Relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method, using the control cultures as reference samples and the *rpb2* gene as the endogenous control (Jacob et al., 2012). The primers used are listed in Table 1.

2.4. T. rubrum ap1 gene deletion

The deletion of the *ap1* gene (TERG_02940) from the CBS 119892 *T. rubrum* strain was carried out by homologous gene replacement (Fig. 1A). According to a previous report, the replacement cassette was obtained by fusion PCR (Yu et al., 2004), using the primers listed in Table 1. Briefly, upstream and downstream flanking regions of the *ap1* gene were amplified (5'UTR – 1.8 kb; 3'UTR – 1.7 kb) and fused with the 2.6 Kb hygromycin B phosphotransferase (*hph*) gene, amplified from the pCSN43 plasmid, using M13 forward and reverse universal primers. The fusion fragment was then amplified using nested primers, generating a product of 6.1 Kb (Fig. 1C), and purified with Wizard® SV Gel and PCR Clean-Up System (Promega).

The linear fusion fragment was used to transform *T. rubrum* protoplasts, obtained by growing 1×10^9 *T. rubrum* conidia in 100 ml liquid

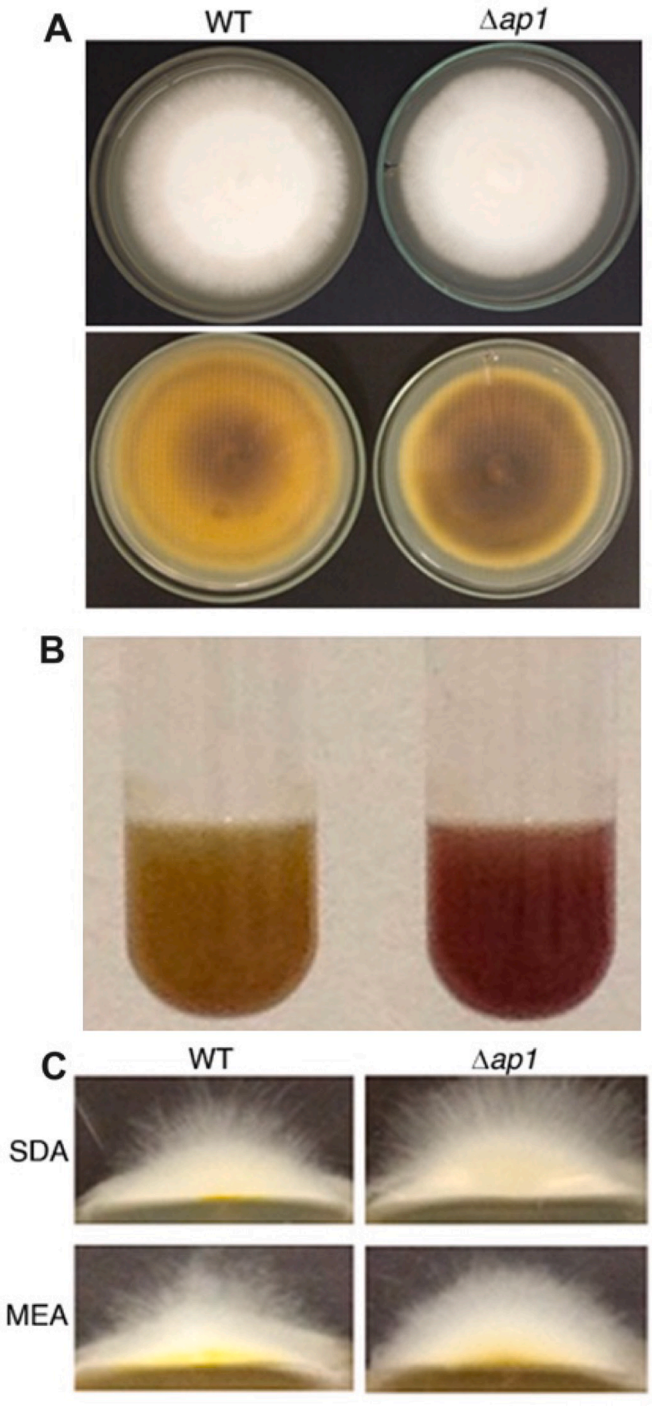

Fig. 3. *ap1* gene deletion alters *T. rubrum* pigmentation and aerial hyphae formation. (A) Growth of wildtype (WT) and Δ*ap1* strains in Sabouraud agar (SDA) for 21 days at 28 °C. (B) Conidial suspension of the strains after SDA growth. (C) Aerial hyphae formation in SDA and MEA, side views of the colonies were observed after 5 days at 28 °C.

Sabouraud for 40 h at 28°C, under agitation. After filtration, the resulting mycelium was transferred to 25 ml of a lysing buffer (1.1 M KCl, 0.1 M citric acid, pH 5.8; containing 100 mg lytic enzymes from *Trichoderma harzeanum* - Sigma Aldrich, 100 mg lysozyme - Sigma Aldrich, and 100 mg bovine serum albumin - Sigma Aldrich), incubated at 30 °C for 5 h, and then filtered through Miracloth (Calbiochem®). The supernatant containing the protoplasts was added to 20 ml cold STC solution (1.2 M sorbitol, 50 mM Tris, 10 mM $CaCl_2$; pH 7.2) and centrifuged at 2000 × g for 10 min in a swing-out rotor. Protoplasts were washed twice with 20 ml cold STC, centrifuged at 2000 × g for 10 min, and resuspended in 1 ml of cold STC buffer. Subsequently, 100 µg of transforming DNA was diluted in 100 µl of cold STC, added to the protoplast solution, and incubated for 20 min at room temperature. The negative control consisted of the same procedure without adding DNA. Next, 1 ml polyethylene glycol solution (25% diluted in STC) was added to the protoplasts and incubated at room temperature for 20 min. The protoplasts were inoculated onto SDA plates containing 1.2 M sorbitol and 450 µg/ml hygromycin B (hygB) to select transformed fungal cells, followed by incubation for 15 days at 28°C. Hygromycin-resistant colonies were transferred to fresh SDA plates containing hygB.

To evaluate homologous recombination and gene replacement, we extracted genomic DNA (gDNA) from stable resistant colonies and used them for PCR with the primers listed in Table 1. gDNA was isolated by grinding mycelia in 1 ml lysis buffer (5 mM EDTA, 1% SDS (w/v), pH 8.0), followed by incubation for 10 min at 68°C and centrifugation at 11,000 × g for 5 min. The supernatant was collected, and 30 µl of 200 mM potassium acetate was added and incubated on ice for 1 h. After extraction with phenol:chloroform:isoamyl acid (24:25:1) extraction, gDNA was precipitated with ethanol, washed twice with 70 % ethanol, and air-dried. gDNA was resuspended in Tris-EDTA buffer (1 mM EDTA; 10 mM Tris-HCl (pH 7.5)) containing RNase A (50 µg/ml) and incubated at 65°C for 10 min. Southern blotting of the WT and mutant strains was performed after digesting the gDNA with PstI restriction enzyme, as shown in Fig. 1D, f using the DIG DNA labeling and detection kit, following the manufacturer's procedure (Sigma-Aldrich).

2.5. Drug susceptibility test

These experiments aimed to compare WT and mutant strains concerning the susceptibility profile to antifungals and other cytotoxic drugs. Sabouraud agar plates were incubated with 5 to 100 µg/ml fluconazole (FLZ), 0.01 to 0.4 µg/ml itraconazole (ITZ), 0.01 to 0.4 µg/ml NYT, 0.01 to 0.4 µg/ml AMB, 0.002 to 0.2 µg/ml TRB, 0.002 to 0.2 µg/ml 4-nitroquinoline 1-oxide (4-NQO), 0.1 to 2 µg/ml griseofulvin (GRI), 0.1 to 2 µg/ml benomyl (BEN), 5 to 100 µg/ml caspofungin (CASP), or 0.5 to 10 µg/ml acriflavine (ACR). Each well of the 24-well plates containing 1.5 ml of Sabouraud agar, with or without each drug, was inoculated with 1×10^5 *T. rubrum* conidia and incubated at 28°C for 7 days. MIC assay by microdilution in RPMI was also performed for the same drugs, according to the M38A2 protocol established by the Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi (CLSI, 2008). Three independent experiments were performed.

2.6. Environmental stress assay

Osmotic, cell wall, and membrane stress were induced by adding NaCl, CFW, CR, or SDS to Sabouraud agar plates before inoculation with 1×10^5 *T. rubrum* conidia (Silva et al., 2020; Martins et al., 2019). Oxidative stress was induced by growing the strains in SDA containing oxygen peroxide (H_2O_2), paraquat, or menadione. All plates were incubated for seven days at 28°C. Drug concentrations are indicated in the respective figures, and they were stablished based on other reports for filamentous fungi and *T. rubrum* (Bitencourt et al., 2020).

2.7. Growth curves and keratinolytic activity assay

We constructed the growth curve by inoculating 1×10^4 *T. rubrum* conidia onto the center of each agar plate (MEA, PDA, SDA, MMA, or KMA) and measuring the colony diameter at three-time points. Conidia (1×10^4) from WT and mutant strains were germinated for 7 and 14 days at 28°C in liquid keratin medium. Subsequently, the dry mycelia were weighed, and the media were recovered by filtration to detect the pH and quantify the keratinolytic activity, as previously described (Lang et al., 2020).

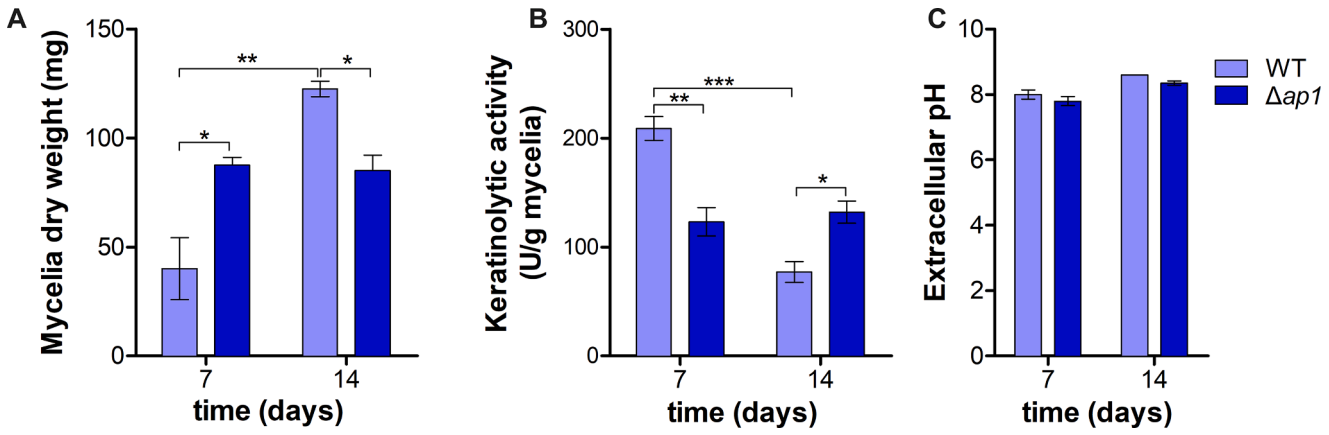

Fig. 4. *ap1* gene deletion alters *T. rubrum* growth on keratin and keratinolytic activity. (A) Mycelia dry weight of the wildtype (WT) and Δ*ap1* strains after growth for 7 and 14 days in Keratin Medium broth (KM) at 28 °C. (B) Extracellular keratinolytic activity after 7 and 14 days of growth in KM. (C) Extracellular pH after 7 and 14 days of growth in KM. Values are the average from three experiments and respective standard deviations. Statistical analysis was performed using ANOVA followed by Tukey's ad hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.8. Ex vivo skin and nail infection

We used a previously described protocol for the growth of *T. rubrum* on human skin or nails (Peres et al., 2016). Briefly, human skin was obtained from healthy donors who underwent abdominal surgery at the University Hospital of the Ribeirão Preto Medical School of the University of São Paulo (HC-FMRP-USP). The adipose layer was removed, and the upper layer (1 cm^2) was infected with 1×10^4 *T. rubrum* conidia and incubated in a humid chamber maintained at 28°C for 72 h. Fungal growth was evaluated by scanning electron microscopy (SEM), as previously described (Lang et al., 2020). For nail infection, 1×10^4 *T. rubrum* conidia were inoculated on healthy human nail fragments, and analyzed by optical microscopy after 72 h. For extended periods of growth, nail fragments were placed in a water agar plate, and 1×10^4 *T. rubrum* conidia were inoculated on each nail fragment and incubated at 28°C for 21 days, with subsequent analysis by SEM. These experiments were approved by the local ethics committee (Protocol No.8330/2009).

2.9. Microscopy

The conidial morphology of both strains was analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and *ex vivo* human skin infection was analyzed by SEM. The cell wall thickness was measured at 10 points for each conidium, in a total of 20 conidia from each strain, using ImageJ software.

Briefly, for SEM, conidia and skin fragments were fixed with 3% glutaraldehyde in 0.1 % phosphate buffer (v/v) (pH 7.2) at 4 °C for 2 h. After washing in 0.1 % phosphate buffer (pH 7.2), samples were post-fixed with 1% osmium tetroxide (v/v) for 2 h and dehydrated in an ethanol gradient. Samples were sputter-coated with gold and analyzed using a JEOL JSM-6610 LV scanning electron microscope at an acceleration of 25 kV. For TEM, conidia were harvested from 15-days Sabouraud agar plates and fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH 7.2) for 24 h following post-fixation with 1% osmium tetroxide for 1 h at room temperature. After washing in water, conidia were treated with 0.5% uracil acetate, dehydrated in an ethanol gradient, and embedded in epoxy resin at 60°C for 48 h. After washing with propylene oxide, the samples were cut into ultrathin sections and analyzed using the JEOL JEM-100 CXII electron microscope and a Hamamatsu ORCA-HR digital camera.

2.10. Keratinocyte's interaction and cytokine analysis

T. rubrum conidia were co-cultured with the HaCat human keratinocyte cell line for 48 h, as previously described (Bitencourt et al., 2020). First, a monolayer of HaCat cells was cultured in RPMI 1640 supplemented with 5% FBS (v/v) in a 25-cm^2 tissue culture flask at a density of 2.5×10^5 cells/ml, at 37 °C for 24h in a humidified atmosphere with 5% CO_2. Then, 1×10^5 *T. rubrum* conidia from each strain were inoculated into flasks containing the HaCat monolayer. No conidia were added in the control group. After 48 h, infected keratinocytes were stained using May-Grünwald-Giemsa and analyzed by optical microscopy. Cells from human keratinocytes were recovered with TRIzol reagent (ThermoFisher Scientific) for total RNA extraction, as described above. Cytokine analysis of these cells was performed by qPCR using the primers listed in Table 1 for IL-6, IL-1β, IL-8, and TNF-α coding genes and using the *rlp38* gene as an endogenous control (Firat et al., 2014). Primers were designed using the PrimerBank (Wang et al., 2012).

2.11. Statistical analysis

Data represent the mean values from three independent assays and respective standard deviations. Significant statistical differences were determined by one-way ANOVA and Tukey's post-test or Student's t-test, using GraphPad Prism 5 software.

3. Results

3.1. T. rubrum ap1 gene deletion

We deleted the *ap1* gene by employing gene replacement by homologous recombination to investigate the role of the Ap1 TF in *T. rubrum* biology and pathogenicity (Fig. 1). The replacement cassette was obtained by fusion PCR (Fig. 1A, C) and using the hygromycin B resistance gene (Fig. 1B) as a selection marker. PCR and Southern blotting confirmed homologous recombination and gene replacement. PCR analysis (Fig. 1E) showed amplification of distinct fragment sizes using primers flanking the *ap1* locus in the WT and the mutant strain (Δ*ap1*) of 5.7 kb and 6.3 kb, respectively (Fig. 1E, left panel). We also showed a lack of amplification of the 126 bp corresponding to the *ap1* gene fragment in the mutant strain (Fig. 1E, right panel). Moreover, we observed different band sizes after hybridization with the same probe, 4.0 kb in the WT and 2.5 kb in the Δ*ap1* (Fig. 1F). We tested the knockout strain for mitotic stability based on hygromycin B (hyg B) resistance after five passages in the absence of the drug (Fig. 1G).

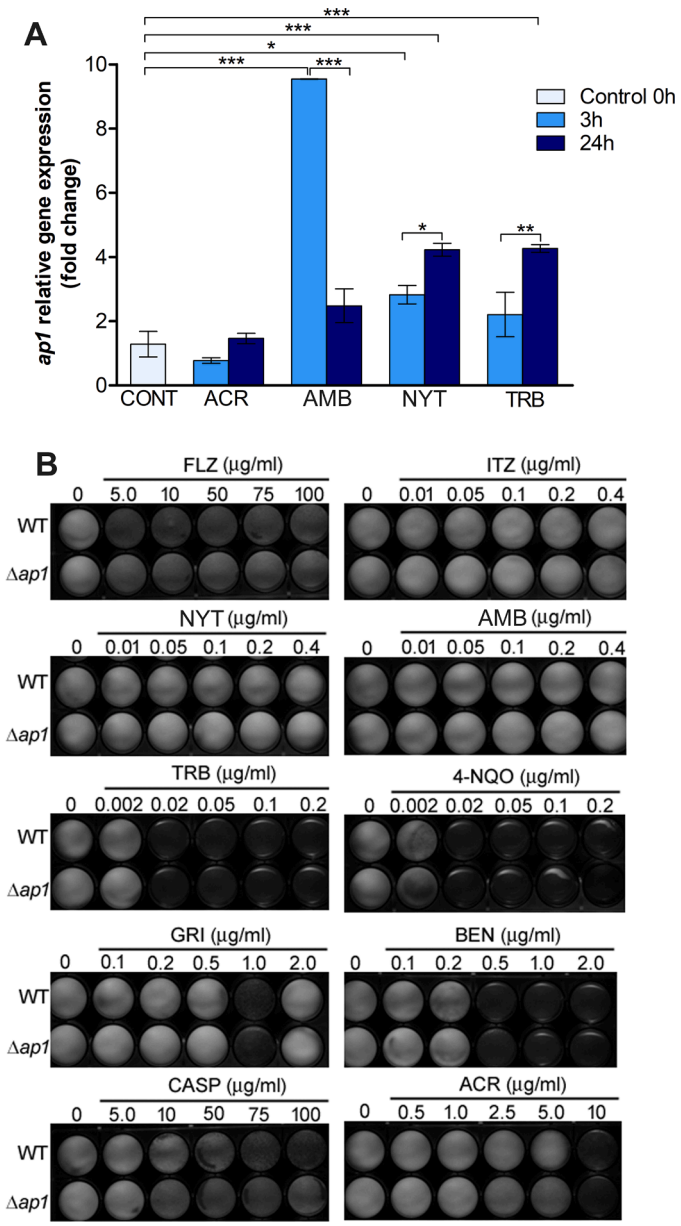

Fig. 5. *ap1* is involved in *T. rubrum* antifungal drug response but not susceptibility. (A) qPCR of the *ap1* gene after exposure of wild type strain to indicated antifungal drugs. Values are the average from three experiments and respective standard deviations. Statistical analysis was performed using ANOVA followed by Tukey's ad hoc test (*$p < 0.05$; ***$p < 0.001$). (B) Growth of the strains in Sabouraud agar containing the indicated antifungal and cytotoxic drugs. Strains were grown for 7 days at 28 °C. ACR: acriflavine; AMB: amphotericin B; NYT: nystatin; TRB: terbinafine; FLZ: fluconazole; ITZ: itraconazole; 4-NQO: 4-nitroquinoline 1-oxide; GRI: griseofulvin; BEN: benomyl; CASP: caspofungin.

3.2. Deletion of ap1 causes reduced growth rate in agar media, induces more pigment formation, and affects keratinolytic activity

We analyzed the impact of *ap1* gene deletion on *T. rubrum* growth on MEA, PDA, SDA, minimal medium agar (MMA), and keratin medium agar (KMA) for 21 days. There was a delay in the growth curve of the *ap1* mutant strain in these agar media (Fig. 2). However, conidia germination and hyphal extension in liquid medium were not affected by the mutation (Supplementary Fig. 1), and no significant difference was observed between the wild type (WT) and Δ*ap1 T. rubrum* strains in conidia formation.

The pigmentation in the Δ*ap1*strain was more intense than that in the WT, as observed on the reverse side of SDA plates (Fig. 3A) and in conidial suspensions (Fig. 3B).

The mutation also led to increased aerial hyphae formation (Fig. 3C).

When the fungi were grown in KM broth, we observed an inverse growth between the WT and mutant strains over time, and this was also observed for keratinolytic activity (Fig. 4). The higher the mycelial mass, the lower the keratinolytic activity. Interestingly, after 7 days of culture in keratin broth, the mutant strain presented a lower extracellular keratinolytic activity compared to the WT. However, after 14 days of growth, the keratinolytic activity of the WT strain decreased, in contrast to the mutant strain, revealing that Ap1 contributes to regulating keratinolytic activity and controlling fungal growth in keratin (Fig. 4B). There was no difference in the extracellular pH after growth (Fig. 4C); thus, the mutation did not affect culture alkalinization.

3.3. ap1 gene is not involved in T. rubrum drug susceptibility, but its expression is modulated in response to antifungal drugs

We further evaluated the role of *ap1* in the antifungal drug response and susceptibility. The analysis of *ap1* gene expression in response to antifungal drugs was upregulated when challenged with AMB, NYT, or TRB, compared to the control (before antifungal addition). By comparing 3h and 24h exposure, the *ap1* gene was downregulated in response to AMB and upregulated in response to NYT and TRB (Fig. 5A). However, we did not observe differences in the susceptibility to these and other antifungal and cytotoxic drugs between the strains in the SDA plate assay (Fig. 5B) or microdilution in RPMI (not shown).

3.4. ap1 gene deletion causes sensitivity to calcofluor-white and menadione and increases cell wall thickness of the conidia

We then evaluated the role of *ap1* in response to stress caused by cell wall disturbances and osmotic and oxidative agents. *T. rubrum* did not significantly modulate *ap1* gene expression in response to osmotic and oxidative stress conditions compared to the control (Fig. 6A). However, the presence of CR or SDS decreased *ap1* gene expression after 3h compared to 1h (Fig. 6A). Deletion of the *ap1* gene in *T. rubrum* impaired conidia germination in SDA containing CFW but did not affect hyphal growth when mycelium was inoculated in the same medium (Fig. 6B). In the presence of oxidative stress, no difference in the growth of either strain in H_2O_2 or paraquat was observed, but the mycelium of the Δ*ap1*strain was more sensitive to menadione than the WT (Fig. 6C). Transmission electron microscopy (TEM) showed that the conidia of the Δ*ap1*strain had a thicker cell wall than the WT (Fig. 7A, B). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) showed no significant differences in the shape and size of the conidia from either strain (Fig. 7A, C).

3.5. ap1 gene deletion increased T. rubrum growth on nail fragments and on keratinocytes

We evaluated the involvement of the *ap1* gene in controlling virulence traits of *T. rubrum* using *ex vivo* skin and nail infection models and co-cultivation with keratinocytes. There was no difference between the strains grown on nails and skin at 48 h and 72 h post-inoculation, showing that Δ*ap1* deletion did not impair hyphal growth on skin or nails (Fig. 8A, B) or skin invasion (Fig. 8A), in early periods of the infection. However, after 21 days of growth on nail fragments, there was a more pronounced growth of the Δ*ap1* strain than the WT (Fig. 8C, D). Co-cultures of the strains with keratinocytes for 48 h showed a more vigorous growth of the Δ*ap1* strain and consequently fewer keratinocytes on the culture plates (Fig. 9A). Furthermore, keratinocytes infected with *T. rubrum* strains *in vitro* modulated the expression of genes encoding proinflammatory cytokines IL-6, IL-1β, and TNF-α, and the chemokine IL-8. The transcript levels of IL-6 and IL-1β were significantly reduced in keratinocytes infected with the Δ*ap1* strain compared to

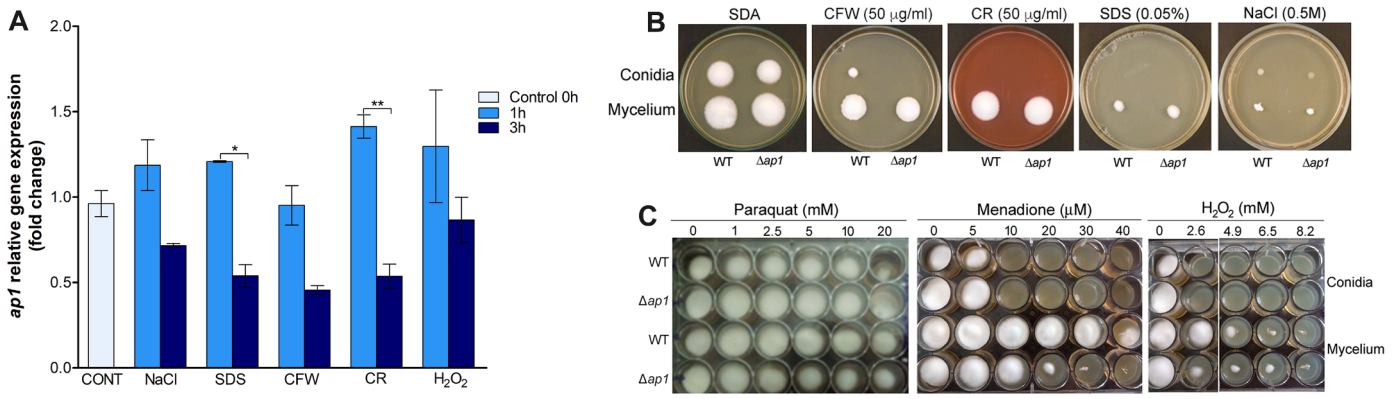

Fig. 6. *ap1* gene deletion alters the susceptibility to calcofluor-white and menadione. (A) qPCR of the *ap1* gene after exposure of wild type strain to each stress agent. Values are the average from three experiments with the respective standard deviations. Statistical analysis performed using ANOVA followed by Tukey's ad hoc test (*$p < 0.05$; **$p < 0.01$). (B) Growth of the strains in Sabouraud agar (SDA) containing stress agents. (C) Growth of the strains on Sabouraud (SDA) containing different concentrations of paraquat, menadione, and H_2O_2. Strains were grown for 7 days at 28 °C. SDS: Sodium Dodecyl Sulfate; CFW: Calcofluor-white; CR: Congo Red.

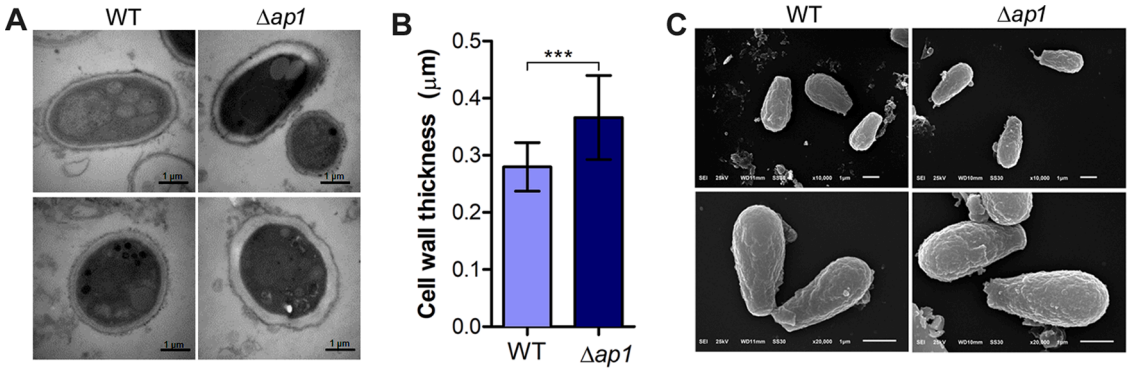

Fig. 7. *ap1* gene deletion alters the cell wall thickness of *T. rubrum* conidia but not its morphology and size. (A) Transmission electron microscopy of the conidia of wildtype (WT) and Δ*ap1* strains. (B) Comparison of the cell wall thickness between the strains, measured at ten random points from 20 conidia from each strain, obtained by ImageJ. Values are the average from the measured points, with the respective standard deviations. Statistical analysis was performed using Student's t-test (***$p < 0.001$). (c) SEM of the conidia of the wildtype (WT) and Δ*ap1* strains.

those in the WT (Fig. 9B).

4. Discussion

Fungi seek nutrients in the host tissue throughout the infection process while simultaneously avoiding recognition by the defense mechanisms of the host. They activate a broad range of transcription factors to modulate the expression of genes required during infection. A previous study showed that the *ap1* gene, which codes for the bZIP TF Ap1, is highly expressed during growth in keratin and *ex vivo* human skin and nail infections (Peres et al., 2016), suggesting its involvement in *T. rubrum* pathogenicity. Here, qPCR analyses showed the upregulation of *ap1* when challenging the fungus with the inhibitors AMB, NYT, or TRB. Moreover, this gene is downregulated upon prolonged exposure to AMB and is upregulated in response to NYT and TRB. Although we did observe no difference in *ap1* expression under the oxidative or osmotic stress conditions analyzed in this study, compared to the control, prolonged cell wall stress caused by CR or SDS decreased its expression. We generated a knockout strain, Δ*ap1,* to further investigate the role of the *ap1* gene in *T. rubrum.*

The *ap1* gene deletion in *T. rubrum* did not affect osmotic or oxidative stress susceptibility by hydrogen peroxide, as reported for *C. albicans, A. fumigatus,* and *C. neoformans* (Nagahashi et al., 1998; Paul et al., 2015; Qiao et al., 2008). However, the mycelium of the Δ*ap1* mutant strain was more susceptible to oxidative stress caused by menadione, indicating that it may trigger different pathways in response to oxidative stress agents. Modulation of several genes and metabolic pathways have been reported in *Aspergillus nidulans ap1* mutant strain, which was also more sensitive to menadione than hydrogen peroxide (Mendoza-Martínez et al., 2017). In *Magnaporthe oryzae*, deletion of the *ap1* gene leads to mild susceptibility to H_2O_2 (Guo et al., 2011). In *T. marneffei*, the *ap1* mutant was sensitive to both stressors but less virulent during *in vitro* macrophage infection (Dankai et al., 2016). Moreover, menadione induces apoptosis by mitochondrial depolarization, leading to anion superoxide production and lipid peroxidation in *Aspergillus flavus* (Zaccaria et al., 2015).

Although the *ap1* gene was upregulated in response to some antifungal drugs, its absence did not interfere with drug susceptibility, revealing that other TFs or other metabolic pathways can control the expression of genes involved in drug resistance. Indeed, exposure of *T. rubrum* to cytotoxic agents and clinical antifungal drugs induces the expression of general mechanisms of drug adaptability, such as detoxification enzymes, and multiple mechanisms act simultaneously to allow fungal viability during drug exposure (Martinez-Rossi et al., 2018; Mendes et al., 2018; Peres et al., 2010; Persinoti et al., 2014). In *Saccharomyces cerevisiae,* Ap1 is involved in the susceptibility to fluconazole (FLZ) by modulating the expression of FLR1, a plasma membrane transporter (Sá-Correia et al., 2009).

Interestingly, *ap1* deletion affected various aspects of *T. rubrum* pathogenicity, including pigmentation, growth on nail fragments, keratinolytic activity, and cell wall thickness. We also observed the *in vitro* expression of keratinocyte proinflammatory genes and cell destruction.

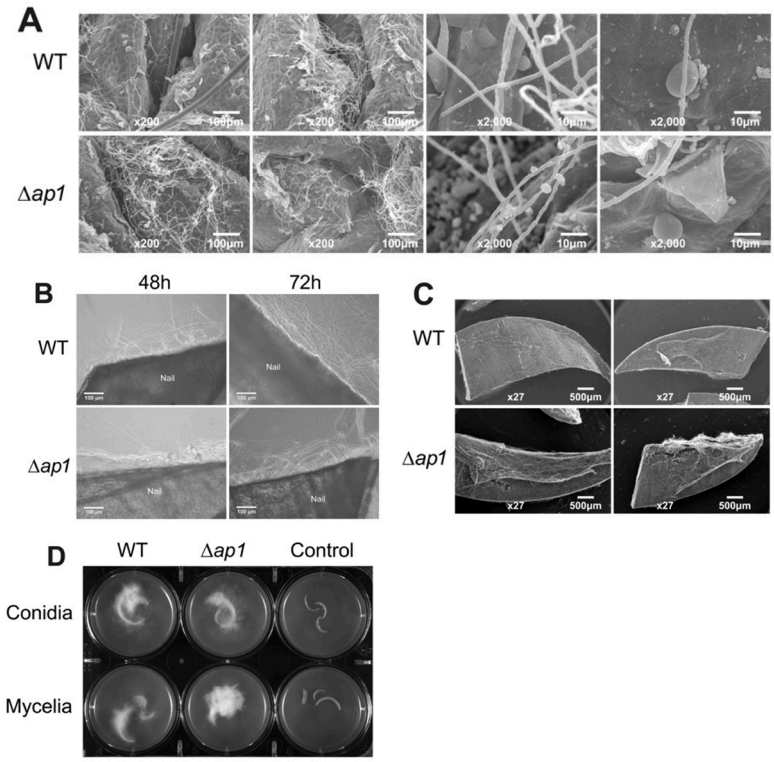

Fig. 8. *ap1* gene deletion alters the growth of *T. rubrum* on human nails. (A) Scanning electron microscopy of the strains grown on human skin fragments *in vitro* for 72 h. (B) Optical microscopy of the strains grown on human nail fragments for 48 and 72 h. (C) SEM of the strains grown on human nail fragments for 21 days. (D) Mycelial plugs or conidia were inoculated on human nail fragments and photographed after 21 days.

Altogether, these results revealed that the *ap1* gene is involved in the negative regulation of genes responsible for virulence in *T. rubrum*. The higher loss of keratinocytes co-cultivated with the Δ*ap1* strain may be due to death induction, leading to cell detachment. Zoophilic dermatophytes cause acute infection by triggering a high inflammatory response, whereas anthropophilic species cause chronic infections (Martinez-Rossi et al., 2017). This difference partly explains why different species produce different cytokine profiles by keratinocytes. However, the molecular responses that participate in this process remain unclear. We hypothesize that the more severe effect of the *ap1* mutant strain on keratinocytes might be a consequence of differential expression of molecules in the cell wall or by differential secretion of molecules or extracellular vesicles, which Ap1 might regulate in *T. rubrum*. This effect also reflects a different modulation of the expression of some proinflammatory genes in response to the fungus. However, further studies are crucial to unraveling the role of *ap1* in inducing keratinocyte death and maintenance of infection.

Adhesion and initial hyphal extension of the mutant strain on the human nail were similar to the WT; however, its growth increased after 21 days. This similar adhesion, accompanied by the higher keratinolytic activity of the mutant strain in later stages of keratin growth, suggests that *ap1* is required to maintain the infection rather than its initiation. Moreover, while the keratinolytic activity of the WT decreased over time, it remained stable in the mutant, indicating a lack of regulation in the absence of Ap1. In the zoophilic dermatophyte Microsporum canis, higher keratinolytic activity is related to the severity of the symptoms of guinea pigs (Viani et al., 2001). In addition, less keratinolytic activity of *M. canis* was correlated with chronicity and asymptomatic cases, while greater activity was correlated with acute infection and fungal clearance (Viani et al., 2007). The keratinolytic activity of the WT strain was inversely proportional to the growth rate, which agrees with previous findings (Kadhim et al., 2015). In contrast, deletion of the *ap1* gene altered the growth pattern and resulted in identical keratinolytic activity over time. There are no animal models to evaluate onychomycosis. Therefore, nail *in vitro* and keratinolytic activity are suitable assays to evaluate this critical aspect of dermatophytes infection. These results led us to hypothesize that Ap1 regulates growth on hard keratin and plays an essential role in the chronicity of nail infection caused by *T. rubrum*.

The cell wall is the first fungal component that makes contact with the host. Its morphology, composition, and structure are essential for maintaining cellular integrity and survival; tissue invasion is also involved. CFW is a dye that interferes with cell wall assembly by binding to nascent chitin chains, inhibiting the enzymes involved in linking β-1,3-glucan and β-1,6-glucan to chitin and weakening the cell wall (Ram and Klis, 2006). CFW activates the cell wall response pathway, increasing chitin levels; in general, fungal strains sensitive to CFW have more chitin in their cell walls than resistant strains (Heilmann et al., 2013). The chitin synthase defective strain of *S. cerevisiae* (*chs3*) is resistant to CFW and presents less chitin deposition. Furthermore, there are increased chitin levels in mutants with defects in the synthesis β of -1,3-glucan and β-1,6-glucan, mannosylation process, and glycosylphosphatidylinositol (CPI-) anchors, which are also hypersensitive to CFW (Ram and Klis, 2006; Roncero et al., 1988).

The role of pigments, especially melanin, in fungal virulence and protection from environmental stress and host defense mechanisms is well known for other pathogenic fungi. However, this trait remains unclear for dermatophytes (Smith and Casadevall, 2019). The main pigment identified in *T. rubrum* is the mycotoxin xanthomegnin, which is associated with the reddish color on the reverse of the colonies in agar plates (Wirth et al., 1965), and was also detected in clinical samples from infected patients (Gupta et al., 2000). Additionally, *T. rubrum* synthesizes melanin or melanin-like proteins both *in vitro* and during infection (Youngchim et al., 2011). Hyperpigmentation, the increased susceptibility of the conidia of the *T. rubrum* Δ*ap1* strain compared to CFW, and its thicker cell wall, suggests that Ap1 regulates cell wall assembly in dermatophytes, among other genes involved in the

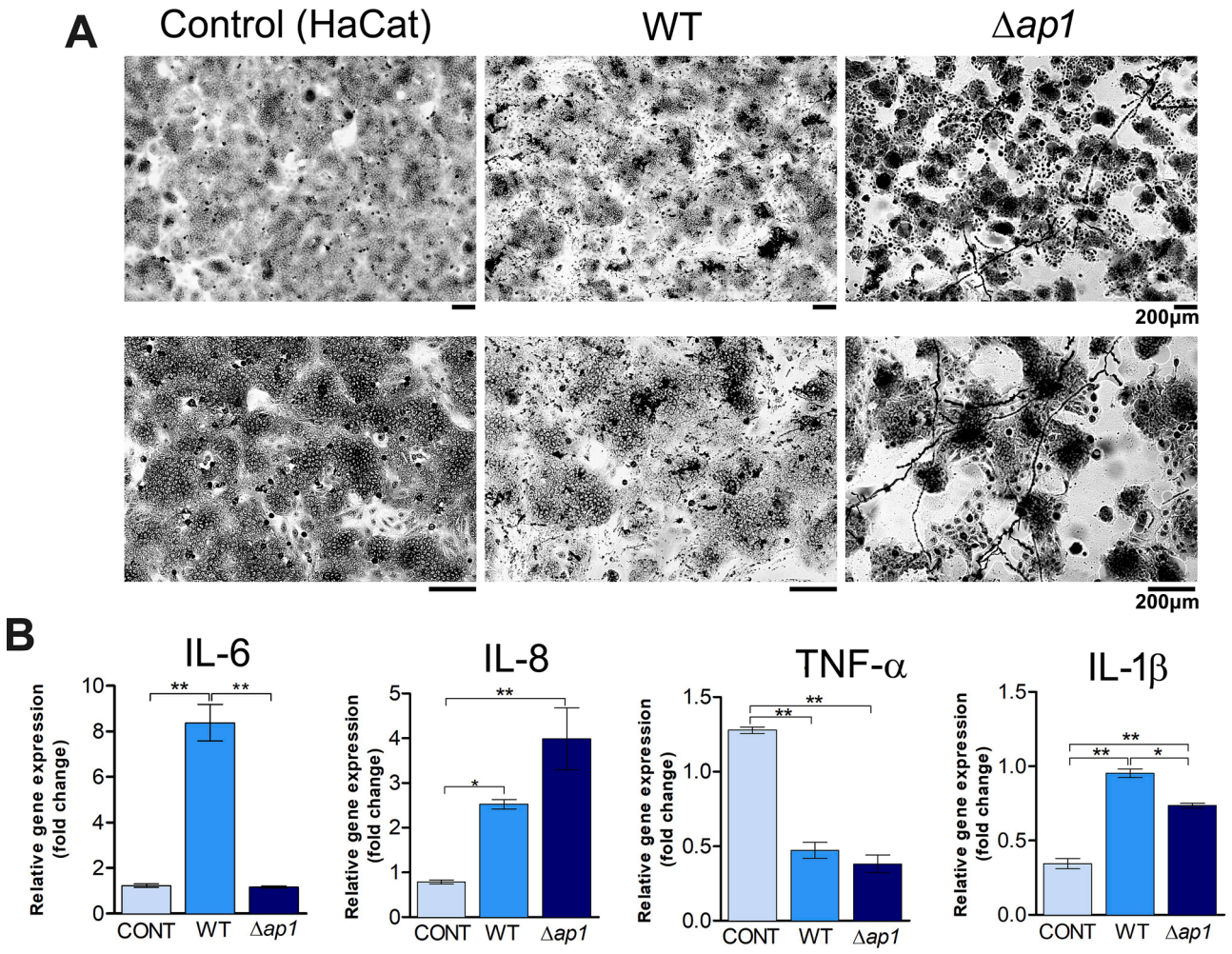

Fig. 9. *ap1* gene deletion in *T. rubrum* causes a more severe reduction of keratinocytes and affects the modulation of IL-6 and IL-1β genes in a co-cultivation assay. (A) Wildtype (WT) or Δ*ap1*conidia were co-cultivated with keratinocytes (HaCat human cell line) for 48 h. No conidia were added to the control. (B) Gene expression of cytokines from keratinocytes after co-cultivation with *T. rubrum* strains for 48 h. Values are the average from three experiments, with the respective standard deviations. Statistical analysis was performed using ANOVA followed by Tukey's ad hoc test (*$p < 0.05$; **$p < 0.01$).

stabilization of the cell wall.

5. Conclusion

In this work, we described the first functional characterization of the *ap1* gene in a dermatophyte, showing the role of this TF in *T. rubrum,* a fungus that causes cutaneous mycoses. Considering our results, we propose that Ap1 plays a role as a negative regulator of some virulence-related attributes, possibly regulating aspects that contribute to the chronicity of onychomycosis. Onychomycosis is the most frequent lesion caused by *T. rubrum* worldwide. Our findings concerning the cellular and molecular aspects of fungal growth in nails help us understand the pathogenic process, which is essential to delineate more efficient methods to control, avoid, and treat these infections.

Appendix

Supplementary figure 1. *ap1* gene deletion does not affect *T. rubrum* conidia germination and hyphae extension. Wild-type (WT) and Δ*ap1* strains were grown on Sabouraud broth media for 3h, 20h, and 48h at 28 °C, and visualized by light microscopy.

CRediT authorship contribution statement

Nalu T.A. Peres: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Elza A.S. Lang:** Investigation, Formal analysis, Writing – original draft. **Tamires A. Bitencourt:** Investigation, Formal analysis, Writing – original draft. **Vanderci M. Oliveira:** Investigation, Formal analysis. **Ana Lucia Fachin:** Formal analysis. **Antonio Rossi:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing – original draft, Writing – review & editing. **Nilce M. Martinez-Rossi:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Ethical Approval

Ex vivo experiments were approved by the University Hospital of the Ribeirão Preto Medical School of the University of São Paulo (HC-FMRP-USP) ethics committee (Protocol No.8330/2009).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2022.100132.

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