

Requirement of a Tsp2-Type Tetraspanin for Laccase Repression and Stress Resistance in the Basidiomycete *Cryptococcus neoformans*

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Fungal laccases have been widely used in industry. The expression of laccase often is repressible by the primary carbon source glucose in many fungi. The underlying basis is largely unclear. We demonstrate here that a gene, *TSP2-1*, was required for laccase repression by glucose in the basidiomycete *Cryptococcus neoformans*. *TSP2-1* encodes a Tsp2-type tetraspanin. The disruption of *TSP2-1* resulted in constant melanin formation and the expression of the laccase gene *LAC1*. This derepression phenotype was restorable by 10 mM exogenous cyclic AMP (cAMP). A capsule defect in the mutant *tsp2-1Δ* also was restored by cAMP. The results indicate an interaction of Tsp2-1 with the cAMP-dependent protein kinase A (PKA) pathway that has been shown to modulate laccase repression and capsule biosynthesis in this fungus. Other roles of *TSP2-1*, e.g., in maintaining cell membrane integrity and stress resistance, also were defined. This work reveals a Tsp2-1-dependent glucose repression in *C. neoformans*. The function of Tsp2-type tetraspanin Tsp2-1 is described for the first time.

Laccase (EC 1.10.3.2), a blue copper-containing phenolic oxidase, is widely distributed in higher plants and fungi, and it also is found in bacteria and insects (7, 23, 30, 37). Laccase was first discovered by Yoshida in the lacquer tree (*Rhus* spp.) (48). The enzyme has been used in a broad array of industrial and environmental applications (7, 10, 23, 37), e.g., in pulp and paper manufacture, textile whitening, food processing (4, 37), and in the bioremediation of soil and water (2, 41). Laccases produced by the white-rot fungi play a critical part in the degradation of plant litters (29, 30). Recent uses of laccase include the generation of new organic molecules, biomaterials, and biosensors and the production of biofuel ethanol from plant lignocellulose (5, 23, 31, 36). The main source for industrial laccase production is higher fungi. However, reactor-scale production in industry is hampered by several difficulties (10, 23). For instance, levels of laccase produced by native producers or by heterogeneous expression still are too low for industrial production (10, 23). Knowledge of the expression regulation of laccase in fungal hosts is accumulating. Some fungal laccase genes are constitutively expressed, while others are inducible (9). The enzyme usually is repressed during fungal growth and is inhibited by carbon sources, such as glucose, and on some occasions it is inactivated by certain compounds (i.e., proteases) (10).

Cryptococcus neoformans produces abundant laccase and has served as a system to investigate laccase expression (42, 51). *C. neoformans* is a soilborne basidiomycete and is particularly enriched in bird droppings and tropical trees (18). Cryptococcal laccase, a cell wall-associated enzyme, was demonstrated to be encoded mainly by *LAC1* (32, 49, 51). The disruption of *LAC1* results in undetectable laccase activity in glucose starvation (32, 51). Another homolog, *LAC2*, is indicated to be expressed under oxidative stress (32, 51). The function of *LAC2* is unknown. The transcription of *LAC1* is induced by glucose starvation (32, 51). In *C. neoformans*, laccase is the sole enzyme for the formation of the pigment melanin via oxidizing phenolic substrates, e.g., norepinephrine (NE) (42, 51). Mutants of *lac1* do not generate melanin and form albino colonies. This becomes advantageous for the study of laccase expression in *C. neoformans* for comparisons to many other fungi that carry multiple copies of laccase genes (6, 9,

21). The pigmentation can be applicable in laccase mutant screening via colony color alteration (51). It is known that several environmental factors determine laccase expression in *C. neoformans*. The trace element copper is an effective inducer of the enzyme, and high temperature inhibits its expression (19, 20). Glucose is a primary repressor of laccase (21, 51). The repression occurs at the transcription level, i.e., the transcription of *LAC1* is inhibited in the presence of glucose (20, 51). When high concentrations of glucose are present, e.g., 2%, the transcription of *LAC1* is down-regulated (34, 51). The nutrient-sensing heterotrimeric G protein and the cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway is responsible for laccase repression and capsule biosynthesis (1). However, many steps in this pathway leading to laccase repression and capsule modulation have been missing from this fungus (22).

We employed *Agrobacterium tumefaciens*-mediated T-DNA random insertional mutagenesis (ATMT) (40) to generate glucose derepression mutants for laccase in *C. neoformans*. By taking advantage of a color-based screening procedure, transformants of interest are expected to form dark colonies in the presence of 2% glucose due to melanin formation, whereas the wild-type colonies remain white. The genes in the candidates that presumably are disrupted by T-DNA can be cloned by methods like inverse PCR. With this approach, we obtained more than 30 glucose-derepression mutant isolates (unpublished data). Here we describe one, LZM36, in which laccase is not inhibited by glucose. The mutated gene in LZM36 was identified as a tetraspanin-encoding gene, designated *TSP2-1*, which belongs to the fungal Tsp2 tetraspanin family (17, 24). We prove here that *TSP2-1* plays a key role in laccase repression by glucose. Also, we showed that the gene

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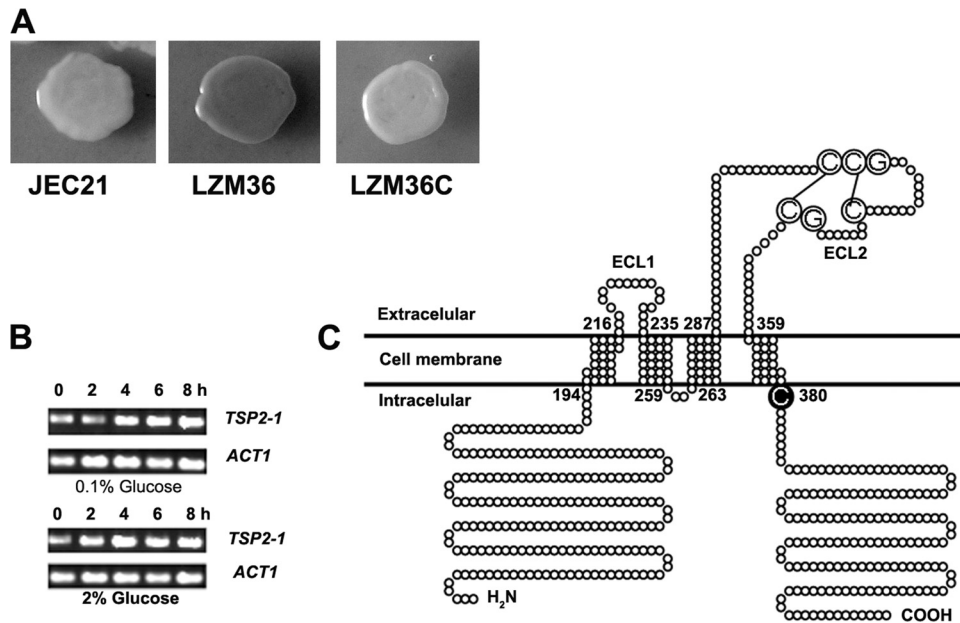


FIG 1 Disruption of *TSP2-1* in the mutant LZM36 is responsible for the laccase derepression phenotype. (A) LZM36 produced melanin in the presence of 2% glucose, the wild-type JEC21 showed an albino colony, and the complemented strain LZM36C restored the repression and produced little melanin. (B) *TSP2-1* is a constitutively expressed gene, as shown by the RT-PCR amplification of *TSP2-1* mRNA. Fresh cells of JEC21 were incubated in 0.1 or 2% glucose-containing Asn liquid medium at 30°C for 2, 4, 6, and 8 h as indicated. (C) Topological structure of Tsp2-1 showing four transmembrane domains (TM1 to TM4), three loops (ECL1, ICL, and ECL2), and a CCG motif on ECL2 (see Results for a description).

TSP2-1 is critical for the fungus to respond to various environmental conditions.

MATERIALS AND METHODS

Strains, media, and culture conditions. *C. neoformans* serotype D strain JEC21 (*MAT α*) was the wild type in this study (33, 47). JEC21FOA, a uracil auxotrophic mutant of JEC21 (47), was used as a recipient for making the targeted disruption of *TSP2-1*. Strain LZM36 was a T-DNA insertional mutant created from JEC21FOA which produced melanin on 2% glucose-containing agar. *C. neoformans* was grown in YPD (2% glucose, 2% Bacto peptone, 1% yeast extract, pH 6.0). Asparagine (Asn) salt agar (0.1% asparagine, 0.3% KH_2PO_4 , pH 5.1) supplemented with 2% glucose or other carbon sources was the medium for melanin production or *LAC1* induction in the presence of the laccase substrate norepinephrine (NE) (100 mg/liter) (42, 47). Cells were incubated at 30°C unless indicated otherwise.

Identification of *TSP2-1* and its targeted disruption. *Agrobacterium tumefaciens*-mediated T-DNA random insertional mutagenesis (ATMT) in *C. neoformans* was carried out by following a previous protocol (40). Dark transformants on 2% glucose-containing Asn agar were picked. One mutant, LZM36, out of 32 dark transformants was chosen for further analysis (Fig. 1A). Genomic sequences flanking the T-DNA integration site in LZM36 was retrieved by inverse PCR. Briefly, 2.0 μg genomic DNA of the mutant was digested with *Xba*I and ligated with T4 DNA ligase overnight at 14°C. Approximately 1 μl of the ligation reaction mixture was taken as the template in inverse PCR with two pairs of primers located on the T-DNA left and right boundaries: p1-S/p1-A, 5'-ACTGGAACAA CACTCAACCCTA-3' and 5'-TGGGGTTTCTACAGGACG-3'; and p2-S/p2-A, 5'-TGGCGGGTAAACCTAAGAG-3' and 5'-CGCACAATCCC ACTATCC-3'. PCR products were subject to sequencing and were used for blast searches against GenBank databases.

To create a deletion mutant of *TSP2-1* via homologous recombination, two pairs of primers, i.e., Primer-969/Primer+54 (5'-TCTCTCGA GGGGATGGAAGAAGTGGAA and 5'-GGGATCGATCGAATTTGGAT GCGAAG, respectively) and Primer+1752/Primer+2762 (5'-CCTGAA

TTCGTATCAGGCGGACGAGT and 5'-TTAGGATCCCCTTTCTCC GCCACCATT, respectively), were designed to amplify 1-kb fragments of the 5' and 3' ends of *TSP2-1* (Fig. 2A). The selection marker *URA5* (1.9-kb) was ligated to the PCR products to create the disruption cassette pBS-*TSP2-1*-*URA5*. The disruption cassette (3.9 kb) then was amplified with primers Primer-969/Primer+2762 to make enough DNA to be transformed into JEC21FOA by electroporation. Transformants were screened by a PCR screening procedure with Primer-1859 (5'-TACGG ATCCGCGTTGTGAGATTGCCTTGAG), located at 890 nucleotides upstream of the disruption cassette, and primer *URA5* (5'-GCTGAATCC GGTACTATGGTTCGGTGC), located in the open reading frame (ORF) of *URA5* (Fig. 2A, bottom). A 2.8-kb PCR product was expected in the positive candidates that carried disrupted *TSP2-1*, while no product was expected from the wild type. The PCR product then was sequenced for verification. One positive candidate, strain *tsp2-1 Δ* , was chosen for Southern blotting and reverse transcription-PCR (RT-PCR) verification with the primers Primer+943 (5'-GCCCTGGCTACATTACCTACA) and Primer+1753 (5'-ACTTCCGCTGGCGATATGAG), generating a 0.81-kb product. The mutant was tested for melanin production on 2% glucose agar.

The complemented strains were made by the cotransformation of a 3.3-kb PCR fragment of the wild-type *TSP2-1* with primers Primer-753/Primer+2584 (ATAGGTACCTCGTTAGCTTGGGAGGCA/ATAGGAT CCGCCCCTAAGGATTTGTTG) and a 2.3-kb *HYG* expression cassette into the mutant LZM36 and *tsp2-1 Δ* strains on hygromycin-containing agar (200 $\mu\text{g}/\text{ml}$). Transformants were incubated on Asn salt agar containing 2% glucose and NE. The complemented strains exhibiting white colonies were subjected to Southern blotting and PCR confirmation together with the *tsp2-1 Δ* mutant.

DNA preparation and Southern blotting. *C. neoformans* cells were grown in 100 ml YPD to stationary phase at 30°C. DNA extraction was carried out as described previously (47, 50). Digested DNA with the appropriate restriction enzymes was subjected to separation in 0.8% agarose gels, and blotting was conducted on N^+ -Magaprobe nylon transfer membrane by following the manufacturer's instructions (GE Osmonics Inc.,

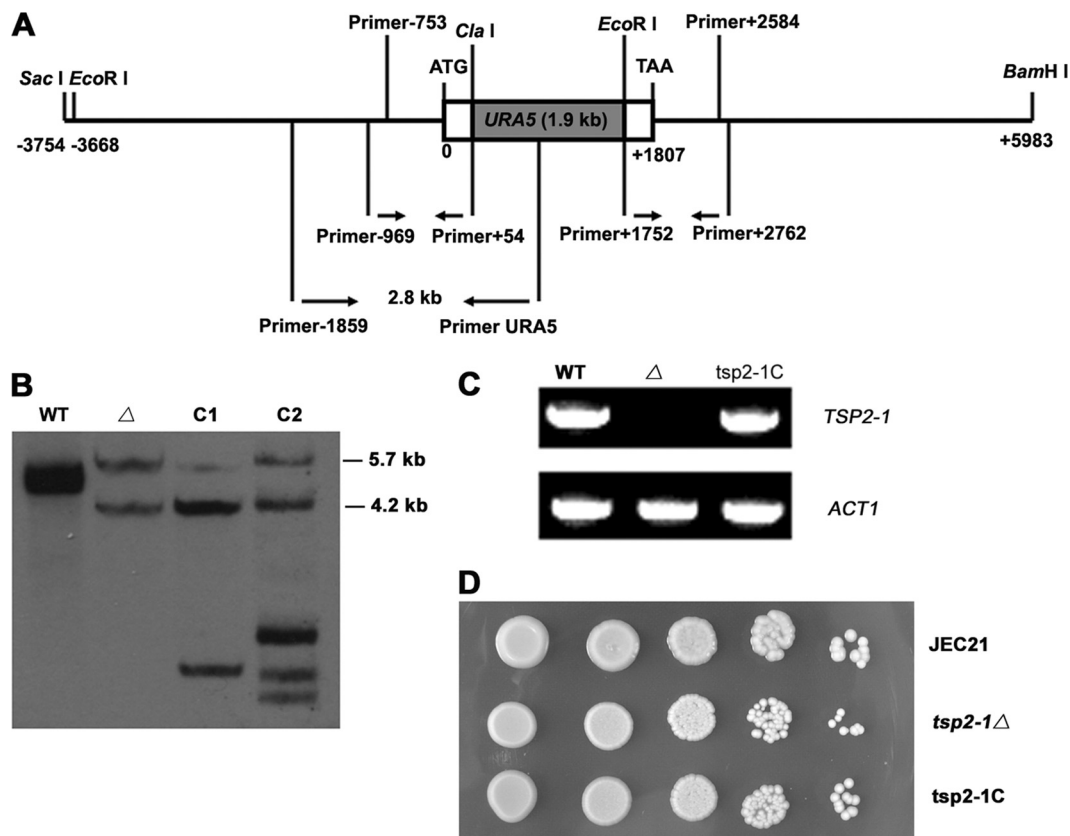


FIG 2 Targeted disruption of *TSP2-1*. (A) Schematic diagram of the replacement of *TSP2-1* by *URA5* through homologous recombination. The locations of primers for making the disruption mutant and for PCR screening are indicated. Primer -969/Primer +54 were used to amplify the upstream fragment of *TSP2-1*, while Primer +1752/Primer +2762 were used to amplify the 3' end of *TSP2-1*. PCR products were ligated to *URA5* with *Cla*I and *Eco*RI restriction. Primer -1895/Primer *URA5* were used for mutant screening. (B) Southern analysis on the insertion site of *URA5* in strain *tsp2-1Δ* (Δ). Genomic DNA was double digested with *Bam*HI and *Eco*RI for wild-type JEC21 (lane 1), the targeted disruption mutant *tsp2-1Δ* (lane 2), and two complemented candidates (lanes 3 and 4; designated C1 and C2, respectively). The membrane was probed with a DIG-labeled 3.3-kb PCR fragment of the wild-type *TSP2-1* gene (see Materials and Methods). As expected, the wild type had two bands, 4.9 and 4.7 kb. The mutant generated two bands of 5.7 and 4.2 kb in size. The complemented strains obtained extra *TSP2-1* bands plus the two original bands, 5.7 and 4.2 kb, in *tsp2-1Δ*. C2 was used in the experiments depicted in panels C and D. (C) *TSP2-1* mRNA was not detected in *tsp2-1Δ*. The reconstituted strain *tsp2-1C* restored the expression of *TSP2-1*. (D) *TSP2-1* is dispensable for growth. Cells were spotted in 10-fold serial dilutions and grown on Asn agar with 2% glucose at 30°C.

MN). The 3.3-kb PCR fragment of *TSP2-1* DNA was used as the probe and was labeled with digoxigenin (DIG) High Primer DNA labeling and detection starter kit II (Roche China, Shanghai, China).

RNA preparation and RT-PCR. To examine *LAC1* transcription, yeast cells were grown in 2% glucose-containing Asn salt liquid at 30°C for 8 h. Total mRNA was prepared by following the protocol provided by the RNAiso plus kit (TaKaRa Biotech Co. Ltd., Dalian, China). The first chain of cDNA was synthesized by Moloney murine leukemia virus (M-MLV) reverse transcriptase with approximately 1 μ g mRNA as the template and oligo(dT)₁₅ as the primer (TaKaRa Biotech Co. Ltd., Dalian, China). The level of *LAC1* cDNA was determined by PCR amplification with primers *LAC1-S* (5'-ATGAAATCTCCATCGACCCC) and *LAC1-A* (5'-CGTCCTATCACCTGGAAGCTCG). A 0.57-kb *LAC1* fragment was expected. The mRNA of *ACT1*, the actin-encoding gene, served as an internal control. A 0.52-kb *ACT1* fragment was generated by the primers *ACT-S* (5'-ACCTCTTCACCACCTCTGC) and *ACT-A* (5'-GGTGGACGATTGAGGGAC).

Assays for melanin production and capsule biosynthesis. Strains were examined for pigmentation on 2% glucose. Fresh cells were grown overnight in liquid YPD, washed three times with sterile double-distilled water (ddH₂O), and resuspended in ddH₂O to 2 \times 10⁸ cells/ml. Approximately 5 μ l of the suspension was dropped onto Asn agar containing 2% glucose and NE at 30°C for 2 days for melanin formation. For capsule observation, fresh cells taken from YPD culture were transferred to malt-

ose agar and incubated at 30°C for 5 days. Cells were subject to India ink staining microscopy (47). To examine the effects of different carbon sources on melanin production in the mutant strain *tsp2-1Δ*, fresh cells were spotted on Asn salt agar containing NE. Tested carbon sources included 2% sucrose, maltose, D-fructose, D-galactose, D-mannose, and lactose (in the place of dextrose) at 30°C for 16 or 6 h according to their type of pigmentation.

For the measurement of laccase enzymatic activity, fresh yeast cells were harvested from Asn salt plates (2% glucose) after incubation at 30°C for 16 h. The enzymatic activity was measured by the oxidation of NE (100 mg/liter) with 10⁷ cells/ml in 0.05 M sodium phosphate (pH 6.5) at 37°C for 30 min. The OD at 475 nm was recorded. One enzymatic unit equals an A₄₇₅ of 0.001. The assay was conducted in triplicate, and the standard deviations were calculated (42).

Stress tolerance assays and CFW staining microscopy. Cells were cultured on YPD agar at 30°C for 2 days, washed by sterile water twice, and resuspended in sterile ddH₂O again. The cell concentration was determined by a hemocytometer. Starting with 10⁵ cells in 5 μ l, cells were spotted on YPD agar in 10-fold serial dilutions to the last spot containing 10 cells. YPD plates were supplemented with 0.01% SDS, 1% Congo red, 1.0 M KCl, 2.0 M NaCl, 2.0 M sorbitol, 1 mM H₂O₂, or 1 mM NaNO₂ at 30°C for 1 to 3 days. To examine chitin biosynthesis, aliquots of the cells were stained with calcofluor white (CFW; Sigma, St. Louis, MO) as de-

scribed previously (47). Cells were examined with a Nikon Eclipse 80i fluorescence microscope using a 4,6-diamidino-2-phenylindole filter.

RESULTS

A *Tsp2* tetraspanin gene was disrupted in LZM36. LZM36 was a T-DNA insertional transformant that produced melanin in the presence of 2% glucose, while under this condition melanin production was inhibited in the wild-type strain JEC21 (Fig. 1A). Probing with T-DNA and Southern blotting verified that only one copy of T-DNA was inserted in the genome of LZM36 (data not shown). Inverse PCR revealed that T-DNA was integrated 54 bp downstream of the start codon of an ORF (AAW45945) that putatively encodes a fungal *Tsp2* tetraspanin (17). Thus, we named the gene *TSP2-1*. The product of *TSP2-1* consists of 533 amino acids (aa). *TSP2-1* is the only tetraspanin gene in the genome of *C. neoformans* (17, 24). *TSP2-1* is constitutively expressed regardless of glucose concentration in the medium (Fig. 1B).

A targeted disruption mutant, strain *tsp2-1Δ*, was created to confirm the role of *TSP2-1* in the laccase derepression phenotype in LZM36. The ORF of *TSP2-1* was replaced by *URA5* via homologous recombination as a routine practice in this fungus (Fig. 2A; also see Materials and Methods). By screening with PCR, one *TSP2-1* disruptant candidate, strain *tsp2-1Δ*, was obtained. Mutant strains LZM36 and *tsp2-1Δ* were complemented with a 3.3-kb PCR fragment of the wild-type *TSP2-1* gene. LZM36, *tsp2-1Δ*, and their complemented strains (LZM36C and *tsp2-1C*, respectively) were verified by Southern blotting (only the *tsp2-1Δ* strain is shown in Fig. 2B; data not shown for LZM36). The reconstituted strain *tsp2-1C* (designated C2 in Fig. 2B) expressed *TSP2-1* mRNA again (Fig. 2C). The disruption of *TSP2-1* did not have an obvious effect on yeast growth at 30°C (Fig. 2D).

Consistently with the phenotype of LZM36, the targeted mutant strain *tsp2-1Δ* formed a dark colony on 2% glucose (Fig. 3A). The complemented strain *tsp2-1C* restored the repression of melanin production in a white colony (Fig. 3A). A laccase enzymatic activity assay confirmed the derepression of laccase in the mutant *tsp2-1Δ* (Fig. 3B). Laccase activity in 10^8 cells produced 291 ± 8 U in the *tsp2-1Δ* strain, whereas the values for JEC21 and the complemented strain were 109 ± 3 and 141 ± 41 U, respectively. These results clearly verify that the repression of laccase activity by 2% glucose is abolished by the disruption of *TSP2-1*.

Laccase derepression in *tsp2-1Δ* mutant takes place at the transcriptional level. To see at what stage the derepression of laccase takes place in the *tsp2-1Δ* mutant strain, RT-PCR was adopted to amplify the mRNA of *LAC1* in the mutant (9). JEC21 and the complement served as controls in parallel. *LAC1* was expressed in *tsp2-1Δ*, while no mRNA of *LAC1* was detected in wild-type JEC21, and the complemented strain obviously restored the inhibition of *LAC1* transcription (Fig. 3C). Further, *LAC1* was constantly expressed from 0 to 8 h in the mutant strain *tsp2-1Δ* in a time course test (Fig. 3D). As a control, the mRNA of the actin-encoding gene *ACT1* displayed a close level of transcription in each assay. These results manifest that the derepression of laccase in *tsp2-1Δ* occurs at the transcriptional level.

More sugar inhibition on laccase depends on *TSP2-1*. We further found that the inhibition of melanin production by some other carbon sources depended on *Tsp2-1* as well. As shown in Fig. 4, five of the six tested sugars (lactose is the exception) exhibited a repression effect on laccase/melanin production in wild-type JEC21 (the spots on the left in each panel). Repression by

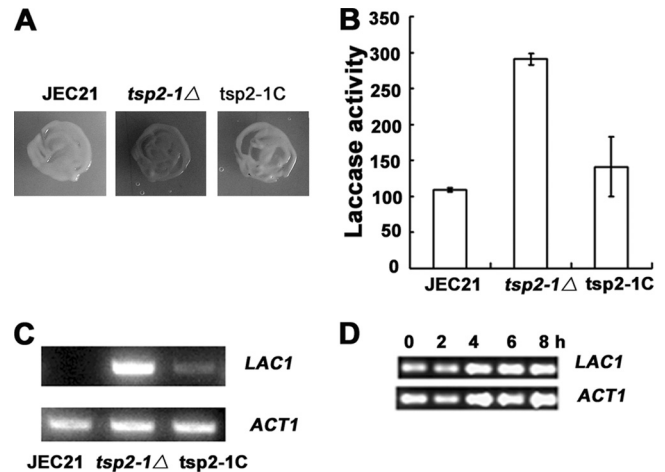


FIG 3 *TSP2-1*-mediated repression of laccase on *LAC1* transcription. (A) The targeted knockout mutant strain *tsp2-1Δ* exhibited a phenotype similar to that of LZM36, producing melanin in 2% glucose (30°C, 2 days). The wild-type JEC21 was used as a control. The reconstituted strain *tsp2-1C* restored the repression of laccase and formed an albino colony. (B) Laccase enzymatic activity increased dramatically in strain *tsp2-1Δ* in 2% glucose (30°C, 16 h) compared to that of the control JEC21 and the complement. Laccase activity in strain *tsp2-1Δ* was 291 ± 8 U (10^8 cells), whereas the wild-type JEC21 and the complemented strain *tsp2-1C* had 109 ± 3 and 141 ± 41 U (10^8 cells), respectively. (C and D) *TSP2-1*-mediated laccase inhibition at the transcription level by RT-PCR. (C) In the mutant *tsp2-1Δ*, *LAC1* transcription was released from repression and expressed at a high level at 8 h. JEC21 and *tsp2-1C* served as controls. (D) Time course for *LAC1* transcription reinforced the observations for panel C. *LAC1* was constantly expressed from 0 to 8 h at a similar level. Total RNA was prepared from cells grown in 2% glucose Asn salt liquid at 30°C for 8 h (C) or in a time course at 0, 2, 4, 6, or 8 h (D). *ACT1* mRNA served as the control. RT-PCR was carried out as described in Materials and Methods.

these sugars required *TSP2-1* function, as the mutant strain *tsp2-1Δ* produced significantly more melanin than JEC21 did (the spots in the middle of each panel). The complemented strain restored the repression of melanin formation (the spots on the right in each panel). As a control, laccase was highly expressed when there is no sugar in the medium (second panel from the top). This result suggests that sugar repression of laccase activity/melanin production shares the common *Tsp2*-associated step in *C. neoformans*.

Exogenous cAMP restores glucose repression of melanin production in *tsp2-1Δ* strain. The disruption of the G protein α subunit gene *GPA1* causes a defect in melanin/laccase activity. This defect can be restored by the addition of cAMP (1, 12). We thus tested whether exogenous cAMP could restore melanin repression in mutant strain *tsp2-1Δ*. When cAMP was added to Asn salt agar, melanin production in *tsp2-1Δ* was inhibited. At 10 mM cAMP, melanin production in *tsp2-1Δ* was inhibited to a level similar to that of the wild type (Fig. 5A). This observation suggests that cAMP homeostasis is critical for laccase repression. The homeostasis likely is impaired in the mutant strain *tsp2-1Δ*.

Capsule defect in *tsp2-1Δ* and its restoration by exogenous cAMP. Since the $G\alpha$ -cAMP-PKA pathway also controls capsule production in *C. neoformans* (1, 12), we examined the biosynthesis of capsule in the mutant strain *tsp2-1Δ* by India ink staining microscopy. As shown in Fig. 5B, capsule was significantly diminished in the *tsp2-1Δ* strain compared to that in the wild-type strain JEC21. A copy of wild-type *TSP2-1* restored the production of

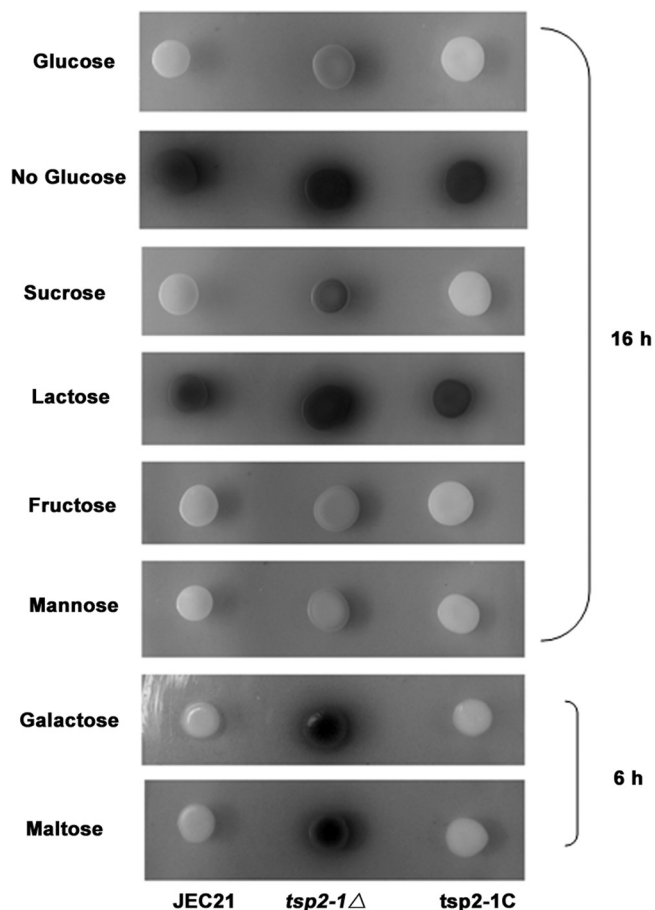


FIG 4 Laccase inhibition by other sugars also depends on *TSP2-1*. Sucrose, fructose, mannose, galactose, and maltose, but not lactose, exhibited a repression effect on melanin production in JEC21 (spots on the left in each panel). The disruption of *TSP2-1* released the repression (central spots in each panel). The complemented strain was restored to the repression status (spots on the right in each panel). Yeast cells were dropped on Asn salt agar containing NE and 2% of each carbon source at 30°C for 16 or 6 h (for galactose and maltose only, due to fast pigmentation) for melanin production.

capsule in the complemented strain (Fig. 5B and C). Again, 10 mM cAMP recovered capsule production in mutant strain *tsp2-1Δ*. This result supports the view that Tsp2-1 modulates capsule biosynthesis likely via the $G\alpha$ -cAMP-PKA pathway.

Sensitivity of *tsp2-1Δ* to SDS, osmotic and oxidative/nitrosative stress, and heat. Considering that Tsp2-1 is a transmembrane protein, we examined the growth of *tsp2-1Δ* in the presence of several cell-disturbing agents. As shown in Fig. 6A, *tsp2-1Δ* displayed a severe sensitivity to 0.01% SDS. The complemented strain *tsp2-1C* had restored growth on SDS agar. This result suggests that defects were present in the cell membrane in *tsp2-1Δ* cells (14). All the strains had similar growth on 1% Congo red agar, indicating that the cell wall is intact in the mutant strain *tsp2-1Δ* (38). Cell morphology remained normal under examination by CFW microscopy (Fig. 6B and C). The mutant *tsp2-1Δ* exhibited an apparent sensitivity to 2 M sorbitol, 1 M KCl, and 2 M NaCl (Fig. 6D to F) and to 1 mM H_2O_2 and $NaNO_2$ (Fig. 6G and H). We also found that *TSP2-1* was required for growth at elevated temperatures, as *tsp2-1Δ* grew significantly more slowly than the wild type did at 37°C or higher temperatures (Fig. 6I). The wild-

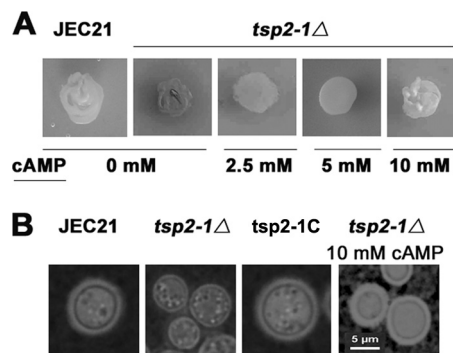


FIG 5 Restoration of melanin repression and capsule production by exogenous cAMP in *tsp2-1Δ*. (A) Addition of cAMP to the media restored the inhibition of melanin in the mutant *tsp2-1Δ*. Less pigmentation was achieved with increasing concentrations of cAMP added. Cells were incubated on 2% glucose Asn agar containing NE, with or without cAMP, at 30°C for 24 h (0, 2.5, 5, or 10 mM). (B) Likewise, 10 mM cAMP restored capsule production in *tsp2-1Δ*. The mutant strain *tsp2-1Δ* produced significantly less capsule (second image from the left). The complemented strain restored capsule production. Cells were incubated on 2% glucose Asn agar supplemented with 10 mM cAMP at 30°C for 5 days. Capsules were observed by India ink staining microscopy.

type strain JEC21 served as the control in these tests. The complemented strain restored the growth (at least partially restored in the case of 2 M NaCl) to the wild-type level under these stress conditions. The results demonstrate that *TSP2-1* plays a crucial role in stress resistance in *C. neoformans*.

DISCUSSION

In this study, we identified a gene, *TSP2-1*, from the mutant LZM36 of *C. neoformans* which exhibited a phenotype of laccase derepression in 2% glucose (Fig. 1A and 3A and B). *TSP2-1* was previously suggested, based on sequence homology, to be a Tsp2-type tetraspanin-encoding gene (17). The putative product of Tsp2-1 harbors characteristic features shared by members of the tetraspanin family (11, 17, 24). These include four transmembrane domains, a small extracellular loop (ECL1; 19 aa), a small intracellular loop (ICL; 4 aa), and a large extracellular loop (ECL2; 72 aa) with a typical cysteine-based motif [CCGY-x(12)-CY-x(5)-GCK] (Fig. 1C). One palmitoylation site is located at the end of TM4, proximal to the inner side of the membrane (a cysteine residue in Fig. 1C). Additionally, Tsp2-1 has long N-terminal (194 aa) and C-terminal cytoplasmic tails (153 aa) that were found only on Tsp2 tetraspanins (11, 17, 24). Tsp2 homologs are found in basidiomycetes by searching the genome project databases (17, 24). Tsp2-1 is the only tetraspanin in *C. neoformans* (17, 24). Although they share common features in three-dimensional structure, members from the different fungal tetraspanin families Pls1, Tsp2, and Tsp3 share little homology in peptide sequences (17, 24).

TSP2-1 is the first glucose-dependent repressor of laccase identified so far in *C. neoformans*. A significant finding for *TSP2-1* is that the disruption of *TSP2-1* activates *LAC1* transcription (Fig. 3C and D). Considering that Tsp2-1 is a membrane protein, it is unlikely that it is involved in *LAC1* transcription. We further found that exogenous 10 mM cAMP restored the inhibitory phenotype of laccase in the *tsp2-1Δ* mutant strain (Fig. 5A). Capsule defect in the *tsp2-1Δ* strain also was restored by cAMP in the mutant (Fig. 5B). It has been established in *C. neoformans* that the

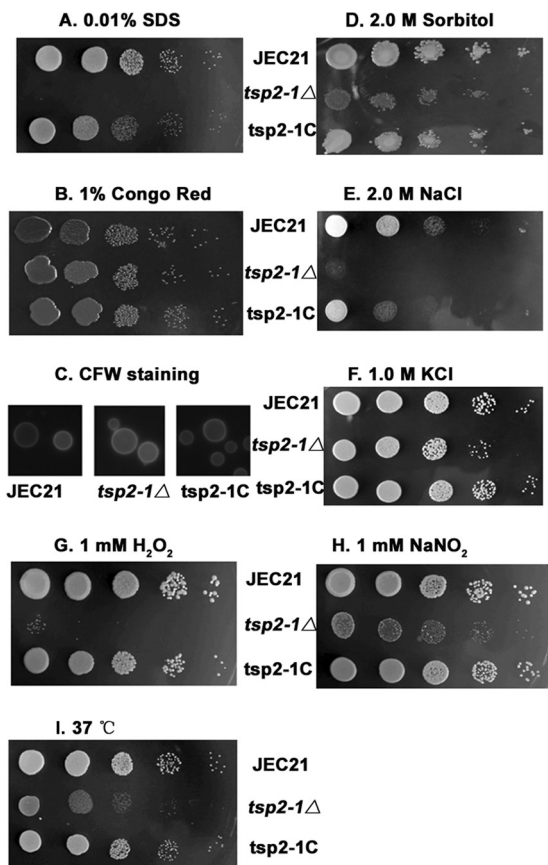


FIG 6 Requirement of *TSP2-1* for stress resistance. Yeast cells were serially diluted in 5 μ l containing 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 cells and separately spotted on 2% glucose agar supplement with testing agents at 30°C for 2 days. The mutant strain *tsp2-1Δ* was highly sensitive to SDS (0.01%) (A) but not to 1% Congo red (B). (C) CFW staining epifluorescence microscopy showed a normal cell wall chitin distribution pattern in the mutant. (D to F) *tsp2-1Δ* displayed hypersensitivity to osmotic stress from 2.0 M sorbitol, 2.0 M NaCl, or 1.0 M KCl. (G and H) *tsp2-1Δ* is sensitive to H_2O_2 (1 mM) and $NaNO_2$ (1 mM). H_2O_2 was added to the medium at a temperature of 55 to 60°C after being autoclaved. (I) Disruption of *TSP2-1* resulted in a growth defect at 37°C.

classic G protein-cAMP-PKA pathway controls capsule production and laccase induction in *C. neoformans* (1, 12, 22). Likewise, laccase and capsule defects caused by mutations in this pathway are restorable by cAMP (1, 12). In this respect, we speculate that Tsp2-1 interacts with the pathway at a step upstream of cAMP biosynthesis. It is intriguing to think that Tsp2-1 senses glucose on the plasma membrane and initiates a signal to the cAMP-PKA pathway. This speculation is supported by evidence from mammalian studies. Human tetraspanins provide molecular scaffolds for other proteins to form complexes and microdomains that are required for cellular signaling (16, 26, 39). A G protein-coupled receptor in human kidney cells, GPR56/TM7XN1, and the heterotrimeric G protein subunits, $G\alpha_q$, $G\alpha_{11}$, and $G\beta$, interplay specifically with tetraspanins CD9 and CD81 (27).

It should be noted that a canonic G protein-coupled receptor for glucose sensing (GPCR) is missing from *C. neoformans* (22). In *Saccharomyces cerevisiae*, a GPCR Gpr1, as a sugar receptor, senses glucose and sucrose to activate Gpa2, which further activates adenylyl cyclase for cAMP biosynthesis (25, 45, 46). In the genome of *C. neoformans*, seven GPCR-like proteins with structural similar-

ity to Gpr1 have been reported (44). Unfortunately, none of them played a role in glucose sensing. The deletion of these GPCRs generates no obvious phenotype, except for Gpr4, which is an amino acid sensor. The finding of *TSP2-1* in laccase repression may facilitate the finding of a glucose sensor in *C. neoformans*.

Other findings include roles of Tsp2-1 in stress resistance and in maintaining the integrity of the plasma membrane in *C. neoformans*. The mutant strain *tsp2-1Δ* displayed hypersensitivity to 0.01% SDS, suggesting a defect in the plasma membrane (14, 32). *tsp2-1Δ* was not sensitive to Congo red, which interacts specifically with β -D-glucan (37), implying that the cell wall integrity did not require *TSP2-1* (14, 43). Consistently, no detectable changes were seen in chitin distribution in *tsp2-1Δ* by calcofluor white staining epifluorescence microscopy (Fig. 6C). The mutant *tsp2-1Δ* displayed growth retardation on plates supplemented with high concentrations of osmotic reagents, e.g., 2.0 M sorbitol, 2.0 M NaCl, and 1.0 M KCl (Fig. 6D, E, and F). Since the high-osmolarity glycerol (HOG) pathway controls the resistance to osmotic stress in *C. neoformans* (3), sensitivity to osmotic stress implies an interaction between Tsp2-1 and the HOG pathway.

Furthermore, *tsp2-1Δ* exhibited a hypersensitivity to 1 mM H_2O_2 or a nitrosative pressure and elevated temperature (37°C) (Fig. 6G, H, and I). Sensitivity to H_2O_2 indicates that catalase function was demolished in the mutant. Hypersensitivity to elevated temperature may be caused by various reasons. For example, mutations in the PKC pathway caused defects in the cell wall heat sensitivity of the yeast (14). A calcineurin gene, *CNA1*, is essential for heat resistance (35). The vacuolar H^+ -ATPase subunit *VPH1* and the 5'-activated protein kinase subunit *SNF1* also are required for growth at high temperatures (13, 47). The action of Tsp2-1 in heat resistance and oxidative/nitrosative stress remains to be illustrated.

In this work, we identified a tetraspanin-encoding gene, *TSP2-1*, from the basidiomycetous yeast *C. neoformans*, and we demonstrated that it is a glucose-dependent repressor of laccase. Tsp2-1 is also a key component for the integrity of the plasma membrane. The characterization of Tsp2-1 reveals a significant divergence in functions among fungal tetraspanins (8, 15, 24, 28). Questions are raised by this work, e.g., whether tetraspanin-dependent laccase repression is present in other fungi. The work may help to illustrate the molecular basis of laccase repression by glucose in *C. neoformans*.

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