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Fungal Biology

journal homepage: www.elsevier.com/locate/funbio



Role of the putative transcription factor Rim101 in pH/oxidative stress response and cell wall structure formation in the white-rot fungus *Pleurotus ostreatus*

Saori Endo^a, Moriyuki Kawauchi^{a,*}, Yuitsu Otsuka^a, Junxian Han^a, Rio Sato^a, Daishiro Koshi^a, Kenya Tsuji^a, Akira Yoshimi^{a,b}, Chihiro Tanaka^{a,b}, Shigekazu Yano^c, Takehito Nakazawa^a, Yoichi Honda^a

^a Graduate School of Agriculture, Kyoto University, Kitashirakawaoiwakecho, Sakyo-ku, Kyoto, 606-8502, Japan

^b Graduate School of Global Environmental Studies, Kyoto University, Kitashirakawaoiwakecho, Sakyo-ku, Kyoto, 606-8502, Japan

^c Graduate School of Sciences and Engineering, Yamagata University, Jonan, Yonezawa, Yamagata, 992-8510, Japan

ARTICLE INFO

Keywords:

Rim101
Oxidative stress
Cell wall
White-rot fungi
Pleurotus ostreatus

ABSTRACT

The transcription factor Rim101 regulates various cellular functions in fungi, including maintaining cell homeostasis, regulating redox processes, and transmembrane transport, in response to environmental pH changes and redox stress. White-rot fungi encounter various environmental stresses during wood degradation. In this study, the role of Rim101 in the stress response of white-rot fungi was investigated by analysing the phenotype of *rim101*-disrupted strains of *Pleurotus ostreatus*. The $\Delta rim101$ strains exhibited approximately a 15 % decrease in growth rate on agar medium and impaired growth under culture conditions at pH 5, compared to the wild-type (WT) strain. Disruption of Rim101 also increased sensitivity to oxidative stress induced by H₂O₂. Notably, RT-PCR analysis showed reduced expression of oxidative stress-related genes (*cat1*, *cat2*, *gsh-px*, and *sod4*), suggesting that Rim101 is essential for the normal oxidative stress response in *P. ostreatus*. Furthermore, the $\Delta rim101$ strains exhibited reduced resistance to cell wall synthesis inhibitors, such as Calcofluor White and Micafungin. While the $\Delta rim101$ strains showed no significant differences in the expression of cell wall polysaccharide synthesis enzyme genes or cell wall thickness compared to the WT strain, the content of α -glucan in the dried mycelia and the signal of α -glucan detected by a fluorescent protein probe in the inner layer of the cell wall were increased. Therefore, disruption of *rim101* appears to affect cell wall integrity. This study provides evidence for the conserved function of Rim101 in pH and oxidative stress responses among basidiomycetes and ascomycetes. Additionally, a novel function of Rim101 was revealed, wherein it modulates the formation of the inner α -glucan layer in the cell wall of *P. ostreatus*.

1. Introduction

Fungi are exposed to various stresses while growing in natural environments, including oxidative stress. Oxidative stress is induced by excessive amounts of reactive oxygen species (ROS), which include hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and hydroxyl radicals (•OH). ROS oxidise cell membranes, proteins, and nucleic acids, and their excessive generation causes an unstable state, which can induce pathological processes and eventually lead to cell death (Belozerskaya and Gessler, 2007). White-rot fungi can be exposed to high oxidative stress during the efficient degradation of wood. Wood is mainly

composed of cellulose, hemicellulose, and lignin. White-rot fungi are particularly capable of decomposing lignin with their lignin-degrading enzymes (Martínez, 2002). Because of lignin's complex and redox-stable chemical structure, its decomposition involves strong oxidative reactions. White-rot fungi degrade lignin efficiently using oxidative processes involving highly reactive hydroxyl radicals (Hammel et al., 2002). In addition to these extracellular decomposition reactions, ROS are involved in a variety of physiological properties, including responses to abiotic factors such as changes in temperature and humidity, light and radiation, the presence of harmful chemicals and metals, and nutrient deficiencies, as well as interactions with other

* Corresponding author. Graduate School of Agriculture Kyoto University Kitashirakawaoiwakecho, Sakyo-ku, Kyoto, 606–8502, Japan.

E-mail address: kawauchi.moriyuki.8c@kyoto-u.ac.jp (M. Kawauchi).

<https://doi.org/10.1016/j.funbio.2025.101713>

Received 20 February 2025; Received in revised form 11 December 2025; Accepted 17 December 2025

Available online 18 December 2025

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microorganisms and host organisms (Mattila et al., 2022). White-rot fungi also survive in this oxidative state where ROS are continuously generated. In addition to oxidative stress, for example, the pH condition in wood can be elevated or depressed by the wood-degrading activity of the white-rot fungi (Koenigs, 1972). This results in pH changes that they must adapt to during the process of wood degradation. It is essential to control these stress responses to degrade wood efficiently while adapting to such wood decomposition-related stressful environments. White-rot fungi are therefore thought to have unique signalling pathways that activate antioxidant enzymes to protect cells and regulate the structure and strength of cell walls.

Rim101 was identified as a pH-responsive transcription factor in the ascomycete *Aspergillus nidulans* (Tilburn et al., 1995). Changes in pH can affect numerous cellular processes, including maintaining cellular homeostasis and morphogenesis by regulating redox processes, stabilising, and regulating the function of proteins such as hydrolases, and controlling transmembrane transport, membrane stability, and nutrient absorption (Davis, 2003; van der Rest et al., 1995). In ascomycetes, pH responses by Rim101 and the associated control of various cellular functions have been studied mainly in model organisms such as *A. nidulans*, *Saccharomyces cerevisiae*, and *Candida albicans*. In these fungi, Rim101 is an important transcription factor that plays a central role in the pH signal transduction pathway called the Pal/Rim pathway. This pathway mainly responds to environmental stresses, including pH changes, and plays an important role in controlling the adaptive functions of cells (Peñalva et al., 2008, 2014). Rim101 has been reported to have various other roles as a transcription factor in ascomycete fungi. In pathogenic species such as *Aspergillus fumigatus* and *C. albicans*, the function of the *rim101* gene also affects the induction of virulence because the fungi often induce their virulence-related phenotypes in response to the pH of the host body tissues (Bertuzzi et al., 2014; Lotz et al., 2004). In addition, Rim101 has been found to control a variety of other functions, including cell wall remodelling, which is necessary to adapt to different environments. In *A. fumigatus*, mutation in *pacC*, a *rim101* homolog, leads to dysregulated expression of secreted protein genes, defined as cell wall synthesis enzymes and gliotoxin biosynthetic genes, during infection (Bertuzzi et al., 2014). In *Neurospora crassa*, deletion of *pac-3*, a homolog of *rim101*, induced abnormal control of gene expression involved in cell wall biosynthesis, redox processes, hydrolase activity, and transmembrane transport when grown under conditions with both low and high concentrations of phytates (Martins et al., 2019).

In yeast-like basidiomycetes, the role of Rim101 in controlling various cellular functions in response to environmental stress is conserved. For example, Rim101 in *Cryptococcus neoformans* is essential for growth under alkaline conditions, and *rim101* knockout strains showed growth defects under iron-deficient and salt-stress conditions (O'Meara et al., 2013, 2014). In *Ustilago maydis*, disruption of *rim101* caused reduced secretion of pH-dependent proteases, reduced resistance to Na⁺, Li⁺ ions, and osmotic stress, increased secretion of polysaccharides, and abnormal septum formation (Aréchiga-Carvajal and Ruiz-Herrera, 2005). Furthermore, in basidiomycetes, some direct involvement of Rim101 in cell wall polysaccharide biosynthesis has been reported. For example, it has been reported that in *C. neoformans*, the expression of α -1,3-glucan, β -1,3/1,6-glucan, and chitin/chitosan biosynthesis-related genes was abnormally regulated in the *rim101* knockout strain, and the amount of chitin/chitosan and α -glucan in the cell wall increased (O'Meara et al., 2010). In *U. maydis*, disruption of *rim101* affected the expression levels of cell wall biosynthesis-related genes (Aréchiga-Carvajal and Ruiz-Herrera, 2005; Franco-Frías et al., 2014).

On the other hand, the function of Rim101 in filamentous basidiomycetes, including white-rot fungi, has not yet been elucidated in detail. Among white-rot fungi, genetic transformation methods and genome editing systems have been well established for *Pleurotus ostreatus*, and research has been conducted using various gene mutants to understand

the wood decomposition mechanism and cell wall structure/synthesis (Nakazawa et al., 2024). In this study, the function of the transcription factor Rim101 in *P. ostreatus* was analysed and its role in the regulation of cell wall synthesis, response to environmental stresses such as pH changes and oxidative stress, and lignin degradation, the unique function in white-rot fungi, was investigated.

2. Materials and methods

2.1. Strains, culture conditions, and genetic techniques

The *P. ostreatus* strains used in this study are listed in Table S1. Yeast extract, malt extract, and glucose (YMG) medium (Rao and Niederpruem, 1969), solidified with 2 % (w/v) agar in 90 mm Petri dishes, was used for routine culture. For the growth test on wood sawdust medium, beech wood (*Fagus crenata*) sawdust (BWS) as described by Wu et al. (2021) were used: BWS-II was used. The medium was prepared in 60 mm glass Petri dishes. In each experiment, mycelium from each strain was inoculated as a plug with a cork borer (Φ 6 mm) taken from a culture on YMG agar plates for 10 days, because *P. ostreatus* strains generally do not form asexual spores. The plug was inoculated into the centre of each plate. All cultures were maintained at 28 °C in continuous darkness. The *rim101* gene-disrupted strains were generated through gene targeting with homologous recombination using the 20b strain as a host (Salame et al., 2012). For *P. ostreatus* transformation, the 20b strain was cultured in YMG liquid medium with shaking for 4 days, after which protoplasts were prepared from the mycelia using cellulase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA), Yatalase-Plus (Takara Bio, Shiga, Japan), and β -glucanase from *Trichoderma longibrachiatum* (Sigma-Aldrich, St. Louis, MO, USA). DNA disruption cassettes were introduced into the protoplasts using the polyethylene glycol/calcium chloride (PEG/CaCl₂) method as described previously (Nakazawa et al., 2016; Salame et al., 2012).

2.2. Construction of the gene disruption cassette

The *rim101* disruption cassette was constructed by fusion PCR (Szewczyk et al., 2006) of the three mutually primed DNA fragments: the upstream and downstream regions of the *rim101* gene (protein ID: 71928 in the JGI genome database for *P. ostreatus* strain PC9) and the hygromycin B-resistance marker gene (*hph*). KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan) was used for the amplification and fusion of each fragment. Genomic DNA of the *P. ostreatus* 20b strain was used as a template to amplify approximately 1.5 kb of the 5' and 3' flanking regions of *rim101*, and the pPHT1 plasmid (Cummings et al., 1999) to amplify the hygromycin resistance marker gene (*hph*). The fused and amplified fragment was purified using the Nucleospin PCR clean-up and gel extraction kit (Takara Bio, Shiga, Japan) and used as a disruption cassette. A schematic diagram of the *rim101* disruption cassette is shown in Fig. S2A, and the primers used for PCR are listed in Table S2.

2.3. Confirmation of the gene disruption

Crude genomic DNA was extracted according to a previous study (Izumitsu et al., 2012) and used as templates for genomic PCR to confirm gene disruption. PCR was performed using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). The integration of the *rim101* disruption cassette was confirmed through gene length change PCR (Fig. S1C), and the presence or absence of the *rim101* gene was verified using PCR amplification (Fig. S1D). Genomic PCR schematics are shown in Fig. S1B, and the gel electrophoresis results are presented in Fig. S1C and S1D. The primers used are listed in Table S2.

To confirm the absence of ectopic integration of the gene disruption cassette, Southern blot analysis has also been performed. About 9 μ g of genomic DNA was extracted from the 20b strain and Δ *rim101* strains

using the cetyltrimethylammonium bromide (CTAB) method, as described previously (Muraguchi et al., 2003; Zolan and Pukkila, 1986). Genomic DNA was digested using restriction enzymes followed by size fractionation in 1 % agarose gel and transferred to a positively charged Hybond-N+ membrane (Cytiva, Marlborough, MA). Probe labelling, hybridization, and detection were conducted, as described previously (Schiphof et al., 2024). Details of Southern blotting experiments are shown in Fig. S3.

2.4. Growth and morphological observations

For observations of mycelial morphology and growth rate, 20 mL of YMG 2 % agar medium was dispensed into Petri dishes (Φ90 mm), and a mycelial plug made with a cork borer (Φ8 mm) was inoculated into the centre of each plate. After 10 days of static cultivation in the dark at 28 °C, images of the mycelia were captured using a scanner, and the average diameters for each mycelial colony were measured using ImageJ (Schneider et al., 2012), for the three biologically independent replicates.

For mycelial dry weight measurements, 40 mL of YMG liquid medium was dispensed into 100 mL flasks. A mycelial plug was made with a cork borer (Φ8 mm) and inoculated into each flask. After 14 days of static cultivation in the dark at 28 °C, the mycelium was washed with water, hand-pressed with a paper towel, and freeze-dried. The dried mycelial weight was measured using an electronic balance.

2.5. pH response observations

For pH response observation, YMG 2 % agar medium was supplemented with 6 N HCl until the pH reached 5.0 and 6.0, and 6 N NaOH until the pH reached 8.0, 9.0, and 10.0, and then autoclaved. YMG agar without HCl and NaOH was used as a control. For pH 4.0, an equal quantity of 2 × YMG (adjusted to pH 4.0 with HCl) and 4 % agar water was autoclaved and then mixed. YMG agar medium mixed in the same manner but without adjusting the pH was used as a control. A mycelial plug made with a cork borer (Φ8 mm) was inoculated into the centre of each plate. After 10 days of cultivation in the dark at 28 °C, images were captured using a scanner to measure the diameter of the colonies. The ratio of the colony diameter of the *rim101* disrupted strains to that of the wild-type (WT) under the same pH condition was calculated, with the colony diameter of the WT set to 1.

2.6. Stress resistance assay

YMG agar medium was supplemented with 5 mM H₂O₂ to test resistance to oxidative stressors. Additionally, 500 µg/mL Calcofluor White (CFW), 100 µg/mL Micafungin (MF), and 0.02 % sodium dodecyl sulphate (SDS) were used to test resistance to cell wall synthesis inhibitors. Fluorescent Brightener 28 disodium salt solution (Sigma–Aldrich) and Funguard (Astellas, Tokyo, Japan) were used as CFW and MF, respectively. YMG agar without the stressors was used as a control. A mycelial plug made with a cork borer (Φ8 mm) was inoculated into the centre of each plate. After 10 days of cultivation in the dark at 28 °C, images were captured using a scanner, and the relative inhibitory rates were calculated from the colony diameters as previously described (Kojima et al., 2024).

2.7. Quantitative reverse-transcription PCR (qRT-PCR)

YMG agar (20 mL) was dispensed into Petri dishes (Φ90 mm). After solidifying, a single layer of cellophane was placed to cover the entire plate. A mycelial plug made with a cork borer (Φ8 mm) was inoculated into the centre of each plate and incubated for 10 days in the dark at 28 °C. After incubation, mycelia were peeled from the cellophane and used for RNA extractions using a FastGene RNA Premium Kit (NIPPON Genetics, Tokyo, Japan). As previously described (Kojima et al., 2024),

the RNA was reverse transcribed to cDNA with PrimeScript RT Master Mix (Takara bio, Shiga, Japan) and then quantitative PCR was performed with the PowerTrack SYBR Green Master Mix and QuantStudio 5 (Thermo Fisher Scientific, Waltham, MA, USA). The Pfaffl method was used for quantification (Pfaffl, 2001). The primer sets used for the amplification of cDNA fragments and their amplification efficiencies are listed in Table S3.

For assays of the transient response to oxidative stress through the oxidative stress-related gene transcriptional regulation, each strain was cultured on the mixed cellulose ester membrane filter (pore size 5.0 µm, ADVANTEC, Tokyo, Japan) on YMG plates for 10 days and then transferred to YMG plates and H₂O₂-supplemented ones, followed by mycelium collection and RNA extraction as described above after 60 min.

2.8. Glucan and chitin measurements

For glucan and chitin measurements, 20 mL of YMG agar was dispensed into Petri dishes (Φ90 mm), and a single layer of cellophane was placed to cover the entire plate. A mycelial plug made with a cork borer (Φ8 mm) was then inoculated into the centre of each plate and incubated for 10 days in the dark at 28 °C. After incubation, the mycelia were peeled from the cellophane using a sterile spatula, washed with reverse osmosis water, ground into powder using liquid nitrogen, and a mortar and pestle, and then freeze-dried.

As previously described (Schiphof et al., 2024), glucan and chitin assays were conducted as follows. A β-Glucan Assay Kit (Yeast and Mushroom) (Megazyme, Bray, Ireland) was used for glucan measurement. Total glucan measurements were conducted using freeze-dried mycelia hydrolysed with sulfuric acid (H₂SO₄) and reagents in the assay kit, and α-glucan was measured using the freeze-dried mycelia treated with 1.7 N NaOH and the amyloglucosidase, invertase, and trehalase in the assay kit. A D-Glucosamine Assay Kit (Megazyme, Bray, Ireland) was also used for chitin measurement. It was conducted using the freeze-dried mycelia hydrolysed with 4 N HCl.

2.9. Cell wall and hyphal observations

Transmission electron microscopy (TEM) was performed to observe the cell wall thickness and structure. The 20b and *rim101*-disrupted strains were cultivated on Petri dishes (Φ90 mm) with 20 mL of YMG agar medium at 28 °C for 10 days. Observations were made after fixation and dehydration, as previously described (Han et al., 2023).

For hyphal observations, 40 mL of YMG medium was dispensed into 100 mL flasks. Eight mycelial plugs made with a cork borer (Φ8 mm) were inoculated into each flask and incubated at 28 °C for 5 days under shaking at 120 rpm. Samples were homogenised using a Polytron homogeniser (Kinematica AG, Malers, Switzerland), and 200 µL of the homogenised solution was inoculated into 40 mL of fresh YMG liquid medium and incubated at 28 °C and 120 rpm for 2 days. Subsequently, 1.5 mL of the solution was washed with 1 M Tris–HCl. In the β-glucanase treatment condition, the appropriate amount of β-glucanase from *T. longibrachiatum* (Sigma–Aldrich, St. Louis, MO, USA) was added to achieve a concentration of 10 µg/mL. Samples were then incubated at 37 °C for 5 h, and hyphal surface glucans were subsequently observed. To probe the hyphal surface α-glucan and β-glucan, 7 µL of 10 nmol/mL α-1,3-glucan-binding domain with an attached red fluorescent protein (DCD-tetraRFP) and 30 µL of 10 nmol/mL β-glucan-binding domain with an attached green fluorescent protein (BGBD-GFP), respectively (Otsuka et al., 2022a, 2022b), were used. Samples were stained with them on ice for 2 h, and confocal microscopic observation was conducted as previously described (Kojima et al., 2024).

2.10. Statistical analysis

All statistical significance tests of differences were performed by calculating *p*-values with a two-tailed equal variance *t*-test.

3. Results

3.1. Disruption of *rim101* affects mycelial growth and acidic pH response

To predict the *rim101* gene in *P. ostreatus*, a BLASTP search was performed in the JGI genome database of *P. ostreatus* PC9 strain (Nordberg et al., 2014). As a reference sequence, the amino acid sequence of the transcription factor Rim101 (XP_012053767.1) in the basidiomycete yeast *C. neoformans* var. *neoformans* JEC21 on NCBI (Schoch et al., 2020) was used. As a result, one Rim101 homolog (Protein ID: 71928) was predicted in *P. ostreatus*. Based on these amino acid sequences, the domain structures were analysed using InterPro (Blum et al., 2025) and demonstrated that the predicted protein has a similar domain structure with *C. neoformans* Rim101 (Fig. S1). We concluded that this gene is the ortholog of *rim101* in *P. ostreatus*.

To analyse the function of Rim101 in *P. ostreatus*, the *rim101*-disrupted strains were generated. The *rim101* gene disruption cassette was designed to knock out the entire region of the predicted *rim101* gene in *P. ostreatus*. Using this construct, *rim101* was disrupted by gene targeting via homologous recombination using the 20b strain, a *ku80* disruptant that lacks non-homologous end-joining of which was generated from PC9 strain, as a host (Fig. S2A and B). As a result of the transformation, five transformants were obtained, and the integration of the *rim101* disruption cassette and the absence of the *rim101* gene were confirmed in these strains through genomic PCR (Fig. S2C and D). Among these strains, two independent strains ($\Delta rim101$ #11 and $\Delta rim101$ #12) were randomly selected. These strains, along with the parental strain 20b, were used for subsequent experiments. First, the complete lack of the *rim101* mRNA expression in these strains was confirmed by qRT-PCR (Fig. S2E). In addition, Southern blot analysis confirmed that the disruption cassette was inserted at a single site and that there was no ectopic insertion (Fig. S3).

On YMG agar medium, the mycelial colony size of $\Delta rim101$ strains was reduced by approximately 15 % compared to that of the WT strain (Fig. 1A and B). On YMG liquid medium, the mycelial dry weight of $\Delta rim101$ strains was approximately 30 % lower than that of the WT (Fig. 1C). These findings indicate that the $\Delta rim101$ strains exhibited reduced growth rates, suggesting that Rim101 is essential for the normal growth of *P. ostreatus* in both solid and liquid environments. However, the disruptants and WT did not exhibit significant morphological differences, such as the density of hyphae in the colony or the formation of aerial hyphae.

Additionally, $\Delta rim101$ strains exhibited a reduced growth rate on media adjusted to acidic pH levels. On YMG media adjusted to pH 4 and 5, the disruptants showed a colony diameter 10–20 % smaller than that of the WT (Fig. 2). This result suggests that Rim101 requires normal adapting responses to acidic pH conditions.

3.2. *rim101* is required for oxidative stress response

Resistance assays to oxidative stressors were conducted to investigate the role of Rim101 in mediating responses to environmental stresses in *P. ostreatus*. The $\Delta rim101$ strains showed approximately 50 % growth inhibition under 5 mM H_2O_2 , compared to the WT, which had a growth inhibition rate of approximately 20 %. Furthermore, the *rim101* disruptants under oxidative stress conditions displayed morphology with highly dense and long aerial hyphae (Fig. 3A and B).

The expression levels of oxidative stress-related genes were examined by qRT-PCR, and the relative expression levels of four genes—*cat1*, *cat2*, *gsh-px*, and *sod4*—were significantly decreased. Specifically, the expression of *cat1* and *cat2* was 80–90 % lower in the $\Delta rim101$ strains compared to the WT (Fig. 3C). Furthermore, in order to ascertain the effect of the *rim101* gene disruption on the early oxidative stress response, a comparison was made of the expression levels of oxidative

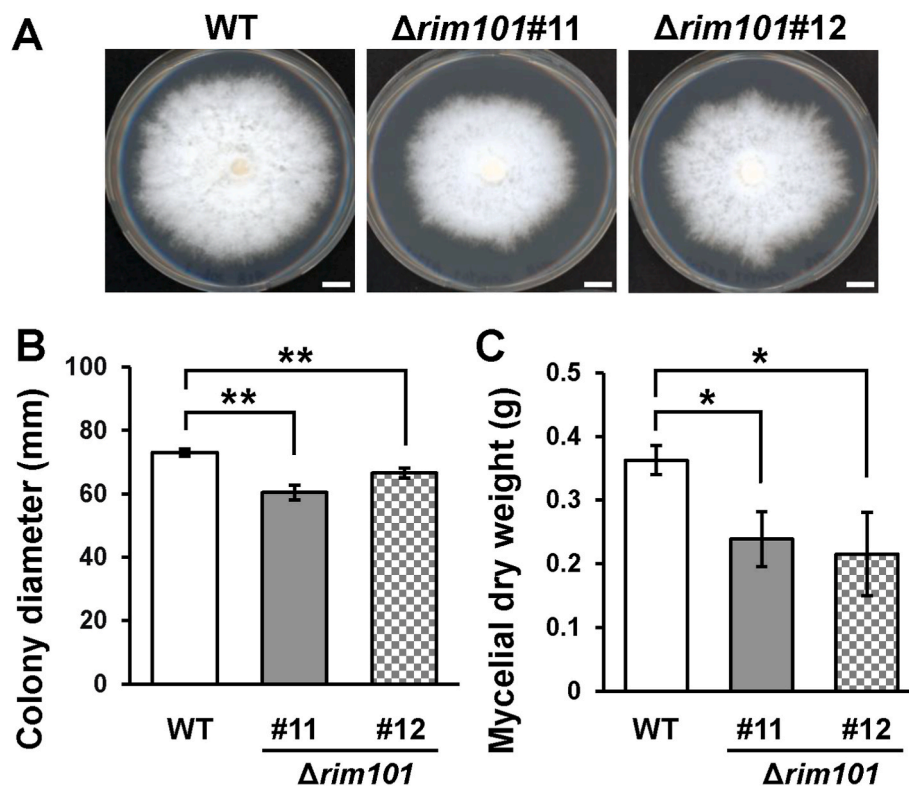


Fig. 1. Morphology and growth rate of the wild-type (WT) and $\Delta rim101$ strains. (A) Effect of *rim101* disruption on the morphology of colonies grown on yeast and malt extract with glucose (YMG) agar (scale bar = 10 mm). (B) Colony diameter of each strain on YMG agar plates. (C) Dry weight of mycelia grown on YMG liquid medium. The bar indicates the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined by a two-tailed equal variance *t*-test (** $P < 0.01$).

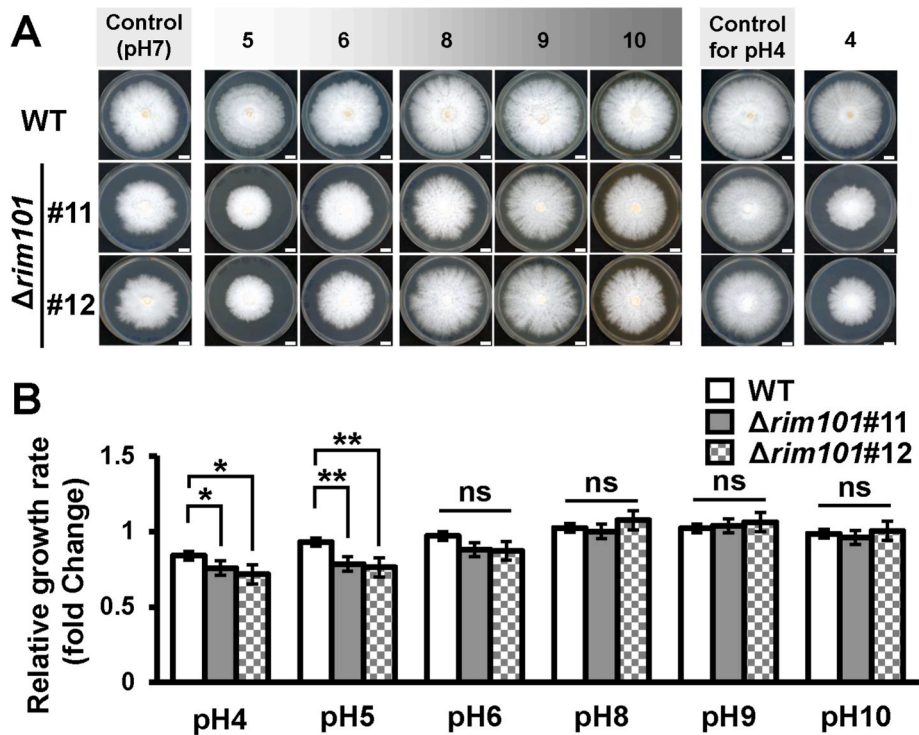


Fig. 2. Morphology and growth rate in the different pH conditions of the wild-type (WT) and $\Delta rim101$ strains. (A) Effect of *rim101* disruption on the morphology of colonies grown on different pH yeast and malt extract with glucose (YMG) agar. Each medium was supplemented with 6 N HCl or 6 N NaOH to achieve the determined pH condition (scale bar = 10 mm). (B) The growth rate of each strain on the varied pH condition compared to the ones on the non-modified YMG agar (pH 7) plates. The bar indicates the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined by a two-tailed equal variance *t*-test (ns: $P > 0.05$, ** $P < 0.01$).

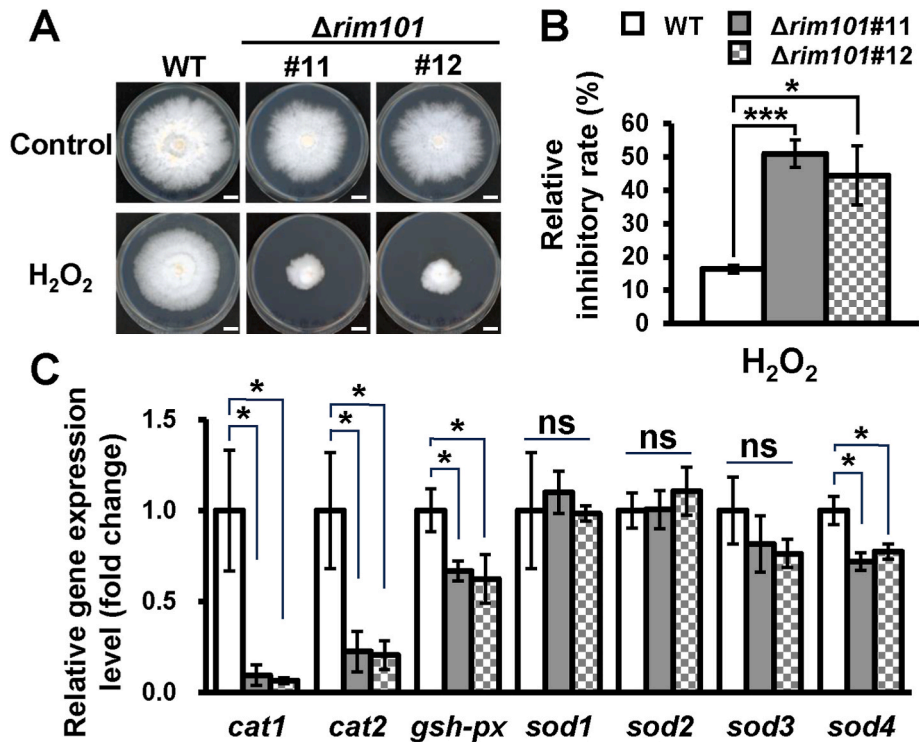


Fig. 3. The oxidative stress response of mycelia and relative gene expression levels in the wild-type (WT) and $\Delta rim101$ strains. (A) Phenotypes of all strains grown on yeast and malt extract with glucose (YMG) agar medium supplemented with 5 mM H_2O_2 for 10 days (scale bar = 10 mm). (B) Bar charts show strains' relative growth inhibition rate in (A). (C) The relative gene expression levels of various oxidative stress response-related genes of the WT and $\Delta rim101$ strains. The bars indicate the standard deviations of three biological replicates ($n = 3$). A two-tailed equal variance *t*-test determined statistical significance (ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

stress-related genes 60 min after the strains were transferred from YMG agar medium to YMG agar medium with/without H₂O₂. Especially for *cat2*, the increase in expression upon H₂O₂ addition observed in the WT was not observed in the *rim101* disruptants (Fig. S4). These results suggest that Rim101 is directly or indirectly involved in the regulation of the expression of oxidative stress response-related genes, and the disruption of *rim101* reduces oxidative stress resistance.

3.3. Rim101 plays an important role in cell wall formation

To investigate the effect of *rim101* disruption on cell wall synthesis, the sensitivities of the WT and $\Delta rim101$ strains to cell wall synthesis inhibitors were first examined. Compared to the WT, $\Delta rim101$ strains exhibited about a two-fold increase in sensitivity to CFW, a chitin synthesis inhibitor, and 3–4-fold increased sensitivity to MF, a β -glucan synthesis inhibitor. Additionally, $\Delta rim101$ strains showed approximately two-fold increased sensitivity to SDS (Fig. 4A and B). A previous study using *P. ostreatus* revealed that disruption of chitin synthase caused increased sensitivity to SDS, a membrane disruptor (Schiphof et al., 2024). Similarly, $\Delta mbp1$ strains, which have a defect in glucan

synthesis, were more sensitive to SDS than the WT (Kojima et al., 2024, 2025). Taken together, these findings demonstrate that cell wall integrity is disturbed in the $\Delta rim101$ strains.

Next, the relative percentages of major cell wall polysaccharides, α -glucan, β -glucan, and chitin (g/100 g dried mycelium) were measured. The relative percentages of β -glucan remained largely unchanged between the WT and the disruptants. However, a significant increase in α -glucan levels was observed, reaching approximately 16 % of the total mycelial dry mass in the $\Delta rim101$ strains, compared to approximately 13 % in the WT (Fig. 5A). A slight increase in chitin content was also observed in the mutant strains, although the difference was minimal. On the other hand, the relative expression levels of cell wall synthase genes were examined via qRT-PCR, and no significant differences were observed between the WT and $\Delta rim101$ strains in the relative expression levels of the α -glucan synthase gene *ags1*, the β -glucan synthase genes *fks1* and *fks2* (Fig. 5B), and the chitin synthase genes *chs1-4* and *chs5-9* (Fig. 5C).

To further investigate the effects of *rim101* disruption on the cell wall structure, microscopic observations of the cell walls and hyphal surface polysaccharides of each strain were conducted. TEM observations revealed no notable differences in the electron images or the thickness of the cell wall between the WT and $\Delta rim101$ strains (Fig. 5D and E). In confocal microscopy, an α -glucan-specific red fluorescent probe DCD-tetraRFP and a β -glucan-specific green fluorescent probe BGBD-GFP (Otsuka et al., 2022a, 2022b) were used to observe the hyphal surface glucans. In the control condition, no significant differences were observed in either red or green fluorescence. To observe the inner layer of the cell wall, mycelia were treated with β -glucanase to peel off the surface-covered β -glucan layer, which was visualised by BGBD-GFP. Consequently, an increase in red fluorescence was observed upon treatment in both the WT and $\Delta rim101$ strains (Fig. 5F). However, a greater increase in red fluorescence was observed in the $\Delta rim101$ strains. This phenomenon may be attributed to alterations in the quantity or spatial distribution of α -glucan within the cell wall. The results observed in TEM and fluorescence imaging suggest that disruption of *rim101* affects cell wall formation without substantially impacting its thickness.

4. Discussion

In this study, to understand the function of the Rim101 transcription factor in the white-rot fungus *P. ostreatus*, *rim101* disruptants were isolated. Subsequently, the role of Rim101 in environmental stress response and cell wall formation was investigated using the $\Delta rim101$ strains. Firstly, the $\Delta rim101$ strains exhibited a decrease in growth rate compared to the WT under acidic pH conditions, especially at pH 4.0 and 5.0 (Fig. 2). In basidiomycetous yeasts, *C. neoformans* and *U. maydis*, Rim101 is known as an environmental pH-responsive transcription factor controlling various cellular functions and has been reported to play an important role, primarily in adaptation to alkaline pH. In these fungi, growth impairment under alkaline pH conditions has been observed in *rim101*-disrupted strains (Franco-Frías et al., 2014; O'Meara et al., 2010). Since such growth inhibition was not observed under alkaline pH conditions in *P. ostreatus*, it is suggested that the function of Rim101 in this fungus may be unique in terms of its role in pH response.

In addition to pH response, increased sensitivity to H₂O₂ was observed after the disruption of *P. ostreatus rim101* (Fig. 3A and B). Oxidative stress is induced by the overproduction of ROS, such as H₂O₂ and hydroxyl radicals. Generally, fungi have several antioxidant enzymes that help them cope with this stress. Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion to H₂O₂, which is then decomposed by catalase (CAT) and glutathione peroxidase (GSH-Px). GSH-Px also catalyses the conversion of lipid hydroperoxides to non-toxic alcohols. These catalytic reactions help maintain redox balance within fungal cells (Ayar-Kayali and Tarhan, 2004). The $\Delta rim101$ strains exhibited significantly reduced expression of four genes encoding antioxidant enzymes, *cat1*, *cat2*, *gsh-px*, and *sod4*, compared to the WT.

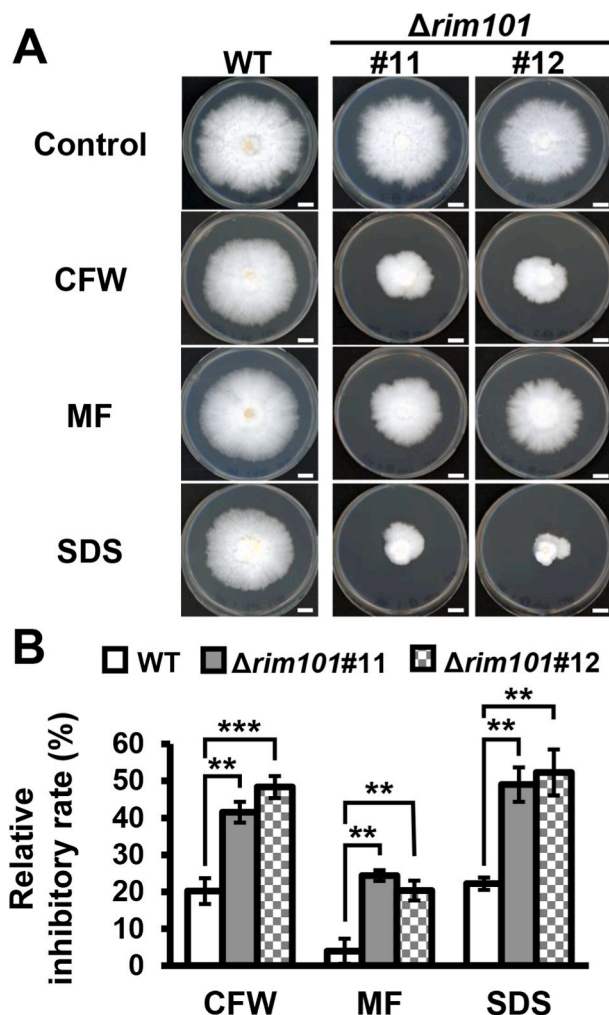


Fig. 4. The cell wall stress resistance of the wild-type (WT) and $\Delta rim101$ strains. (A) Mycelial morphology grown on yeast extract, malt extract, and glucose (YMG) agar media supplemented with 500 μg/mL Calcofluor White (CFW), 100 μg/mL Micafungin (MF), and 0.02 % sodium dodecyl sulphate (SDS) after 10 days (scale bar = 10 mm). (B) The relative growth inhibitory rates of the WT and $\Delta rim101$ mycelia. The bars indicate the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined using a two-tailed equal variance *t*-test (** $P < 0.01$, *** $P < 0.001$).

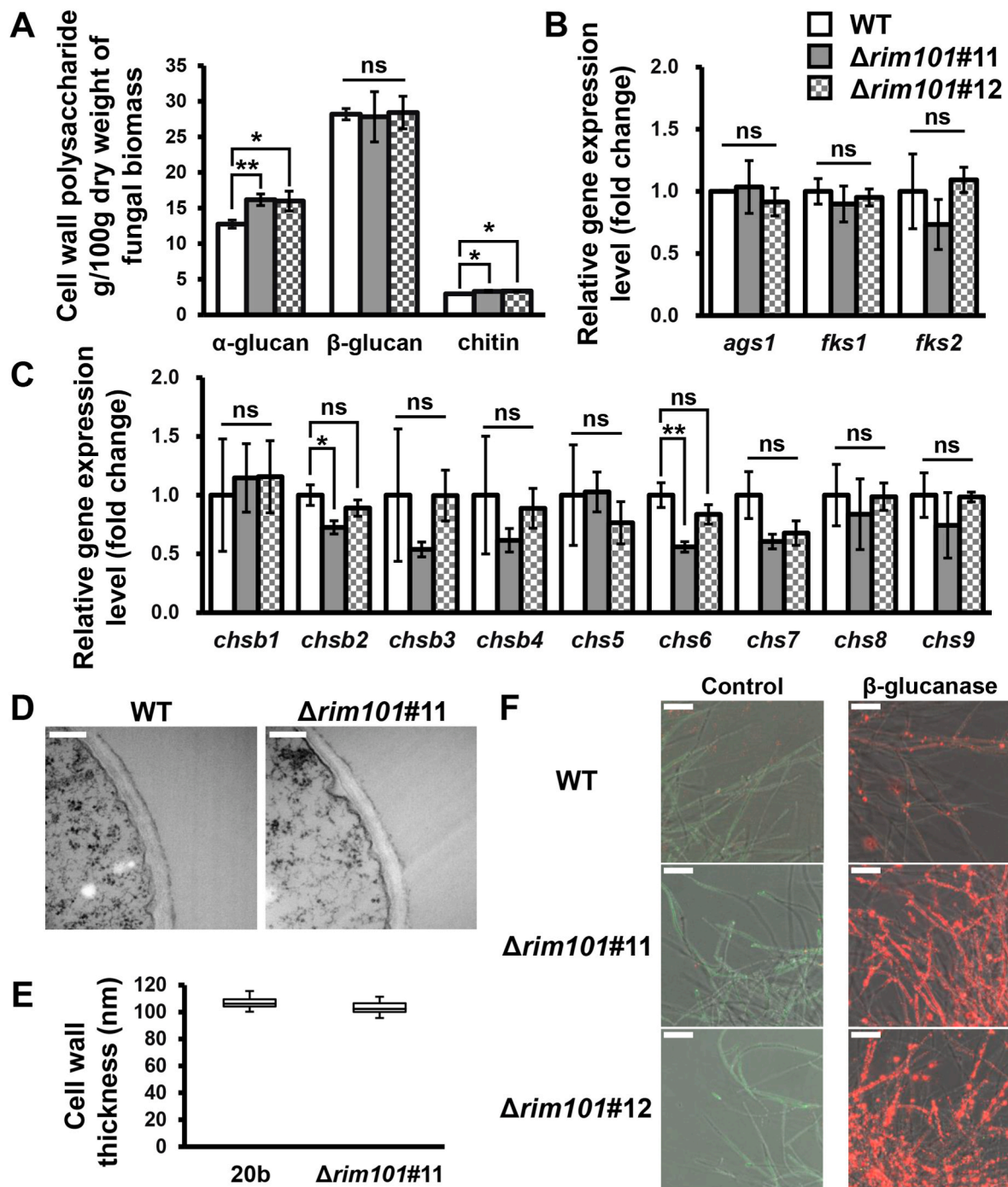


Fig. 5. Cell wall components, structure, and relative gene expression levels of various cell wall synthases of the wild-type (WT) and $\Delta rim101$ strains. (A) Each polysaccharide weight content of dried mycelia. (B, C) Relative expression levels of the cell wall synthase genes ((B) *ags*: α -glucan synthase, *fks*: β -glucan synthase, (C) *chs*: chitin synthase) in the WT and $\Delta rim101$ strains. (D) Cell wall transmission electron microscopy (TEM) at 30,000 magnifications (scale bar = 200 nm). (E) Cell wall thickness. (F) α -glucan and β -glucan staining using the specific fluorescent probes (DCD-tetraRFP and BGBD-GFP, respectively) under a confocal microscope (scale bar = 20 μ m). Images merged fluorescence images and optical microscopy with differential interference contrast. Red fluorescence indicates α -glucans, and green fluorescence indicates β -glucans. β -glucanase: the mycelia were treated for 5 h with β -glucanase from *Trichoderma longibrachiatum*. A two-tailed equal variance *t*-test determined statistical significance (ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Specifically, the expression of *cat1* and *cat2* was 80–90 % lower in the $\Delta rim101$ strains compared to the WT (Fig. 3C). Furthermore, the increase in expression of some of the antioxidant enzyme genes, especially *cat2*, upon H_2O_2 addition observed in the WT was not observed in the disruptants (Fig. S4). These suggest that the expression induction of these genes plays a crucial role in the oxidative stress response, and that *rim101* disruption may impair this normal transient response to

oxidative stress through the regulation of them. To support this observation, we examined whether the promoter region of the oxidative stress-related genes contained a Rim101-binding motif. The consensus Rim101-binding site (5'-GCCARG-3', R = A or G) has been predicted in ascomycete *A. nidulans*, *Metarhizium robertsii*, *Coniothyrium minitans*, and basidiomycete *Ganoderma lucidum* (Peñalva et al., 2008; Huang et al., 2015; Lou et al., 2015; Hu et al., 2020). Among the genes in *P. ostreatus*,

cat2, *sod1*, and *sod3* contain the binding site within 1500 bp upstream of the coding region (Fig. S5), suggesting that the regulation of expression may be mediated by Rim101 DNA binding. In *G. lucidum*, silencing of *GlpacC* reduced oxidative stress resistance (Hu et al., 2020). In the ascomycete *Trichothecium roseum*, the *PacC* mutant was more sensitive to oxidative stress compared to the WT (Wang et al., 2022). In *Botrytis cinerea*, *BcpacC* is involved in ROS generation (Rascole et al., 2018). In *Colletotrichum acutatum*, CAT activity was reduced in the disruptant of *pacC*^{KLAP2}, a homolog of *rim101* (You and Chung, 2007). These results suggest that the function of Rim101 in oxidative stress responses is widely conserved among fungi.

Preliminary experiments were conducted to evaluate the impact of *rim101* disruption on growth in BWS medium (Fig. S6). The $\Delta rim101$ strains appear to exhibit delayed growth in comparison to the WT over the 8-day culturing period (Fig. S6A). After 20 days of culture, the medium surface hyphal appearance exhibited a slight decrease in the $\Delta rim101$ strains in comparison with the WT (Fig. S6B). To verify whether *rim101* plays specific roles in growth on BWS medium, it is necessary to determine whether observed growth delay is comparable to or different from that on YMG medium through biomass measurements. In addition, further investigation is necessary to validate the impact of *rim101* disruption on the gene expression and activity of wood-degrading enzymes, as well as the effect on wood component degradation. These subsequent analyses could elucidate the potential relationship between *rim101*, oxidative stress response, and wood degradation.

The $\Delta rim101$ strains exhibited no significant alterations in the relative gene expression levels of cell wall polysaccharide synthesis enzymes (Fig. 5B and C). Furthermore, TEM observations of the $\Delta rim101$ strains revealed no discernible changes in cell wall morphology or thickness (Fig. 5D and E). However, the α -glucan content increased by approximately 1.2-fold (Fig. 5A), even though the expression of the α -glucan synthase gene *ags1* did not increase (Fig. 5B). Moreover, imaging analysis of cell wall glucans revealed that upon treatment of mycelia with β -glucanase, which removes β -glucan from the cell wall surface, there was a visible increase in the fluorescence intensity of α -glucan in the inner layer of the cell wall in the $\Delta rim101$ strains compared to the WT (Fig. 5F). These findings suggest that *rim101* disruption affected the cell wall content, including α -glucan in the inner layer. Other gene disruption studies in *P. ostreatus* suggest that the α -glucan content does not necessarily correspond to the expression level of *ags1*. For example, in the *P. ostreatus* *hydp16*-disrupted strain, *ags1* expression did not change compared to the WT, but the α -glucan content increased by approximately 1.15-fold (Han et al., 2025). Disruption of *chsb2* and *chsb3* caused an increase in *ags1* expression, but the α -glucan content did not increase significantly (Schiphof et al., 2024). The possible explanation for this discrepancy may lie in the balance between the synthesis and degradation of α -glucan. If the expression of α -glucan-degrading or modifying enzymes is reduced, the measured amount of α -glucan in the cell wall may be higher, even if the expression of *ags1* remains the same. This hypothesis requires further verification through detailed analyses, such as transcriptome analysis. Taken together, it is suggested that *P. ostreatus* Rim101 may be involved in cell wall formation, and the disruption of *rim101* may lead to changes in the content of α -glucan inside the β -glucan layer within the cell wall.

Studies on the Rim101 function have been conducted in other basidiomycete fungi, and direct control of cell wall polysaccharide synthesis has been commonly reported. For example, *C. neoformans* Rim101 is involved in cell wall remodelling under various induction conditions, and genes related to polysaccharide synthesis are differentially transcribed in a Rim101-dependent manner. Under capsule induction conditions, where Rim101 is induced, the expression of β -glucan synthase genes *FKS1* and *KRE6* was approximately five- and two-fold higher in the *rim101* disruptants, respectively, whereas the β -(1,3)-glucan content did not change. On the other hand, chitin and α -glucan levels were increased in the *rim101* disruptants compared to the WT, and similarly, the expression levels of the α -glucan synthase gene *AGS1* and

several genes encoding chitin synthase were increased by two- to three-fold, suggesting an essential role for *rim101* in the normal synthesis of chitin and α -glucan in this fungus (O'Meara et al., 2013, 2014). In *U. maydis*, the disruption of the *rim101* gene resulted in a decrease in the expression levels of chitin synthase genes by two- to three-fold and of β -glucan synthesis-related genes (*KRE6* by 25-fold and *SKN1* by 100-fold). Furthermore, the *rim101* knockout strain exhibited increased cell wall sensitivity to lysing enzymes, suggesting effects on the cell wall structure (Aréchiga-Carvajal and Ruiz-Herrera, 2005; Franco-Frías et al., 2014). In *G. lucidum*, *rim101* disruption resulted in approximately a 20 % decrease in cell wall β -glucan content and a 20–50 % reduction in the expression levels of putative glucan synthase genes (Hu et al., 2020). Our study also suggests that the function of Rim101 is involved in cell wall formation in *P. ostreatus*, although no changes in the expression of polysaccharide synthase genes were observed. Taken together, these studies illustrate involvement of Rim101 in the cell wall structure/formation process in these basidiomycetes, though the mode of action may vary. To elucidate the detailed molecular mechanisms of Rim101 function in cell wall formation in *P. ostreatus*, further studies are needed through more comprehensive analysis, such as transcriptomics using RNA-Seq.

5. Conclusion

Disruption of the gene encoding the transcription factor Rim101 in the white-rot fungus *P. ostreatus* resulted in abnormal acidic pH responses, increased sensitivity to oxidative stress agents, and reduced expression levels of antioxidant enzyme genes. These findings suggest that Rim101 plays an essential role in regulating the response to these environmental stresses in *P. ostreatus*. *rim101* disruption also reduced resistance to the cell wall inhibitors CFW and MF and increased the amount of α -glucan in the inner layer of the cell wall, suggesting that Rim101 also plays a role in cell wall integrity in *P. ostreatus*. This study provides further evidence for the relationship between Rim101 and the pH and oxidative stress response, which are conserved in both basidiomycetes and ascomycetes. It also elucidates the unique function of Rim101 in regulating the process of the cell wall inner α -glucan layer formation in *P. ostreatus*.

CRedit authorship contribution statement

Saori Endo: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Moriyuki Kawauchi:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Yuitsu Otsuka:** Methodology, Investigation, Funding acquisition, Data curation. **Junxian Han:** Investigation, Data curation. **Rio Sato:** Investigation, Data curation. **Daishiro Koshi:** Investigation, Data curation. **Kenya Tsuji:** Resources, Funding acquisition, Data curation. **Akira Yoshimi:** Resources, Funding acquisition, Data curation. **Chihiro Tanaka:** Resources, Project administration, Data curation. **Shigekazu Yano:** Resources, Methodology. **Takehito Nakazawa:** Methodology, Data curation. **Yoichi Honda:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization.

Funding sources

This work was supported in part by the Institute for Fermentation, Osaka, Japan (K-2019-002 to M. K. and K. T.), the Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (22H02238 to A. Y. and M. K., 22K05763, 24KK0119 to M. K.), the Grant-in-Aid for JSPS Fellows (23KJ1191 to Y. O.), and the JSPS Bilateral Program (JPJSBP 120238807 to M. K.).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Prof. Yitzhak Hadar (Hebrew University of Jerusalem) for providing the *P. ostreatus* strain 20b. We also thank the Division of Electron Microscopic Study, Centre for Anatomical Studies, Graduate School of Medicine, Kyoto University, for technical assistance with electron microscopy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funbio.2025.101713>.

Data availability

Data will be made available on request.

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