



Putative transcription factor Nrg1 is involved in the hyphal branching and the cell wall structure formation in the filamentous fungus *Pleurotus ostreatus*

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ABSTRACT

Nrg1 is a C2H2 zinc finger transcription factor that functions in various cellular processes related to environmental responses and cell wall synthesis in yeast. The present study investigated the function of Nrg1 in filamentous development in the white-rot fungus *Pleurotus ostreatus* for the first time. The $\Delta nrg1$ strains exhibited a higher frequency and larger angles of hyphal branching than the wild-type strain, suggesting *nrg1* is essential to hyphal branching regulation. Transmission electron microscopy observations indicated no significant change in cell wall thickness; however, the black layer on the cell wall surface, thought to be composed of proteins, including hydrophobins, was reduced. The reduced hydrophobin gene expression and hyphal hydrophobicity corroborate these observations. The *nrg1* disruption resulted in decreased levels of α -glucan and β -glucan content in the cell wall and the expression levels of their synthase genes. Fluorescent microscopy using protein probes for cell wall surface polysaccharides also suggested that in the $\Delta nrg1$ strains, there was an abnormal localization of chitin and β -glucan. In contrast, the fluorescent signals due to α -glucan and mannan were increased. These data demonstrate that Nrg1 is involved in the cell wall surface and structure formation via the gene expression of hydrophobin and polysaccharide synthases. This study provides the first evidence of the role of Nrg1 in filamentous fungi, especially in hyphal branching. It provides a clue toward a comprehensive understanding of the mechanisms regulating cell wall synthesis in white-rot fungi.

1. Introduction

The transcription factor Nrg1 was initially identified in *Saccharomyces cerevisiae* (Park et al., 1999). The Nrg1 protein is involved in various environmental response processes, including the regulation of numerous stress response genes, control of haploid invasive growth and biofilm formation, mitochondrial function, ion transport, conjugation, and nutrient utilization in this fungus (Kuchin et al., 2002; Vyas et al., 2005). The interactions between Nrg1 and other environmental stress response-related transcription factors have been investigated in *S. cerevisiae* (Lamb and Mitchell, 2003; Rothfels et al., 2005). In *Candida albicans*, Nrg1 has also been shown to function in environmental responses, particularly in negatively regulating the morphological transition from yeast cells to filamentous growth and in response to salt and

oxidative stress (Braun et al., 2001; Murad et al., 2001). In *Cryptococcus neoformans*, Nrg1 is involved in pathways related to the response to various stresses (e.g., osmotic, temperature, and oxidative stress) and in the production of virulence factors (i.e., capsule and melanin formation) (Caza and Kronstad, 2019). Thus, it can be inferred that Nrg1 is a transcription factor that plays an essential and multifaceted role in the adaptation to the environmental response in these yeast fungi. Also, Nrg1 has been reported to be involved in cell wall formation. *C. neoformans* Nrg1 has been found to play a key role in maintaining cell wall integrity (Cramer et al., 2006). The reduced gene expression of chitin synthase, chitin deacetylase, and chitinase, which are thought to be necessary for normal cell wall synthesis, and the increased cell aggregation in the *nrg1* knockout strain support gene function in cell wall formation. In *S. cerevisiae*, Nrg1 is involved in regulating cell wall and

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cell surface formation-related gene expression (Vyas et al., 2005). These studies revealed the various functions of Nrg1 in environmental responses and cell wall formation. However, to our knowledge, these studies have only been conducted on yeast-like fungal species; the function of Nrg1 in filamentous fungi has not yet been investigated.

The fungal cell wall is an essential structure for fungi, and a comprehensive understanding of its structure and regulatory mechanisms is imperative for determining the lifestyle of fungi in response to the environment. The cell wall mainly comprises polysaccharides such as α -glucan, β -glucan, and chitin. Polysaccharide synthase and its upstream genes intricately regulate cell wall biosynthesis (Gow et al., 2017; Ruiz-Herrera and Ortiz-Castellanos, 2019). The basidiomycete *Pleurotus ostreatus* is a model white-rot fungus, and transformation protocols and genome editing systems have been established. Studies on the cell wall of white-rot fungi have been conducted using various gene disruptants of this species (Nakazawa et al., 2024). In *P. ostreatus*, the α -glucan synthase gene *ags1* and β -glucan synthase genes *fgs1* and *fgs2* have been predicted (Nakazawa et al., 2024). Nine chitin synthase genes have been predicted, four basidiomycete-specific (*chs1*–4). Phenotype analysis using knockout strains of the *chs* revealed the functions of each gene in the structural formation of aerial hyphae and cell walls (Schiphof et al., 2024). Functional analysis was performed using gene disruption strains for the transcription factors Mbp1 and Swi6, which regulate the expression of β -glucan and chitin synthesis enzymes (Kojima et al., 2024, 2025). These studies have gradually revealed the complex control of cell wall synthesis in white-rot fungi. However, a complete understanding of the cell wall structure and the mechanisms of its synthesis, regulation, and the genes involved in it has not been elucidated.

Protein kinase A (PKA) is a heterotetrameric protein that is activated depending on cyclic AMP (cAMP) and consists of two catalytic subunits and two regulatory subunits in many fungi. It is an upstream regulator of several transcription factors related to nutrient sensing and cell-wall synthesis (Toda et al., 1987a, 1987b; Caza and Kronstad, 2019). Our previous study revealed that when the gene *pkac2*, which encodes the catalytic subunit of the PKA complex, was disrupted in *P. ostreatus*, the β -glucan content in the cell wall and the transcription level of the β -glucan synthase gene were altered. The hydrophobicity of the hyphal surface and oxidative stress resistance are reduced (Otsuka et al., 2024). These results demonstrate that PKA has multiple functions in cell wall synthesis and environmental stress response in *P. ostreatus*, suggesting that PKA may be the key to understanding cell wall synthesis regulation and environmental stress response in white-rot fungi. However, although disruption of *pkac2* causes multiple phenotypes, it is unclear how the downstream pathway of the *pkac2* gene is related to each phenotype. In *C. neoformans*, Nrg1 is phosphorylated by PKA, which plays a central role in the stress response and cell wall remodeling process (Caza and Kronstad, 2019). This suggests that the *pkac2* gene may also be involved in regulating Nrg1 in *P. ostreatus*. Therefore, analyzing the function of *nrg1* may reveal some of the downstream pathways in which *pkac2* is expressed and provide valuable clues regarding the regulation of cell wall synthesis and environmental stress response in *P. ostreatus*.

This study elucidates the function of the Nrg1 transcription factor in the white-rot fungus *P. ostreatus*, with particular focus on its role in filamentous development and cell wall structure formation.

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 cultures. Cultures were maintained at 28 °C in continuous darkness. The *nrg1* gene-disrupted strains were generated through

gene targeting with homologous recombination using the 20b strain as a host (Salame et al., 2012). These strains are all monokaryotic. For *P. ostreatus* transformation, the 20b strain was cultured in YMG liquid medium with shaking at 28 °C, 120 rpm for four 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 protoplasts using the polyethylene glycol/calcium chloride (PEG/CaCl₂) method as previously described (Salame et al., 2012; Nakazawa et al., 2016).

2.2. Phylogenetic and domain structure analyses

Phylogenetic analysis was performed using Nrg1 and Nrg2 homologs from 40 fungal species. The Nrg1 amino acid sequences of *C. neoformans* var. *neoformans* JEC21 database from the National Center for Biotechnology Information (NCBI; Schoch et al., 2020) were used as references to conduct protein–protein basic local alignment search tool (BLASTP) homolog searches in the NCBI database. The top hit for each BLASTP search was used as a reference to conduct reciprocal BLASTP searches in the *C. neoformans* reference strain to confirm true homologs. The hits with *E*-values below 1.0×10^{-9} were used as predicted homologs and phylogenetically analyzed using the maximum likelihood method with the JTT matrix-based model (Jones et al., 1992) in MEGAX (Kumar et al., 2018). Further information on the databases used for each strain, corresponding strain names, and protein IDs are shown in Table S4.

For domain structural analysis, the predicted amino acid sequences of *C. neoformans* Nrg1 and their homolog candidates in *C. albicans*, *Ustilago maydis* and *P. ostreatus* were used. Predicted domain structures were analyzed using InterPro (Blum et al., 2025) and are shown in Fig. S1.

2.3. Construction of the gene disruption cassette

The *nrg1* disruption cassette was constructed by fusion PCR (Szewczyk et al., 2006) of three mutually primed DNA fragments: the upstream and downstream regions of the *nrg1* gene (Accession number: XP_36632547.1) in the NCBI genome database for *P. ostreatus* strain PC9, Genome assembly ASM1446616v1, and the hygromycin B-resistance marker gene (*hph*). KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan) was used to amplify and fuse 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 *nrg1*, and the pPHT1 plasmid (Cummings et al., 1999) to amplify the hygromycin resistance marker gene (*hph*). The fused and amplified fragments were purified using a Nucleospin PCR Clean-up and Gel Extraction Kit (Takara Bio, Shiga, Japan) and used as disruption cassettes. Schematic representation of the *nrg1* disruption cassette is shown in Fig. S3A, and the primers used for PCR are listed in Table S2.

2.4. Confirmation of the gene disruption

Crude genomic DNA was extracted according to a previous study (Izumitsu et al., 2012) and used as a template for genomic PCR to confirm gene disruption. PCR was performed using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). Integration of the *nrg1* disruption cassette was confirmed by gene length change PCR (Fig. S3C), and the presence or absence of the *nrg1* gene was verified by PCR amplification (Fig. S3D). Schematics of genomic PCR are shown in Fig. S3B, and the gel electrophoresis results are shown in Figs. S3C and S3D. Primers used are listed in Supplementary Table S2.

To confirm the absence of ectopic integration of the disruption cassette, Southern blot analysis has also been performed. About 6 μ g of genomic DNA was extracted from the 20b strain and Δ *nrg1* 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. S4.

2.5. Growth and morphological observations

For observations of mycelial morphology and growth rate, 20 mL of autoclaved YMG agar medium was dispensed into Petri dishes (ø90 mm). A mycelial plug was taken from the periphery of the colony with a cork borer (ø8 mm) and inoculated into the center 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 mycelial colony diameters were measured using ImageJ software (Schneider et al., 2012).

For biomass assay, 40 mL of YMG medium was dispensed into 100 mL flasks and autoclaved. Eight mycelial plugs, each made with a cork borer (ø8 mm), were inoculated into each flask and incubated at 28 °C for five days under shaking at 120 rpm. The samples were homogenized using a Polytron homogenizer (Kinematica AG, Malters, Switzerland), and the homogenized solution was inoculated into 40 mL of fresh autoclaved YMG liquid medium (the mycelium density of each homogenate was measured, and the amount of inoculated homogenate from the two disruptants was made to have the same biomass as 200 µL of the 20b). The flasks were incubated at 28 °C and 120 rpm for 3 days, and the dry weight of the mycelia was then measured.

For aerial hyphae observation, 10 mL of YMG agar medium or YMG liquid medium was dispensed into a ø16 mm test tube and autoclaved. The inoculated mycelium plugs were cut onto the agar medium using a cork borer (ø10 mm). After 10 days of static cultivation under dark conditions at 28 °C, photographs were captured to measure aerial mycelia.

2.6. Stress resistance assay

The autoclaved YMG agar medium was supplemented with 5 mM H₂O₂ to test the resistance to oxidative stressors. Additionally, 500 µg/mL Calcofluor White (CFW), 100 µg/mL micafungin (MF), and 0.02 % sodium dodecyl sulfate (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 for CFW and MF assays, respectively. YMG agar without stressors was used as the control. A mycelial plug made with a cork borer (ø8 mm) was inoculated into the center of each plate. After 10 days of cultivation in the dark at 28 °C, images were captured using a scanner. The colony diameters of the mycelia on the supplemented (D1') and control (D2') plates were measured using ImageJ software (Schneider et al., 2012). The values of D1 and D2 were calculated by subtracting 8 mm, which is the diameter of the cork borer, from D1' and D2'. The relative inhibitory rates were calculated as follows: Relative inhibitory rate (%) = (D2 – D1)/D2 × 100.

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

Autoclaved YMG agar (20 mL) was dispensed into Petri dishes (ø90 mm). After solidification, a single layer of autoclaved cellulose ester sheet was added to cover the entire plate. A mycelial plug made with a cork borer (ø8 mm) was inoculated into the center of each plate and incubated for 10 days in the dark at 28 °C. After incubation, the mycelia of the whole colony were peeled from the cellulose ester membrane, frozen, ground, and used for RNA extraction, followed by quantitative reverse-transcription PCR (qRT-PCR) as previously described (Kojima et al., 2024). The primer sets used for amplifying the cDNA fragments and their amplification efficiencies are listed in Table S3.

2.8. Cell wall and hyphal observations

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to observe the cell wall thickness and structure of the mycelia. The 20b and *nrg1*-disrupted strains were cultivated in Petri dishes (ø90 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). The cell wall thickness was measured from the images at 50 points.

For hyphal observation, 40 mL of YMG medium was dispensed into 100 mL flasks and autoclaved. Eight mycelial plugs, each made with a cork borer (ø8 mm), were inoculated into each flask and incubated at 28 °C for five days under shaking at 120 rpm. The samples were homogenized using a Polytron homogenizer (Kinematica AG, Malters, Switzerland), and 200 µL of the homogenized solution was inoculated into 40 mL of fresh autoclaved YMG liquid medium and incubated at 28 °C and 120 rpm for two days.

The mycelia in each flask were collected for hyphal branching assays, and images of the hyphae were captured using a confocal microscope (STELLARIS 5, Leica Microsystems, Wetzlar, Germany). The images were then analyzed using ImageJ software (Schneider et al., 2012). To evaluate hyphal branching frequency, the L_{hgu} (length of a hyphal growth unit) was calculated by dividing the hyphal length by the number of tips (Zhao et al., 2021). Thirty traceable hyphae of each strain were randomly selected, and their lengths were measured. In addition, the branching angles at 30 random branching sites were measured. Three independent experiments were performed for each assay.

For the fluorescence observation of the hyphal surface polysaccharides, the mycelia taken in the 1.5 mL tube was washed with 1 M Tris-HCl. To probe the hyphal surface chitin, 10 µL of 10 nmol/mL chitin-binding domain with an attached green fluorescent protein (ChBD-GFP) was used (Yano et al., 2011). Concanavalin A at a final 100 µg/mL concentration was used to probe the surface mannan. To probe the surface α-glucan and β-glucan, 8 µL of 10 nmol/mL α-(1,3)-glucan-binding domain with an attached red fluorescent protein (DCD-tetraRFP) and 40 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. The samples were stained on ice for two hours, and confocal microscopy was conducted as previously described (Kojima et al., 2024).

2.9. Glucan and chitin measurements

For glucan and chitin measurements, 20 mL of autoclaved YMG agar was dispensed into Petri dishes (ø90 mm), and a single layer of autoclaved cellulose ester sheet was placed to cover the entire plate. A mycelial plug made with a cork borer (ø8 mm) was then inoculated into the center of each plate and incubated for 10 days in the dark at 28 °C. After incubation, the mycelia were peeled from the cellulose ester sheet using a sterile spatula, washed with RO water, ground into powder using liquid nitrogen and a mortar and pestle, and freeze-dried.

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 hydrolyzed 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 used for the chitin measurements, employing freeze-dried mycelia hydrolyzed with 4 N HCl.

2.10. Determination of hydrophobicity

For the hydrophobicity assay, each strain was cultivated on 20 mL of YMG agar medium for 10 days, and a ø10 mm mycelial plug was cut with a cork borer. SDS/50 mM EDTA droplet diffusion was observed as

described previously (Han et al., 2023).

2.11. Statistical analysis

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

3. Results

3.1. Disruption of *nrg1* affects mycelial growth and aerial hyphae formation

BLASTP analyses were performed using the NCBI genome database of *P. ostreatus* PC9 strain to search for the *nrg1* gene in *P. ostreatus*. As a reference sequence, the amino acid sequence of the identified transcription factor Nrg1 (NCBI accession number XP_567961.1; NCBI annotated this as Nrg2; however, based on homology search and phylogenetic analysis, this annotation is likely an incorrect annotation.) in the basidiomycete yeast *C. neoformans* var. *neoformans* JEC21 was used, and reciprocal BLASTP searches were conducted on the top hits to avoid unidirectional relationships and confirm putative orthology. One Nrg1 ortholog (NCBI accession number XP_36632547.1) was predicted in *P. ostreatus*, and the domain structures were analyzed using InterPro (Blum et al., 2025), revealing the presence of two Zn-finger DNA-binding domains of the C2H2 family, similar to those of *C. neoformans*, *C. albicans*, and *U. maydis* Nrg1 homologs (Fig. S1). In addition, BLASTP predicted Nrg1 homologs were used to conduct phylogenetic analysis among 40 fungal species (Fig. S2). Most species were found to have one homolog, while some Saccharomycotina species had two, Nrg1 and Nrg2. With the exception of the *U. maydis* Nrg1, which is closely related to ascomycete Nrg1, all putative basidiomycete Nrg1 homologs were very closely related to the *C. neoformans* Nrg1, suggesting that these are putative Nrg1 orthologs.

To analyze the function of Nrg1 in *P. ostreatus*, the *nrg1*-disrupted strains were produced. The *nrg1* disruption cassette was designed to knock out the entire region of the predicted *nrg1* gene in *P. ostreatus*. Using this construct, *nrg1* was disrupted by gene targeting via homologous recombination using the 20b strain, a *ku80* disruptant that lacks non-homologous end-joining, as the host (Figs. S3A and B). As a result, two transformants were obtained, and the integration of the *nrg1* disruption cassette and the absence of the *nrg1* gene were confirmed in these strains through genomic PCR (Figs. S3C and D). The complete lack of the *nrg1* mRNA expression in these $\Delta nrg1$ strains was confirmed by qRT-PCR (Fig. S3E). In addition, Southern blot analysis confirmed that the disruption cassette was inserted at a single site and that there was no ectopic insertion (Fig. S4). The two independent $\Delta nrg1$ strains ($\Delta nrg1$ #1 and $\Delta nrg1$ #4), along with the parental strain 20b as the wild-type (WT), were used for subsequent experiments.

On YMG agar medium, the mycelial colony size of $\Delta nrg1$ strains was significantly reduced ($n = 3$) by approximately 50 % compared to that of the WT strain (Figs. 1A and D). The mycelial biomass from 3 days of liquid shaking culture was also reduced in the disruptants (Fig. 1E). In addition, they exhibit morphological abnormalities. The aerial hyphal formation ability of the disruptant was less than half of that of the WT (Figs. 1B and C). These results indicate that Nrg1 is essential for the normal growth, colony extension of vegetative mycelia, and aerial hyphae formation in *P. ostreatus* grown on solid medium.

The effect of *nrg1* disruption on hyphal morphology was examined using confocal microscopy on the mycelia in the liquid shaking culture. They were found to be abnormally branched in $\Delta nrg1$ strains (Fig. 2A). From the captured microscopic images, the calculated values indicating the distance between hyphal branches (L_{hgu} , the length of the leading hyphae per number of hyphal tips; 30 hyphae were measured for each strain) were significantly reduced in the disruptants (Fig. 2B). The average hyphal branching angles were also significantly larger (30 branching points of each strain were measured), approaching

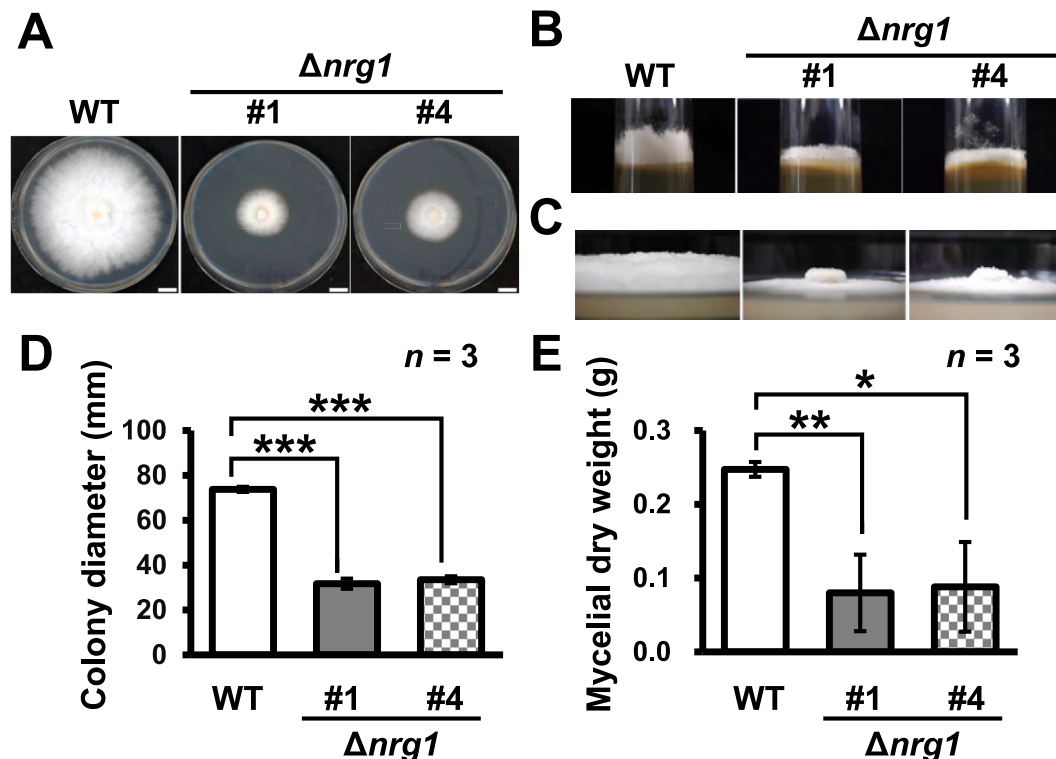


Fig. 1. Morphology and growth rate of the wild-type (WT) and $\Delta nrg1$ strains. (A, B, C) Effect of *nrg1* disruption on the morphology of colonies grown (A) and aerial hyphae formation (B, C) on yeast and malt extract with glucose (YMG) agar plates. Bar = 1 cm. (D) Colony diameter of each strain on YMG agar plates. (E) Mycelial dry weight of each strain in 3 days of liquid shaking culture. The bar indicates the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined by a two-tailed equal variance *t*-test (*** $P < 0.001$).

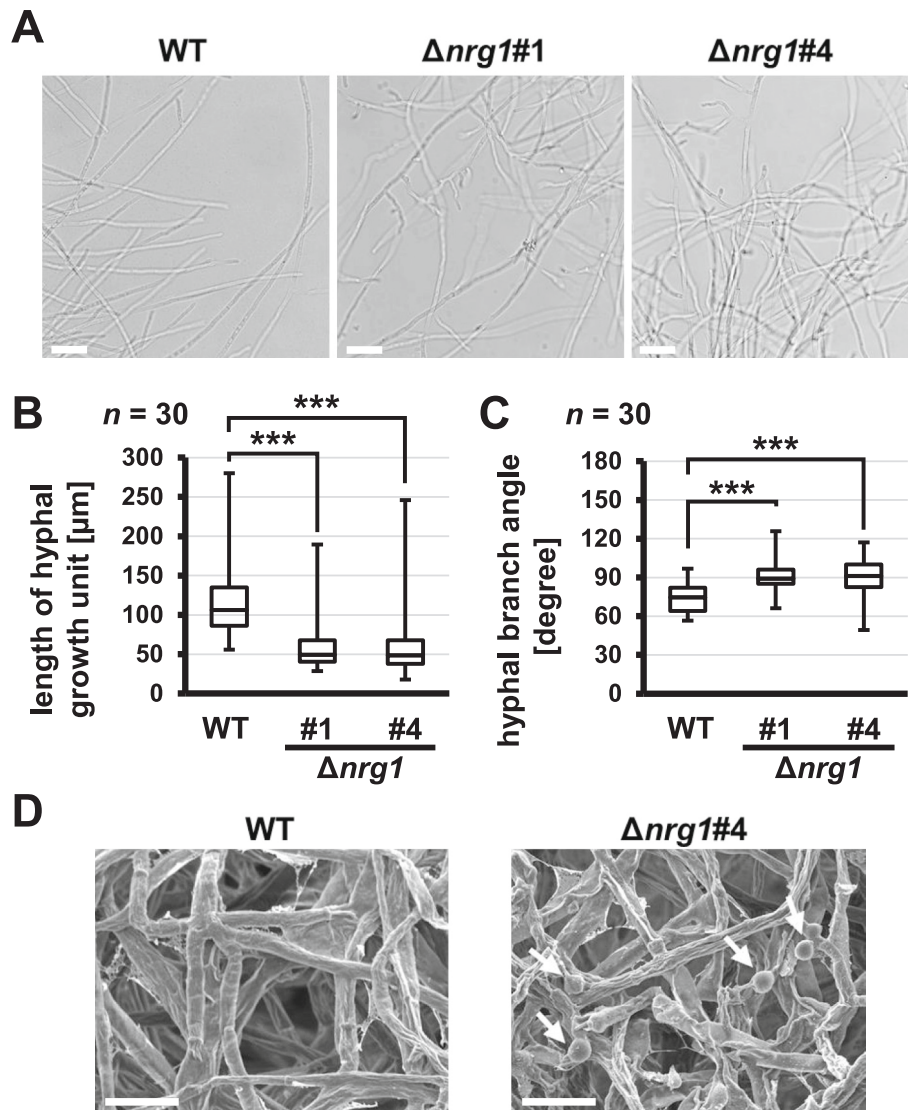


Fig. 2. Hyphal branch in the wild-type (WT) and $\Delta nrg1$ strains. (A) Confocal microscopic image of the mycelia of each strain. The periphery areas of the mycelium pellets during liquid culture were shown. Bar = 100 μm . (B, C) The hyphal length between branches (B) and the hyphal branching angle (C) were measured with (A). The bar indicates the standard deviations ($n = 30$). Statistical significance was determined by a two-tailed equal variance t -test ($***P < 0.001$). (D) Scanning electron microscopy of hyphae at $\times 2500$ magnification. Mycelium was collected from the periphery of the agar culture colony. Arrows indicate abnormal branching sites. Bar = 10 μm .

approximately 90° , compared to the WT, which branched at approximately 75° (Fig. 2C). On the hyphal surface of the disruptant observed using SEM, bulb-like structures swelling from the hyphae, thought to be due to abnormal branching, were often observed (Fig. 2D). These results demonstrated that the function of Nrg1 influences hyphal branching.

3.2. *nrg1* is required for the oxidative stress response

The $\Delta nrg1$ strains exhibited approximately 30–40 % growth inhibition on YMG with 5 mM H_2O_2 , in comparison to the WT, whose growth inhibition rate is approximately 20 % (Figs. 3A and B, $n = 3$). The expression levels of oxidative stress-related genes were examined by qRT-PCR; the relative expression levels of *cat2* (protein ID: 88827), *sod2* (protein ID: 114978), and *sod3* (protein ID: 111564) were significantly decreased ($n = 3$). Specifically, the expression of *cat2* was more than 90 % lower in the $\Delta nrg1$ strains compared to the WT (Fig. 3C). Although the expression levels of several other genes remained unchanged, it remains unclear exactly how Nrg1 contributes to oxidative stress response. However, these findings suggest that Nrg1 plays a critical role in

oxidative stress response, likely due to its direct or indirect influence on the expression of genes encoding antioxidant enzymes related to oxidative stress.

3.3. *nrg1* disruption does not affect the cell wall thickness but affects its surface structure

To investigate the effects of *nrg1* disruption on the cell wall structure, TEM observations of the cell wall were conducted. It revealed no notable differences in cell wall thickness between the WT and $\Delta nrg1$ strains (Figs. 4A and B, 50 points of each strain were measured), whereas it revealed that a black layer of putative protein aggregates on the cell wall surface is thinner and lighter in the $\Delta nrg1$ strain than in the WT (Fig. 4A). A previous study reported that in *P. ostreatus*, disruption of genes coding for the hydrophobin proteins Vmh2 (protein ID: 74127) and Vmh3 (protein ID: 88078), which are thought to be located on the cell wall surface, causes loss of the black layer as well as hyphal hydrophobicity (Han et al., 2023). In $\Delta nrg1$ strains, the expression level of a hydrophobin gene *vmh3* was reduced ($n = 3$) by more than 95 %

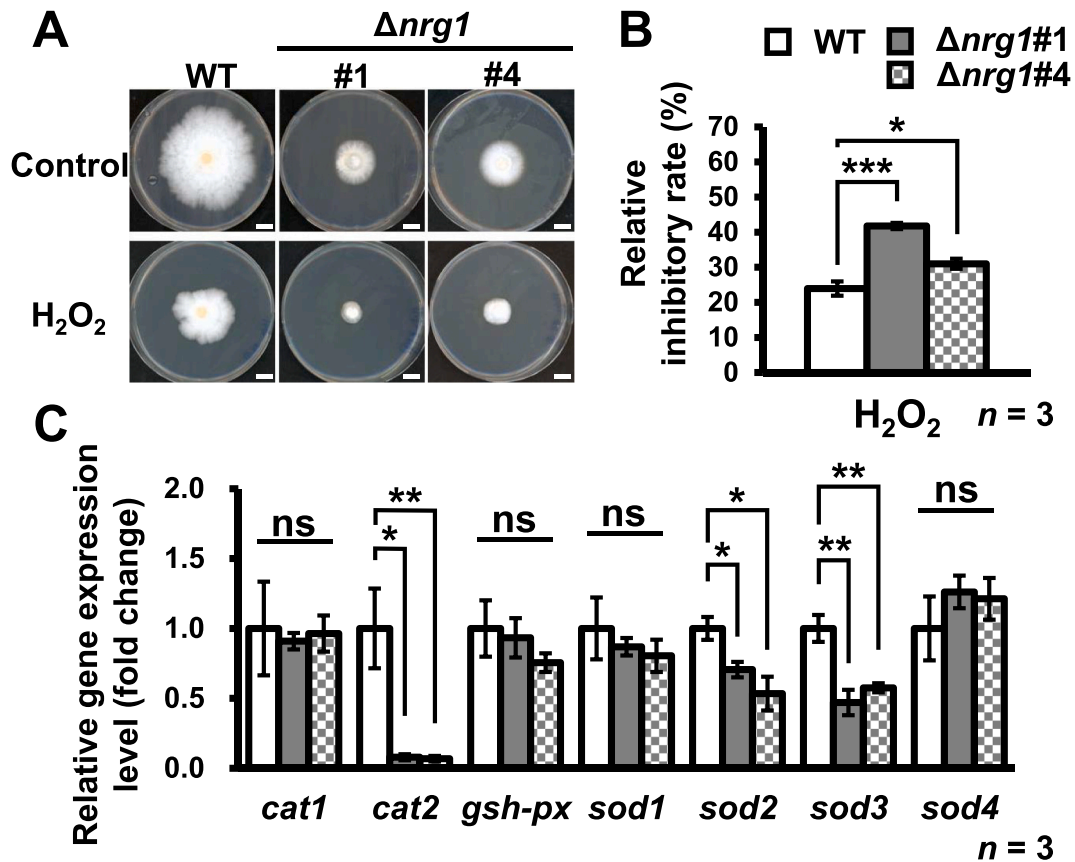


Fig. 3. The oxidative stress response of mycelia in the wild-type (WT) and $\Delta nrg1$ strains. (A) Phenotypes of all strains were grown on yeast and malt extract with glucose (YMG) agar medium supplemented with 5 mM H₂O₂ for 10 days. Bar = 1 cm. (B) Bar charts show strains' relative growth inhibition rate in (A). (C) The relative gene expression levels of various oxidative stress response-related genes of the wild-type and $\Delta nrg1$ strains. The bars indicate the standard deviations of three biological replicates ($n = 3$). A two-tailed equal variance t -test determined statistical significance. (ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

compared to the WT (Fig. 4C), while the other genes of main hydrophobins, *vmh2* and *hydph16*, remained unchanged. In a hydrophobicity test using 0.2 % SDS, the droplets on the mycelial plugs were absorbed more quickly and disappeared within approximately 20 min in the disruptant, indicating a significant decrease in hydrophobicity compared to that of the WT (Fig. 4D). These results suggest that *nrg1* can be involved in the regulation of hydrophobin gene expression and that disruption of *nrg1* reduces the amount of hydrophobins on the cell wall surface, resulting in a decrease in hydrophobicity of the hyphal surface.

3.4. *nrg1* is crucial for the normal regulation of cell wall synthesis

The sensitivities of the WT and $\Delta nrg1$ strains to cell wall synthesis inhibitors were examined, and demonstrated that $\Delta nrg1$ strains exhibited approximately a 1.4-fold and 2-fold increased sensitivity to CFW, a chitin synthesis inhibitor, and SDS, respectively, compared to the WT. However, no such increase in sensitivity to MF, a β -glucan synthesis inhibitor, was observed (Figs. 4A and B, $n = 3$). These results suggest that cell wall integrity is somehow disturbed in the $\Delta nrg1$ strains. To further investigate the effect of *nrg1* disruption on cell wall structure formation, the relative percentages of the major cell wall polysaccharides, including α -glucan, β -glucan, and chitin, were measured. The approximate α -glucan level was 15 % in the WT and 10 % in the $\Delta nrg1$ strains. With an approximate β -glucan level of 28 % in the WT and 23 % in the disruptants, both glucans were significantly reduced in the $\Delta nrg1$ strains, although the relative percentages of chitin remained unchanged (Fig. 6A, $n = 3$). The relative expression levels of cell wall synthase genes were examined by qRT-PCR. Approximately 30–40 % lower levels of expression were observed in the α -glucan synthase gene

ags1 (protein ID: 82171) and the β -glucan synthase gene *fks1* (protein ID: 114314) between the WT and $\Delta nrg1$ strains (Fig. 6B, $n = 3$). The chitin synthase genes *chs1* (protein ID: 115957) and *chs7* (protein ID: 110811) were also 30–50 % less in the disruptants than in the WT, although the majority of chitin synthase genes were unchanged in expression (Fig. 6C, $n = 3$). These findings suggest that *nrg1* may be involved in the regulation of cell-wall polysaccharide synthesis in *P. ostreatus*.

To investigate the distribution of polysaccharides on the hyphal surface visually, fluorescence microscopy with polysaccharide-specific probes was used. The ChBD-GFP (Yano et al., 2011) was used for the chitin labeling, the concanavalin A (Alexa Fluor Plus 405, Thermo Fisher Scientific, USA) for the mannan labeling, and the DCD-tetraRFP and the BGBD-GFP (Otsuka et al., 2022a, 2022b) for the α -glucan and β -glucan labeling, respectively. In ChBD-GFP staining, chitin was usually localized to the hyphal tips. In the $\Delta nrg1$ strain, chitin is less localized at the tip; some hyphae have chitin scattered around the tip, and some hyphae have no chitin staining, including at the tip (Fig. 7A). With concanavalin A, blue fluorescence was distributed relatively evenly throughout the hyphae of WT. In contrast, some hyphae had strong fluorescence in the $\Delta nrg1$ strains (Fig. 7B), suggesting that these hyphae contain an abnormally large amount of mannan due to the *nrg1* disruption. In double staining of α -glucan and β -glucan, stronger red fluorescence of α -glucan was observed throughout the hyphae of the disruptants than in the WT. The green fluorescence labeling of β -glucan was particularly weak in the abnormally branched hyphae of the $\Delta nrg1$ strains (Fig. 7C). These results demonstrate that *nrg1* disruption influenced the cell wall structure formation, and this may occur via the synthesis of cell wall polysaccharides.

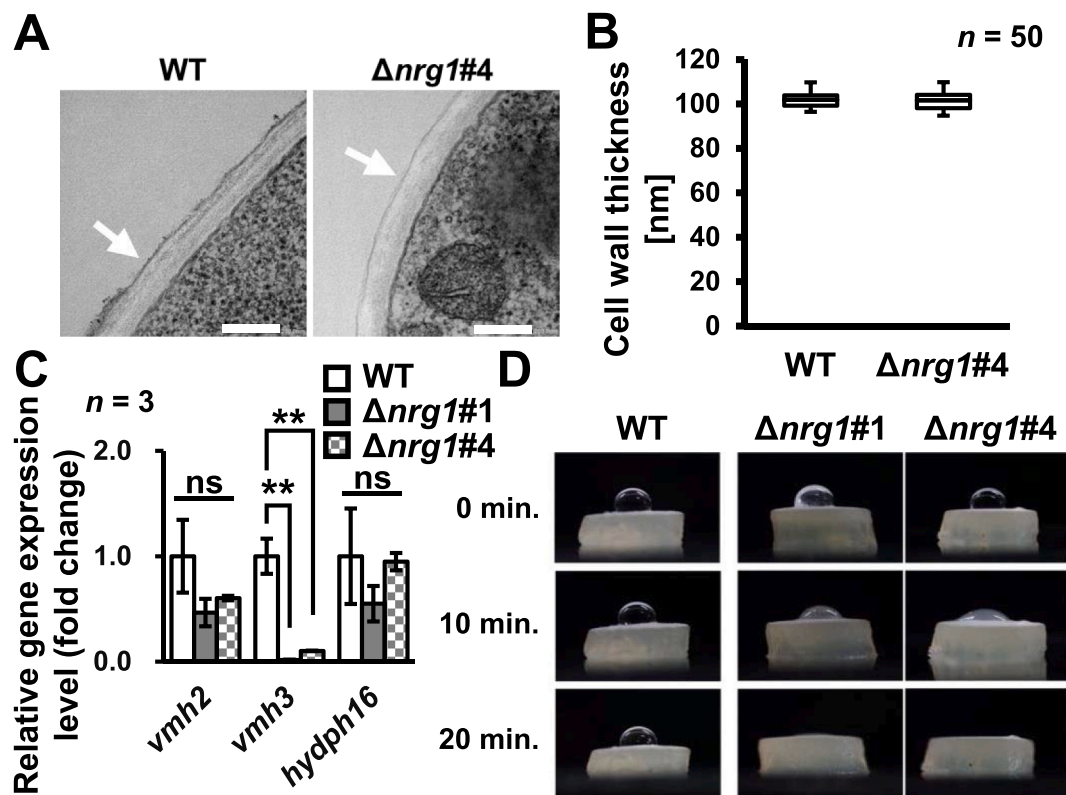


Fig. 4. Cell wall and its surface structure in the wild-type (WT) and $\Delta nrg1$ strains. (A) Cell wall transmission electron microscopy (TEM) at $\times 30,000$ magnification. The arrow indicates the cell wall surface. Bar = 200 nm. (B) Cell wall thickness measured by TEM images. The bars indicate the standard deviations ($n = 50$) (C) Relative gene expression levels of the major hydrophobin genes. The bars indicate the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined by a two-tailed equal variance t -test (ns: $P > 0.05$, $**P < 0.01$). (D) The hydrophobicity of each strain's hyphae affects the disappearance of 10 μ L SDS droplets on the mycelial mat.

4. Discussion

In this study, to understand the function of the Nrg1 transcription factor in the filamentous basidiomycete fungus *P. ostreatus*, *nrg1* disruptants were isolated. Subsequently, multiple roles of Nrg1 in environmental stress response and cell wall formation were investigated using the $\Delta nrg1$ strains. Firstly, the $\Delta nrg1$ strains exhibited a significant decrease in growth rate and shorter aerial hyphae on the YMG agar plates compared to the WT (Figs. 1A, B, C, D and E). These results indicate that the Nrg1 transcription factor in *P. ostreatus* plays a crucial role in the colony extension and growth of vegetative hyphae and aerial hyphae formation. In contrast, the effects of *nrg1* disruption on the growth of basidiomycete yeasts were weaker, and the *nrg1* disruptant in *C. neoformans* grew comparably to the WT strain on YPD, minimal medium, and glucose medium (Cramer et al., 2006). The *nrg1* mutant of *U. maydis* exhibited significantly reduced cell growth compared to the WT, but the reduction was approximately 20–25 %, not as great as more than 50 % in *P. ostreatus* (Sánchez-Arreguin et al., 2021). In addition to such a growth defect, there was an abnormality in hyphal branching in the $\Delta nrg1$ strains of *P. ostreatus* (Fig. 2). Given that research on the function of the *nrg1* gene has been predominantly conducted in yeast-like fungi, such as *S. cerevisiae*, *C. albicans*, and *C. neoformans*, there have been no analogous reports regarding the role of hyphal branching. In *C. albicans*, Nrg1 represses genes specifically expressed during pseudohyphal development. Disruption of *nrg1* in *C. albicans* led to morphological growth switching from a yeast-like to a filamentous state, regardless of the induction conditions. (Braun et al., 2001). It is possible that in *P. ostreatus*, the function of Nrg1 is also involved in essential functions for filamentous growth and hyphal branching, whether directly or indirectly. Studies using other filamentous fungi have also provided suggestions regarding the cause of abnormal hyphal

branching. A study using the white-rot fungus *Ganoderma lucidum* reported that copper stress shortened the hyphal branching distance, which was dependent on the overproduction of ROS (Gao et al., 2018). Another study using the filamentous fungus *Tricoderma reesei* suggested a relationship between hyphal branching and abnormal control of cell wall and surface formation, with abnormally regulated expression of genes encoding multiple cell wall remodeling enzymes and hydrophobins in the *gul1* gene disruptants, which showed an increased hyphal branching frequency (Zhao et al., 2021). In the *nrg1* knockout strain of *P. ostreatus*, (the details will be discussed later), various phenotypes related to the above have been observed. Increased sensitivity to oxidative stress and reduced expression levels of antioxidant enzyme genes suggest that the intracellular ROS concentration may be high in the disruptants (Fig. 2). In addition, the expression level of the hydrophobin gene *vmh3* was reduced (Fig. 3), and the regulation of normal cell wall polysaccharide synthesis was disrupted (Figs. 5, 6, and 7). These results suggest that such complex factors may interact to affect the abnormal hyphal branching in the $\Delta nrg1$ strains.

nrg1 disruption also affected cell wall surface proteins. TEM revealed no notable changes in cell wall thickness (Figs. 3A and B). However, the TEM images showed differences in the outermost layer of the cell wall. A black layer of aggregates was observed on the surface of the WT cell wall, which was thinner and lighter in the presence of the disruptant (Fig. 3A). This layer is thought to be formed by the aggregation of cell wall surface proteins. In a previous study on *P. ostreatus*, the layer was lost in strains with disrupted *vmn2* and *vmn3* genes, which encode hydrophobin proteins on the cell wall surface (Han et al., 2023). In *P. ostreatus*, these two genes are expressed at very high levels and are essential for maintaining cell-wall surface hydrophobicity in vegetative mycelia (Han et al., 2023). Therefore, the reduction in the black layer on the cell wall surface in the TEM images of the $\Delta nrg1$ strains may be

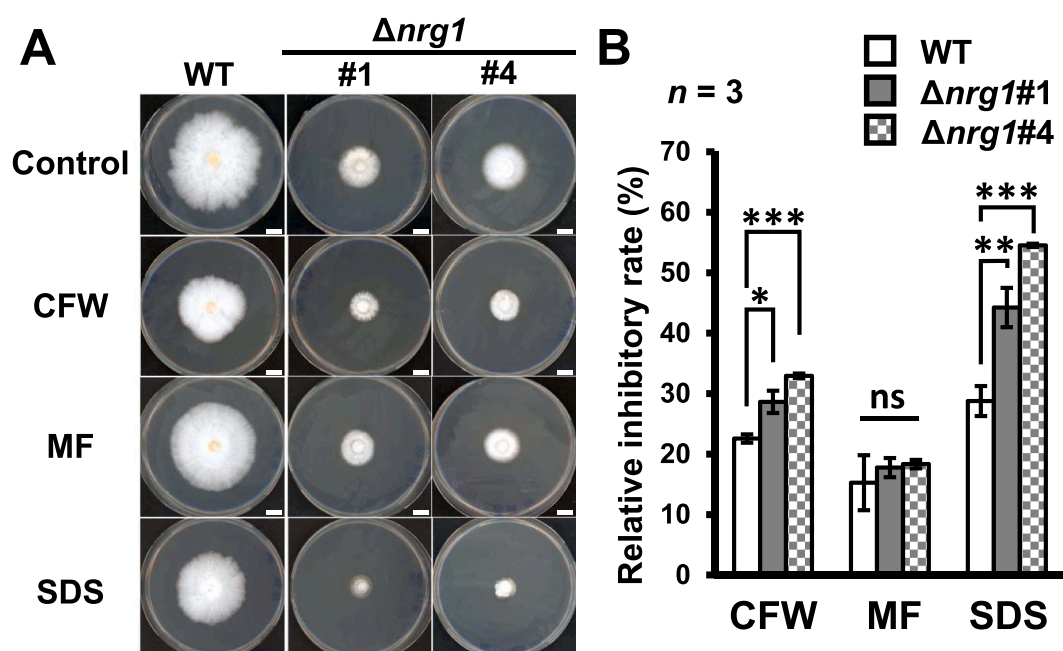


Fig. 5. The cell wall stress response of mycelia in the wild-type (WT) and $\Delta nrg1$ strains. (A) Phenotypes of all strains grown on yeast and malt extract with glucose (YMG) agar medium supplemented with 500 $\mu\text{g}/\text{mL}$ Calcofluor White (CFW), 100 $\mu\text{g}/\text{mL}$ micafungin (MF), 0.02 % sodium dodecyl sulfate (SDS), and 5 mM H_2O_2 for 10 days. Bar = 1 cm. (B) Bar charts show strains' relative growth inhibition rate in (A). The bars indicate the standard deviations of three biological replicates ($n = 3$). A two-tailed equal variance t -test determined statistical significance. (ns: $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

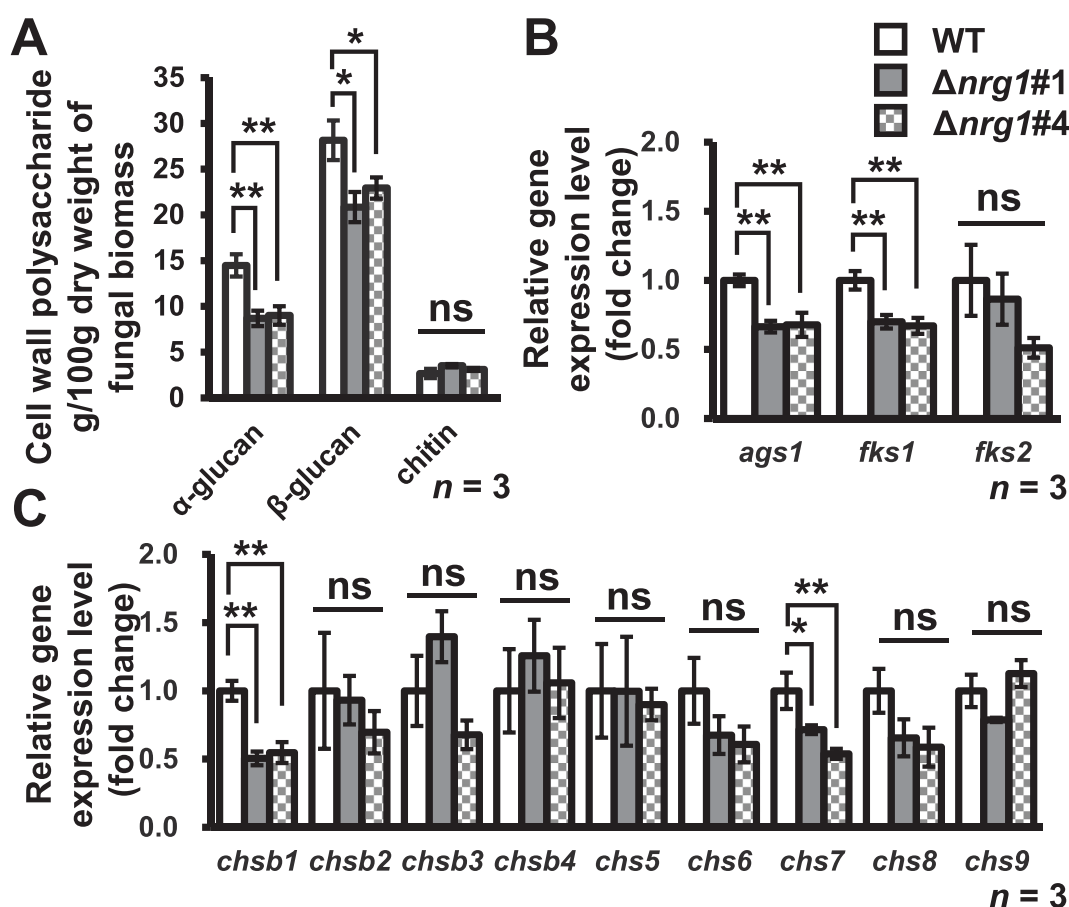


Fig. 6. Cell wall components and relative gene expression levels of various cell wall synthases of the wild-type (WT) and $\Delta nrg1$ strains. (A) The weight content of each polysaccharide in dried mycelia. (B, C) Relative gene expression levels of the α -glucan and β -glucan synthases (B), and chitin synthases (C) in the wild-type and $\Delta nrg1$ strains. The bars indicate the standard deviations of three biological replicates ($n = 3$). A two-tailed equal variance t -test determined statistical significance. (ns: $P > 0.05$, $*P < 0.05$, $**P < 0.01$).

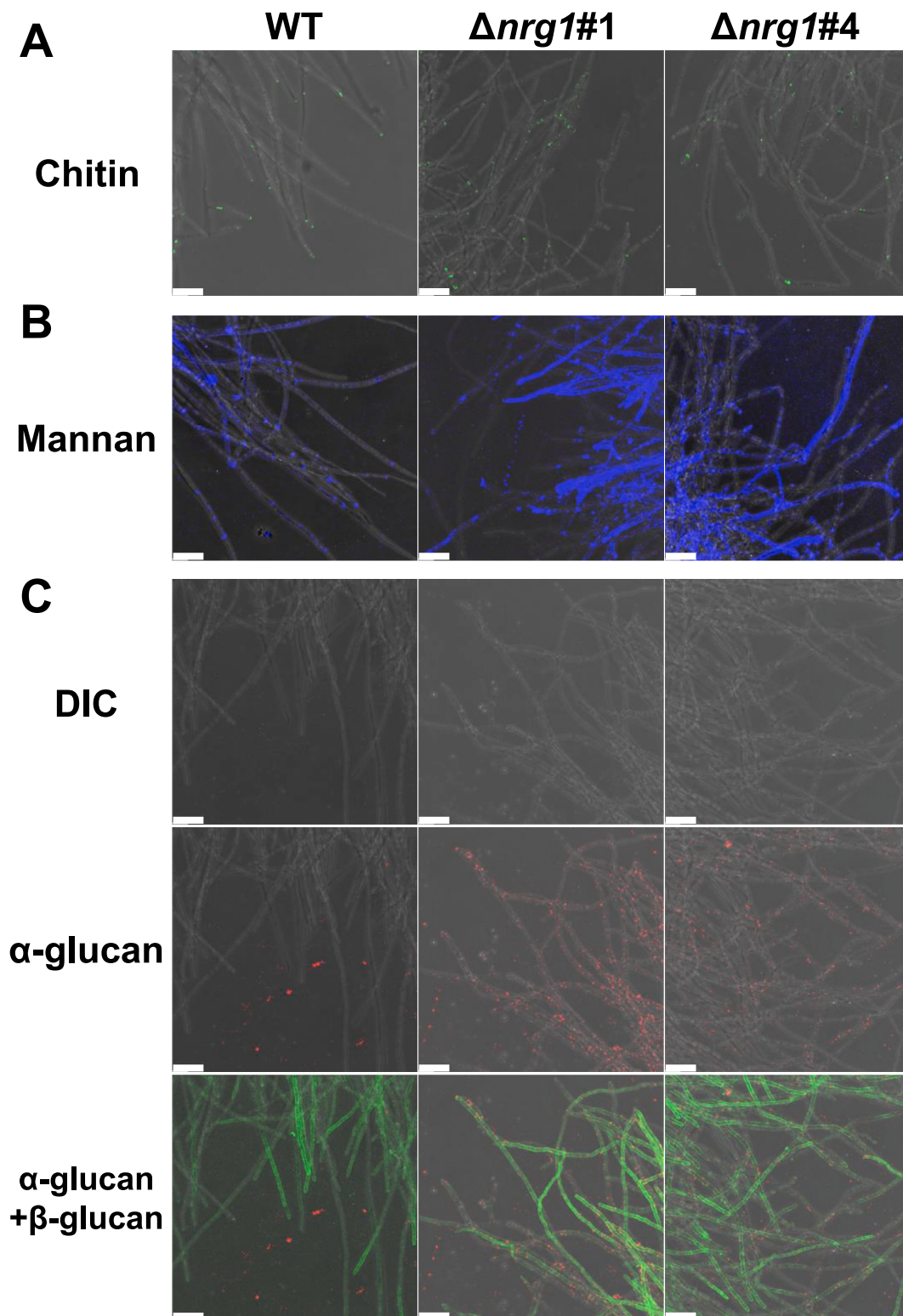


Fig. 7. Cell wall polysaccharide distribution of the wild type and $\Delta nrg1$ strains. Mycelia fluorescence microscopic images were obtained using each cell wall polysaccharide-specific probe with chitin labeled green (A), mannan in blue (B), β -glucans in green, and α -glucans in red (C). Bar = 20 μ m.

explained by the results below. The expression level of *vmh3* in $\Delta nrg1$ strains was reduced by more than 95 % compared to the WT (Fig. 3C). The hydrophobicity test revealed a significant decrease in hydrophobicity due to *nrg1* disruption (Fig. 3D). The SDS droplets on the mycelium were absorbed within 20 min in the disruptant, which is consistent with the results of the $\Delta vmh3$ strains (Han et al., 2023).

It has been suggested that Nrg1 may play an essential role in the formation of cell-wall structures. The decreased resistance to the cell wall stressors CFW and SDS indicated disrupted cell wall integrity due to *nrg1* disruption (Fig. 5). Similarly, the *nrg1* disruptant of *C. neoformans* was more sensitive to SDS than the WT (Cramer et al., 2006). In this study, a series of experiments on polysaccharide content, expression

levels of polysaccharide synthesis enzyme genes, and fluorescence staining demonstrated the effects of *nrg1* disruption on cell wall polysaccharide synthesis in *P. ostreatus*. The *nrg1* disruption significantly reduced α -glucan and β -glucan contents by approximately 30 % and 20 %, respectively (Fig. 6A). Correspondingly, the expression levels of the α -glucan synthase gene *ags1* and β -glucan synthase gene *fks1* were lowered (Fig. 6B), which suggests the possible reason for the decrease in these polysaccharide contents. Fluorescent microscopy using a double-staining method of α -glucan and β -glucan showed the distribution of these glucans on the hyphae visually. The red fluorescence labeling of α -glucan was stronger in the disruptant, whereas the green fluorescence labeling of β -glucan, which is typically abundant on the whole cell wall surface, was weak (Fig. 7C). A previous study using alkaline fractionation methods reported that the outermost layer of the vegetative mycelium of *Schizophyllum commune* was a β -glucan layer containing β -1,3 and β -1,6 linkages (Bartnicki-García, 1999), suggesting that in agaricomycetes, the outermost layer of the cell wall contains β -glucan. In *P. ostreatus*, fluorescent staining experiments have suggested that β -glucan is present in the outer layer of the cell wall, covering the hyphae (Nakazawa et al., 2024). Solid NMR of the *S. commune* cell wall has also revealed that it contains β -glucan in the outer layer and α -1,3-glucan in the inner layer (Ehren et al., 2020). These results suggest that in the *nrg1* disruptants, the amount of β -glucan present on the surface of the cell wall was reduced, resulting in increased exposure of α -glucan. It revealed that the β -glucan fluorescent was weak especially at the abnormally branched hyphae in the $\Delta nrg1$ strain (Fig. 7C). This suggests that *nrg1* may play a crucial role in the normal regulation of glucan synthesis and, at the same time, is further evidence that *nrg1* disruption causes abnormal cell wall formation during hyphal branching.

Interestingly, even with this decrease in glucan content, the cell wall thickness remained unchanged. One possible explanation is that the density of the cell wall, which consists mainly of glucans and chitin, is reduced due to *nrg1* disruption, and that the weight percentage of the parts other than the cell wall increases. Another possible explanation is that the amount of mannan in the cell wall increased, as shown in the concanavalin A-stained image (Fig. 7B). In the $\Delta nrg1$ strains, there were some hyphae with abnormally strong blue fluorescence, thought to be due to mannan. However, it is unclear whether mannan compensates for the lack of α -glucan and β -glucan. In addition, microscopy revealed that the abnormally branched hyphal sites were particularly prone to crushing (data not shown). Because the cell walls observed with TEM withstand dehydration during specimen preparation, it was not possible to measure the cell wall thickness of the abnormally branched parts of the disruptants, which are presumed to be particularly fragile. This may explain why there was no significant difference in cell wall thickness compared to the WT.

In contrast, $\Delta nrg1$ strains exhibited no significant alterations in chitin content, although the chitin synthase genes *chs1* and *chs7* expression levels were lowered (Figs. 6A and C). Changes in chitin synthase gene expression levels do not necessarily lead to changes in chitin content. In *P. ostreatus* *chs2* and *chs3* disruptants, there was no significant change in the chitin content (Schiphof et al., 2024). However, in the *nrg1* disruptants, there was no complementary increase in the expression of chitin synthase genes other than that of the downregulated chitin synthase gene. It is also possible that, because of the decreased rate of chitin decomposition at the same time, the downregulation of chitin synthesis may not have changed the overall chitin content. Focusing on the effects of these changes in chitin synthesis gene expression, the decrease in *chs1* expression may have a particularly large impact on the great decline in the growth rate of the $\Delta nrg1$ strains. A previous study investigated the function of chitin synthases in *P. ostreatus* using the gene disruption method; a *chs1* disruptant could not be obtained, even after repeated transformations (Schiphof et al., 2024). These results suggest that *chs1* is an essential gene for the growth of *P. ostreatus* mycelia and that *nrg1* is involved in its regulation. In *C. neoformans*, there are also chitin synthase genes whose expression

levels are lowered by 3–4 times in the *nrg1* disruptant (Cramer et al., 2006). In addition, *chs7* downregulation can be related to abnormal hyphal branching. It has been reported that chitin synthases Chs5, Chs6, Chs7, and Mcs1 in *U. maydis* are localized and active in the growing regions of yeast-like cells and hyphae and are involved in hyphal tip growth (Weber et al., 2006). It is also possible that, in *P. ostreatus*, the loss of normal cell wall synthesis on the hyphal tip, via the function of *chs7*, causes the abnormal frequency of hyphal branching. Fluorescent microscopy of cell wall chitins revealed the abnormalities in localization of the fluorescent signals of chitin in *P. ostreatus* $\Delta nrg1$ strains (Fig. 7A), which may be due to changes in the localization of chitin itself or the aforementioned changes in β -glucan synthesis. In a previous study (Kojima et al., 2024) using *P. ostreatus*, abnormal chitin localization, similar to that seen in some mycelia of the $\Delta nrg1$ strain, was observed in the disrupted strains of the *mbp1* gene, which is known as a transcription factor mainly controlling β -glucan synthesis. In those $\Delta mbp1$ strains, the relative expression levels of the β -glucan synthesis enzyme genes *fks1* and *fks2* and the β -glucan content were reduced compared to the WT. Given these phenotypes, which suggest β -glucan synthesis is affected by the gene disruption, were also exhibited in $\Delta nrg1$ strains, inhibited β -glucan synthesis may affect the localization or exposure of the chitin.

Finally, in addition to the various other phenotypes described thus far, the disruption of *nrg1* affected the oxidative stress response. Cultures on agar plates supplemented with H_2O_2 showed increased oxidative stress sensitivity after disruption of *P. ostreatus nrg1* (Figs. 3A and B). Oxidative stress is induced by the overproduction of ROS, such as H_2O_2 and hydroxyl radicals. Fungi possess several antioxidant enzymes that help them cope with stress. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to H_2O_2 , which is then decomposed by catalase (CAT) and glutathione peroxidase (GSH-Px). GSH-Px catalyzes the conversion of lipid hydroperoxides into nontoxic alcohols. These catalytic reactions help maintain redox balance within fungal cells (Ayar-Kayali and Tarhan, 2004). The $\Delta nrg1$ strains exhibited significantly reduced expression levels of three genes encoding antioxidant enzymes, *cat2*, *sod2*, and *sod3*, compared to the WT. Specifically, the expression of *cat2* was more than 90 % lower in the $\Delta nrg1$ strains (Fig. 3C), suggesting that Nrg1 plays an essential role in the normal oxidative stress response by directly or indirectly affecting the expression of these antioxidant enzyme genes. In addition, the defective cell wall integrity discussed above, which may reduce the cell wall permeability to H_2O_2 , may also affect the oxidative stress sensitivity of the *nrg1* disruptant. In *C. neoformans*, resistance to oxidative stress caused by H_2O_2 was comparable to that of the WT. In contrast, a gene microarray of the disruptant revealed that the putative target genes of the Nrg1 pathway included genes involved in the oxidative stress response. Similar to the results in *P. ostreatus*, *cat2* was downregulated approximately four-fold in the disruptant (Cramer et al., 2006). Increased sensitivity to H_2O_2 was reported in the *C. albicans* $\Delta nrg1$ strain (Murad et al., 2001). Conversely, increased H_2O_2 resistance has been observed in *S. cerevisiae* and *U. maydis* disruptants (Vyas et al., 2005; Sánchez-Arreguin et al., 2021). Although the role of Nrg1 in controlling the expression of genes involved in the oxidative stress response seems to be conserved among these species, it is speculated that the detailed pathway through which it functions is different.

In this study, we found several genes whose transcription levels were reduced in the $\Delta nrg1$ strain compared to the WT. As Nrg1 has been characterized as a transcriptional repressor in *S. cerevisiae* and *C. albicans* (Park et al., 1999; Braun et al., 2001), it is possible that the transcription levels of the genes are downregulated because the repression control of other genes was disrupted by the *nrg1* knockout. Therefore, the involvement of Nrg1 in the changes in gene expression observed in this study is not necessarily direct.

The phenotypes of the *P. ostreatus nrg1* knockout strains discussed so far include some similarity to those of the *pkac2* knockout strains. Given that PKA1 regulates the activation of Nrg1 by phosphorylation in *C. neoformans* (Caza and Kronstad, 2019), it is possible that Nrg1 activity

in *P. ostreatus* is also dependent on PKA. The *P. ostreatus* $\Delta pkac2$ strains were sensitive to oxidative stress and showed a large decrease in the expression levels of the hydrophobin genes *vmh2*, *vmh3*, and *hdyph16* (Otsuka et al., 2024). The decreased resistance to oxidative stress and the decreased expression of *vmh3* observed in the *nrg1* disruptants are similar to these. On the other hand, the *pkac2* knockout strains showed a decreased β -glucan content and increased expression levels of β -glucan synthase genes compared to the WT (Otsuka et al., 2024). The *nrg1* disruption also caused a decrease in β -glucan content, but since the expression of the β -glucan synthase gene also decreased in $\Delta nrg1$ strains, the function of *pkac2* and *nrg1* in controlling β -glucan synthesis is indicated not to be related. These suggest that also in *P. ostreatus*, PKAc2 is possibly involved in some part of its functions through phosphorylation of Nrg1, such as oxidative stress response and maintaining hydrophobicity. However, further verification is required to determine whether the phosphorylation and activation of Nrg1 by PKA occurs in *P. ostreatus*.

5. Conclusion

In *Pleurotus ostreatus*, *nrg1* plays a crucial role in colony extension and growth on agar medium and aerial hypha formation, and *nrg1* disruption results in the abnormal branching of hyphae. It is also involved in the normal response to oxidative stress induced by H_2O_2 by regulating the expression of oxidative stress-related genes. In addition, *nrg1* may be involved in regulating hydrophobin gene expression and maintaining hydrophobicity on the hyphal surface. Disruption of the *nrg1* reduced the contents of α -glucan and β -glucan in the cell wall without changing the thickness of the cell wall. It also reduced the expression levels of cell wall synthesis enzyme genes *ags1*, *fsk1*, *chs1*, and *chs7*. Further, it altered the localization of β -glucan and chitin, suggesting *nrg1* plays an essential and multifaceted role in cell wall formation in this species. This study provides the first evidence of the diverse functions of Nrg1 in filamentous basidiomycetes and suggests its importance in the environmental adaptation of white-rot fungi.

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:** Methodology, 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.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fgb.2025.104026>.

Data availability

Data will be made available on request.

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