



Functional analysis of the class III chitin synthase genes in the filamentous basidiomycete *Pleurotus ostreatus*

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ABSTRACT

Class III chitin synthases (CHSs) are specific to filamentous fungi and are associated with biosynthesis of chitin, a key structural component of fungal cell walls. While extensively studied in ascomycetes, their roles in basidiomycetes remain poorly understood. In this study, we present the first functional analysis of class III *chs* in the white-rot basidiomycete *Pleurotus ostreatus*. Through targeted single gene disruption of the class III *chs*, *chs7* and *chs9*, as well as double disruption of *chs7chs9*, we demonstrate their distinct and sometimes overlapping roles in regulating hyphal growth, cell wall thickness, and stress resilience. Despite reduced cell wall thickness and decreased growth rates in disruption strains, compensatory upregulation of other chitin and glucan synthase genes maintains polysaccharide composition, revealing a robust regulatory network. Notably *chs9* disruption strains had more severe impacts than those of *chs7*, suggesting that *chs9* is the main class III *chs* and *chs7* provides more of a supporting role. Double disruption strains also revealed straighter hyphae suggesting redundancy and highly specialized functions of clade III *chs* in hyphal straightness in *P. ostreatus*. Our findings highlight the evolutionary divergence and seemingly unique functions of class III CHSs in comparison to the previously analyzed basidiomycete-specific classes, providing foundational insights into filamentous basidiomycete cell wall dynamics.

1. Introduction

Chitin synthases (CHSs) are essential enzymes responsible for the production of chitin, a key structural component of fungal cell walls that confers rigidity, strength, and structure (Gow et al., 2017; Rogg et al., 2012). In filamentous fungi the inner fungal cell wall consists generally of chitin, β -1,3-glucan and α -1,3-glucan, with chitin embedded deep in the cell wall (Gow et al., 2023). However, the cell wall structure and distribution of components can vary significantly between phyla, and compared to ascomycetes, little is understood about basidiomycete cell wall biosynthesis (Nakazawa et al., 2024). Chitin can vary in folding, organization, degree of acetylation, orientation, and length, but generally anchors to the cell membrane and binds to β -glucan to form a chitin-glucan complex providing the scaffolding of the cell wall (Brown et al., 2020; Ehren et al., 2020; Gonçalves et al., 2016; Gow et al., 2023; Rogg et al., 2012). The amount of chitin can also vary greatly between species and phyla ranging from 1% of total dry weight to over 15% (Gow et al., 2017; Gow et al., 2023; Rogg et al., 2012). The size and other properties of chitin are often determined by post-production processes such as

through cleavage by chitinases or deacetylation by chitin deacetylases, into various degrees of deacetylated chitin or chitosan (Abo Elsoud et al., 2019; Alimi et al., 2023; Baker Lorina et al., 2007; Bouregghda et al., 2021; Brown et al., 2020; Gow et al., 2017; Gow et al., 2023; Rogg et al., 2012).

Fungi generally have multiple *chs* genes with ascomycetous yeasts having less and filamentous fungi having more (Nakazawa et al., 2024). CHSs are membrane bound proteins that catalyze the polymerization of cytoplasmic UDP-N-acetylglucosamine (UDP-GlcNAc) into the polysaccharide chitin, which forms the cell wall chitin layer (Chen et al., 2023; Gonçalves et al., 2016; Soria et al., 2021). Although there is no complete consensus regarding the classification of *chs* genes, there are typically two divisions, with classes I-III in division I and classes IV-VII in division II (Gonçalves et al., 2016; Li et al., 2016; Liu et al., 2017; Pacheco-Arjona et al., 2014; Rogg et al., 2012; Roncero, 2002; Ruiz-Herrera et al., 2010; Schiphof et al., 2024), although some studies suggest an additional third division (Li et al., 2016; Liu et al., 2017). Our previous study also identified three clades of basidiomycete specific *chs* genes with clade BI and BII in division I, and clade BIII in division II

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(Schiphof et al., 2024). Class III *chs* are distinctive for their presence exclusively in filamentous fungi. Gene duplication events in some filamentous species have resulted in two class III *chs* which are thought to have undergone sub-functionalization to acquire specialized roles in fungal growth, morphogenesis, and pathogenicity (Gonçalves et al., 2016; Li et al., 2016; Liu et al., 2017). Functional analysis of class III *chs* gene disruption strains have identified vastly diverse roles with some species having severe growth defects in the absence of class III *chs*, and other species having minor or no phenotype changes (Borgia et al., 1996; Cheng et al., 2015; Fukuda et al., 2009; Mellado et al., 1996a; Mellado et al., 1996b; Soulié et al., 2006; Wang et al., 2000; Weber et al., 2006; Werner et al., 2007; Yanai et al., 1994; Yarden et al., 1991). These studies are almost exclusively in ascomycete or yeast-like basidiomycetes, with very few studies having been conducted about the function of *chs* in filamentous basidiomycetes.

Our previous studies identified nine *chs* genes in the white-rot basidiomycete *Pleurotus ostreatus*, and in addition to the seven known clades of *chs* genes, highlighted the presence of three basidiomycete-specific clades containing four basidiomycete-specific *chs* (*chsb* genes) (Schiphof et al., 2024). Functional analysis of all the *chsb* disruption strains highlighted clade-specific functions with the sole gene in clade BI having severe growth defects and altered cell wall component ratios, double disruption of both genes in clade BII additionally exhibiting abnormal septation and the single gene in clade BIII having minor impacts on aerial hyphae production only (Schiphof et al., 2024; Schiphof et al., 2026). To effectively understand the conservation, diversity, and mechanisms of chitin synthases in *P. ostreatus*, it is essential to study both basidiomycete-specific and shared chitin synthase genes to capture their full functional range and evolutionary significance. Additionally most *chs* research has been conducted in ascomycete species, and very little is understood about filamentous or white-rot basidiomycete cell wall biosynthesis, despite known differences in cell wall architecture across phyla (Nakazawa et al., 2024), and their significance in forest ecosystems, agriculture, and sustainable industry (Baeva et al., 2019; Bouregghda et al., 2021; Chen et al., 2014; Haneef et al., 2017; Kojima et al., 2026; Nakazawa et al., 2024; Vetter, 2007; Xu et al., 2022).

In this study, we aimed to investigate the roles of class III *chs* genes, *chs7* and *chs9* in vegetative mycelia for the first time in a basidiomycete. To do so, single disruptants (Δ *chs7*, and Δ *chs9*), and double disruptants (Δ *chs7* Δ *chs9*) of both class III *chs* genes were isolated and functionally analyzed.

2. Materials and methods

2.1. Strains and culture conditions

Routine cultures were conducted using 20 mL of yeast extract/malt extract/glucose (YMG) medium and solidified with 2% (w/v) agar (Rao et al., 1969) and incubated at 28 °C in continuous darkness. Strains were precultured for 7 d and inoculated centrally with a Φ 4 mm plug. All strains used and generated in this study are listed in table S1.

2.2. Construction of gene disruption strains

Δ *chs7* and Δ *chs9* disruption strains were generated by targeting *chs7* and *chs9*, respectively, through a hygromycin resistance cassette and homologous recombination with the 20b strain as the host (Salame et al., 2012). Δ *chs7* Δ *chs9* double disruption strains were generated by targeting *chs9* with a bialaphos resistance cassette through homologous recombination with the Δ *chs7* #1 strain as the host.

Gene disruption cassettes were constructed using polymerase chain reactions (PCR) of three DNA fragments: the upstream and downstream regions of the target gene and the drug resistance marker. *P. ostreatus* PC9 genomic DNA was used as a template to amplify about 1.5 kb of the 5' and 3' flanking regions of *chs7* and *chs9*. The hygromycin resistance marker (*hph*) used for single disruption of *chs7* and *chs9* disruption was

amplified from the pPHT1 plasmid (Cummings et al., 1999). The bialaphos resistance marker (*bar*) used for double disruption of *chs7chs9* was amplified from the pFNCB plasmid (Avalos et al., 1989). Each fragment was amplified using Prime STAR GXL polymerase (Takara bio, Shiga, Japan) and fusion PCR utilized to combine the fragments into a cassette (Szewczyk et al., 2006). The amplified fragments were purified using a Nucleospin PCR Clean-up and Gel Extraction Kit (Takara bio, Shiga, Japan) and used as disruption cassettes. Disruption cassette schematics and primers can be found in Fig. S1 and Table S2.

For *P. ostreatus* transformation, the 20b or the Δ *chs7* #1 strain was cultured in YMG liquid medium at 120 rpm for 4 d and subsequently protoplasted 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 method, as previously described (Nakazawa et al., 2016; Salame et al., 2012).

2.3. Rapid PCR

Rapid PCR was conducted using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan) with crude genomic DNA from candidate transformants as a template (Izumitsu et al., 2012). The integration of the disruption cassettes was confirmed by PCR amplification of the target gene locus to confirm presence or absence of the target genes (Fig. S2, S3). Both drug resistance markers are more than 2 kb shorter than *chs7* and *chs9*, therefore a second PCR was performed using different primer sets to confirm the length change of the gene locus upon integration of the cassette. The PCR schematics, gel electrophoresis results, and primers are shown in Fig. S2, Fig. S3, and Table S2.

2.4. Growth and morphology

Mycelial diameters were measured at 10 d to calculate growth rate of colonies and a Nikon SMZ25 stereomicroscope (Tokyo, Japan) was used to observe hyphal sparsity. For aerial mycelia observation, 15 mL of YMG agar was dispensed into test tubes (Φ 16 mm), inoculated centrally with a mycelium plug, and the medium-air interface observed at 14 d.

2.5. Microscopy

Mycelia at 10 d were prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) following fixation and dehydration as described previously (Han et al., 2023).

For cell wall chitin staining with Calcofluor White (CFW), four mycelium plugs were inoculated into 40 mL YMG liquid medium and incubated at 28 °C, 120 rpm for 5 d. Samples were then homogenized using a polytron homogenizer (Kinematic AG, Malvern, Switzerland) and 200 μ L was reinoculated into 40 mL of fresh YMG liquid medium and incubated at 28 °C, 120 rpm for 2 d. Samples were then washed with 1 M Tris-HCl and stained with 2% Calcofluor White stain (Sigma Aldrich, St. Louis, USA) in Tris-HCl and incubated on ice for 2 h with intermittent mixing. The confocal microscopy platform STELLARIS (Leica Microsystems, Wetzlar, Germany) was used to capture 10 μ m Z-stack images, and hyphal thickness was measured using the Leica LAS X measuring tool.

2.6. Glucan and chitin quantification

A plug of mycelia was centrally inoculated on YMG agar overlaid with cellophane (Φ 90 mm) for 14 d. Mycelia was harvested using a spatula and washed with RO water before being ground into a powder using liquid nitrogen, and freeze-dried. The freeze-dried mycelial powder was subsequently used for cell wall component assays as described previously (Kojima et al., 2024; Schiphof et al., 2024). Briefly, α - and β -glucan measurements were quantified using the Yeast and Mushroom

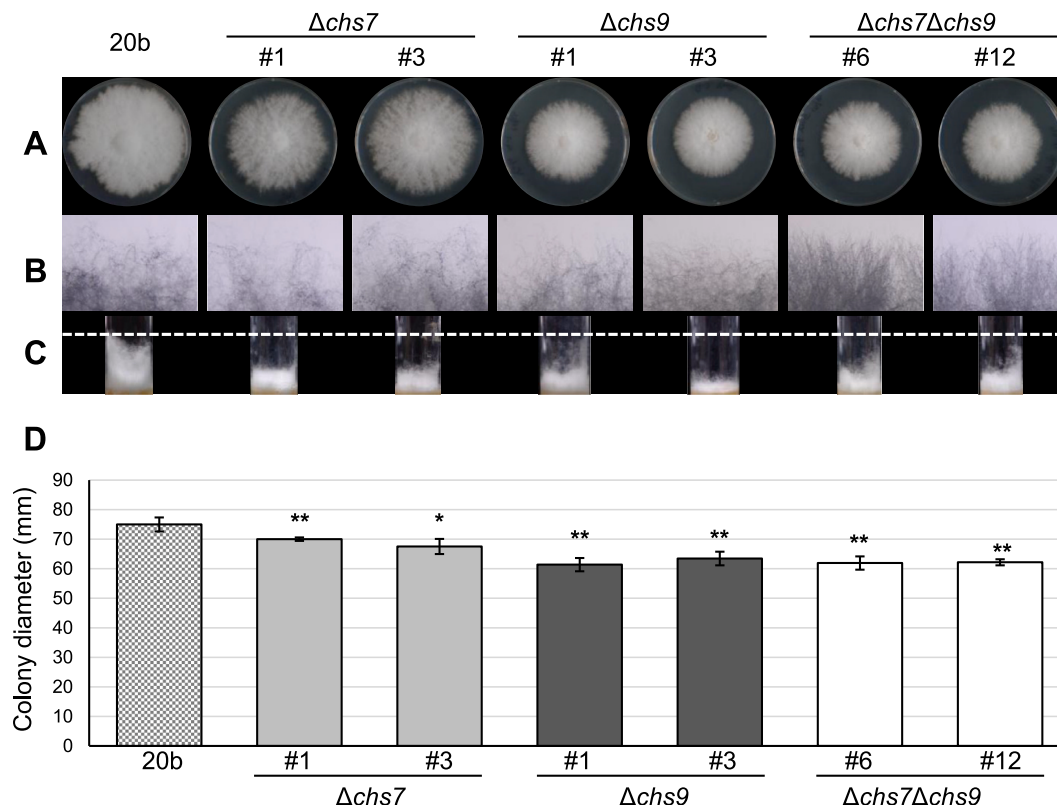


Fig. 1. Growth rate and morphological observations on the parent strain 20b and class III *chs* disruption strains. (A) Colony diameter on yeast, malt, glucose (YMG) agar. (B) Mycelial observations under stereomicroscopy. (C) Aerial hyphae assay, white dotted line indicates aerial hyphae height of the 20b strain. (D) Colony diameter on YMG agar. All bars indicate the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined by a two-tailed equal variance *t*-test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

β -Glucan Assay Kit (Megazyme, Bray, Ireland) with half amounts, in duplicate, following manufacturer protocols. Chitin was determined by acid hydrolysis with 4 N HCl, and incubated at 96 °C for 16 h, followed by the addition of sterile water to cool the reaction, activated charcoal treatment to remove contaminants, centrifugation and neutralization with NaOH. The neutralized solution was quantification using the D-Glucosamine Assay Kit (Megazyme, Bray, Ireland) in accordance with the manufacturer's protocol.

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

Mycelia cultured on YMG agar overlaid with cellophane ($\Phi 90$ mm) were harvested at 14 d, ground in liquid nitrogen, and RNA extracted using the FastGene RNA Premium Kit (NIPPON Genetics, Tokyo, Japan). cDNA synthesis was performed with PrimeScript RT Master Mix (Takara bio, Shiga, Japan). qRT-PCR was conducted using PowerTrack SYBR Green Master Mix and QuantStudio 5 (Thermo Fisher Scientific). Relative expression was calculated by the Pfaffl method (Pfaffl, 2001). Technical duplicates were averaged within each biological replicate. The primer sequences and efficiencies are listed in Table S4.

2.8. Stress and cell wall synthesis inhibitor resistance

YMG agar was supplemented with stress agents: 5 mM H_2O_2 and 0.02% SDS (oxidative/membrane stress), 0.3 M NaCl (osmotic stress), and 100 μ g/mL Micafungin (MF) and 500 μ g/mL CFW to test cell wall stress resistance. Fluorescent Brightener 28 (Sigma-Aldrich, St. Louis, MO, USA) and Funguard (Astellas, Tokyo, Japan) were used as the CFW and MF, respectively. Plates were inoculated with a mycelium plug and incubated for 10 d. Colony diameters on the supplemented plates (D1) and non-supplemented control plates (D2) were measured; relative

inhibitory rate (%) was calculated as: $(D2-D1)/D2 \times 100$.

3. Results

3.1. Disruption of class III *chs* affects mycelial growth

Gene targeting via homologous recombination with a gene disruption cassette was utilized to knockout the entire gene locus of the class III *chs* genes with the *P. ostreatus* 20b strain (*ku80* disrupted monokaryon) as the host (Salame et al., 2012). Single disruptants of *chs7* and *chs9* as well as double disruptants of *chs7chs9* were isolated (Fig. S3). All strains were confirmed using genomic PCR and two independent strains ($\Delta chs7$ #1 and $\Delta chs7$ #3, $\Delta chs9$ #1 and $\Delta chs9$ #3, $\Delta chs7\Delta chs9$ #6 and $\Delta chs7\Delta chs9$ #12) were randomly selected alongside the 20b parent strain for subsequent investigation.

Growth phenotypes on YMG agar plates and aerial hyphae assays highlighted a slight decrease in aerial hyphae height and radial growth in all disruption strains (Fig. 1), with $\Delta chs7$ exhibiting the least severe growth defects, and $\Delta chs9$ and $\Delta chs7\Delta chs9$ exhibiting slightly more severe growth defects. Despite double disruption, the growth rate of $\Delta chs7\Delta chs9$ and $\Delta chs9$ strains were very similar. Mycelial morphology in $\Delta chs7\Delta chs9$ strains had straighter hyphae in comparison to 20b and the single disruption strains (Fig. 1B). The growth defects and morphology changes in the class III disruption strains suggest roles in hyphal formation and mycelial growth.

3.2. Microscopy highlights decreased cell wall thickness in class III *chs* disruption strains

TEM highlights significantly thinner cell walls, with the average 20b strain cell wall being 97.2 nm thick, $\Delta chs7$ 73.5 nm, $\Delta chs9$ 64.2 nm and

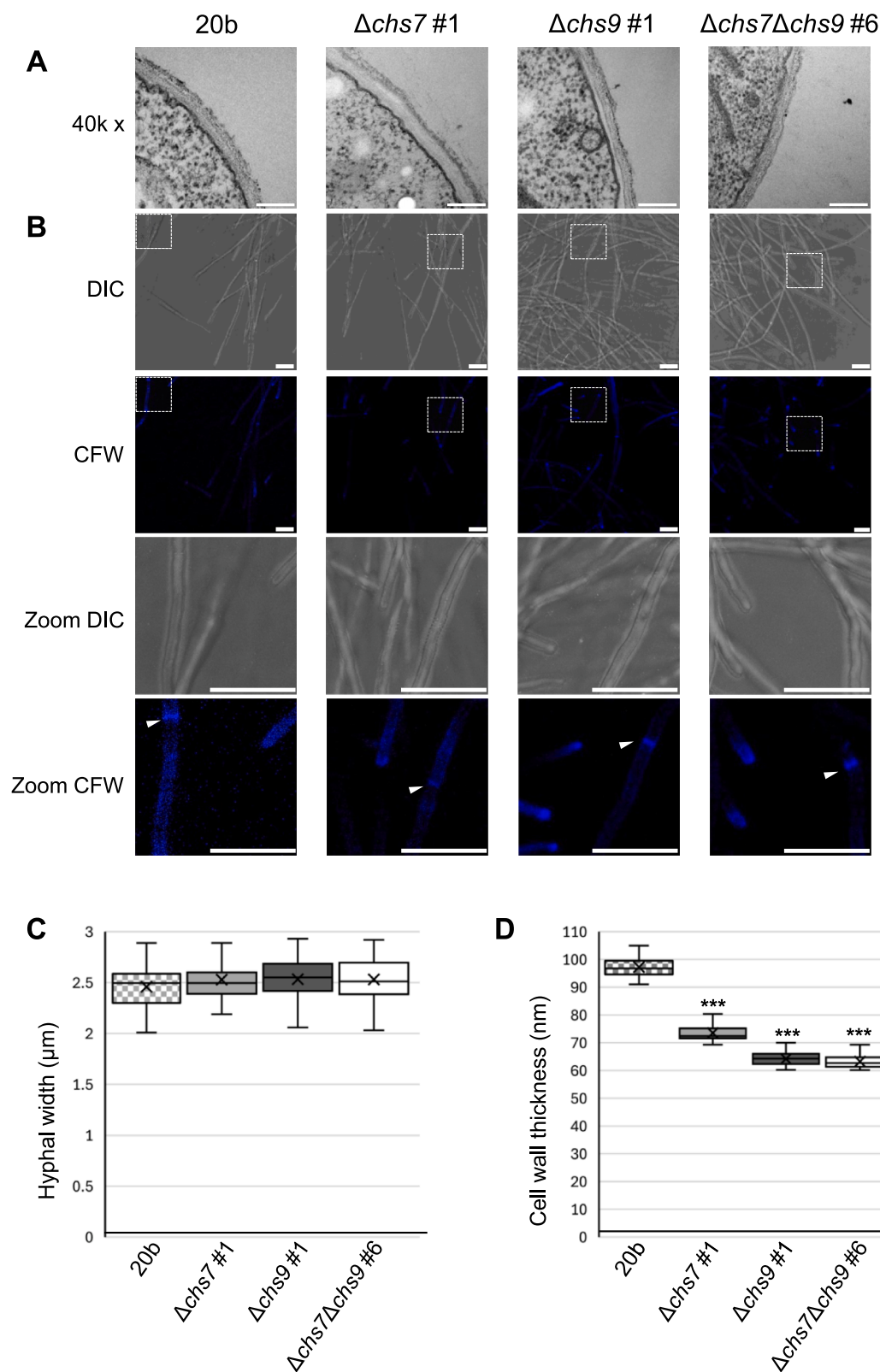


Fig. 2. Microscopy observations of the parent strain 20b and class III *chs* disruption strains. (A) Transmission electron microscopy (TEM), scale bar = 200 nm. (B) Calcofluor White (CFW) staining, arrows indicate septa (scale bar = 20 μm), DIC: Differential interference contrast, Merge: the merged DIC and staining images, Zoom: Enlargement of white dotted boxes from merged images. (C) Cell wall thickness obtained from TEM observations in (A) ($n = 50$). (D) hyphal thickness obtained from confocal microscopy ($n = 50$). Statistical significance was determined using a two-tailed equal variance *t*-test (***) $P < 0.001$.

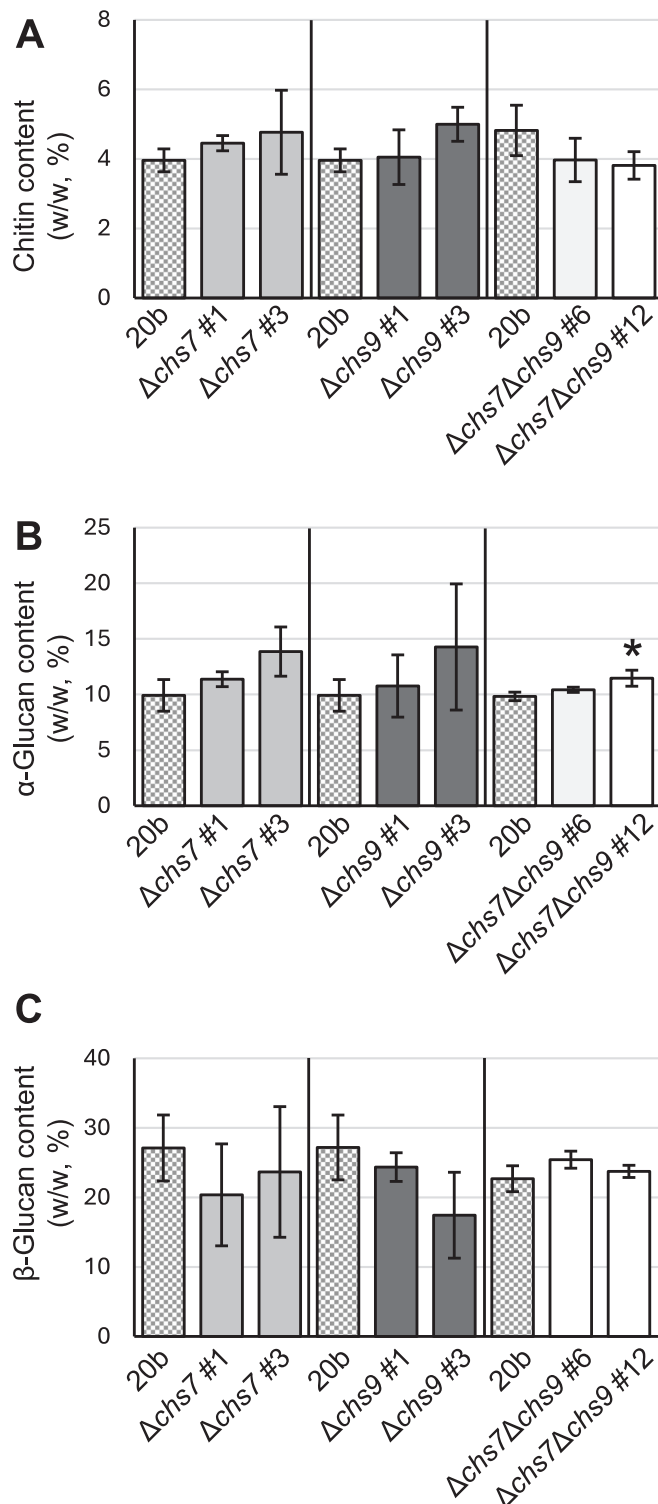


Fig. 3. Relative percentages (g/100 g) of cell wall polysaccharides in the 20b and class III *chs* disruption strains. (A) chitin, (B) α-glucan, and (C) β-glucan from mycelia on yeast and malt extract with glucose (YMG) cellophane at 14 d. The bars indicate the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined using a two-tailed equal variance *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Δ*chs7*Δ*chs9* 63.3 nm (Fig. 2D). Similar trends are observed in growth rate on agar, with Δ*chs7* strains having less severe impacts and Δ*chs9* and Δ*chs7*Δ*chs9* strains having similar results to each other. Suggesting important roles in cell wall formation and that there is some redundancy

within the class III *chs*s. CFW staining to visualize chitin localization under confocal microscopy highlights the presence of chitin in tips and septa of all strains (Fig. 2B). Hyphal thickness measurements under confocal microscopy highlight no differences in hyphal thickness (Fig. 2C).

3.3. Cell wall components remain unchanged and various cell wall synthases were upregulated, suggesting redundancy within class III *chs*s

Relative percentage (g/100 g dried mycelia) of chitin (Fig. 3A), α-glucan (Fig. 3B), and β-glucan (Fig. 3C) remained unchanged in all disruption strains. A slight increase in α-glucan was observed in Δ*chs7*Δ*chs9* #12, however this change is within the assay kit's margin of error.

Relative gene expression levels of all nine *chs* genes, two β-glucan synthases (*fkss*) and single α-glucan synthase (*ags*) were examined (Fig. 4). In Δ*chs7* strains, *chsb1*, *chsb4*, *chs6*, and *chs9*, are upregulated around 1.5–2-fold, and *fkss1* around 2.5-fold. In Δ*chs9* strains *chsb1*, *chsb3*, *chs5*, *chs6*, and *fkss2* are consistently upregulated 1.5–2-fold, *chsb2* 2–3-fold and notably *chs7* is upregulated 3.5–4.5-fold higher than in the 20b strain. In Δ*chs7*Δ*chs9* double disruption strains, all *chs* genes are upregulated 1.5–2-fold higher as well as *fkss1* and *ags1*.

These results suggest redundancy within class III *chs*s, relationships between chitin and glucan synthases, and inter-clade compensatory responses by various chitin synthases, which may account for the lack of changes observed in cell wall component synthesis or mitigate more severe impacts.

3.4. Disruption of class III *chs* genes causes altered sensitivity to various cell wall, oxidative, and osmotic stressors

All disruption strains were significantly more sensitive to CFW, a chitin synthesis inhibitor and MF, a β-glucan synthesis inhibitor, and slightly more sensitive to H₂O₂, an oxidative stressor, and SDS, a detergent that damages the plasma membrane, indicating a decrease in cell wall integrity in the class III *chs* disruption strains (Fig. 5, S4). Additionally, most strains were less sensitive to or not affected by the osmotic stressor NaCl (Fig. 5, S4), suggesting cell wall defects in the class III *chs* disruption strains.

4. Discussion

Class III CHSs have been characterized in ascomycetes and yeast-like basidiomycetes, but their roles in filamentous basidiomycetes remain largely unexplored. By isolating single and double disruptants of the two class III *chs* genes (*chs7* and *chs9*) in *P. ostreatus*, we revealed distinct and sometimes overlapping functions in fungal growth, cell wall integrity and stress responses. Our previous work identified nine *chs* genes in *P. ostreatus*, including the two class III genes, and revealed clade-specific roles of basidiomycete-specific *chs*s in cell wall formation and hyphal development (Schiphof et al., 2024; Schiphof et al., 2026). Building on this, our current study focused on class III *chs*s during vegetative mycelial growth. Disruption of *chs7* and *chs9* reduced radial growth and aerial hyphae height, with *chs9* disruption causing more severe defects (Fig. 1), suggesting a critical role for *chs9* and a supportive role for *chs7*. Single Δ*chs9* and double Δ*chs7*Δ*chs9* disruptant strains showed similar growth defects, however Δ*chs7*Δ*chs9* strains additionally exhibited hyphae which extend more linearly (Fig. 1B), suggesting that class III *chs* genes have roles in growth and hyphal straightness.

TEM observation highlighted significantly thinner cell walls in all single and double class III *chs* disruption strains (Fig. 2A, D, Fig. 6), with *chs9* and *chs7*Δ*chs9* disruption strains having more severe impacts than those of *chs7*, indicating important roles of class III *chs*s in cell wall architecture. Some *chs* genes have specific roles in hyphal septa or tips, and disruption of these genes alters chitin presence in these locations (Brown et al., 2020; Gow et al., 2017; Gow et al., 2023; Rogg et al.,

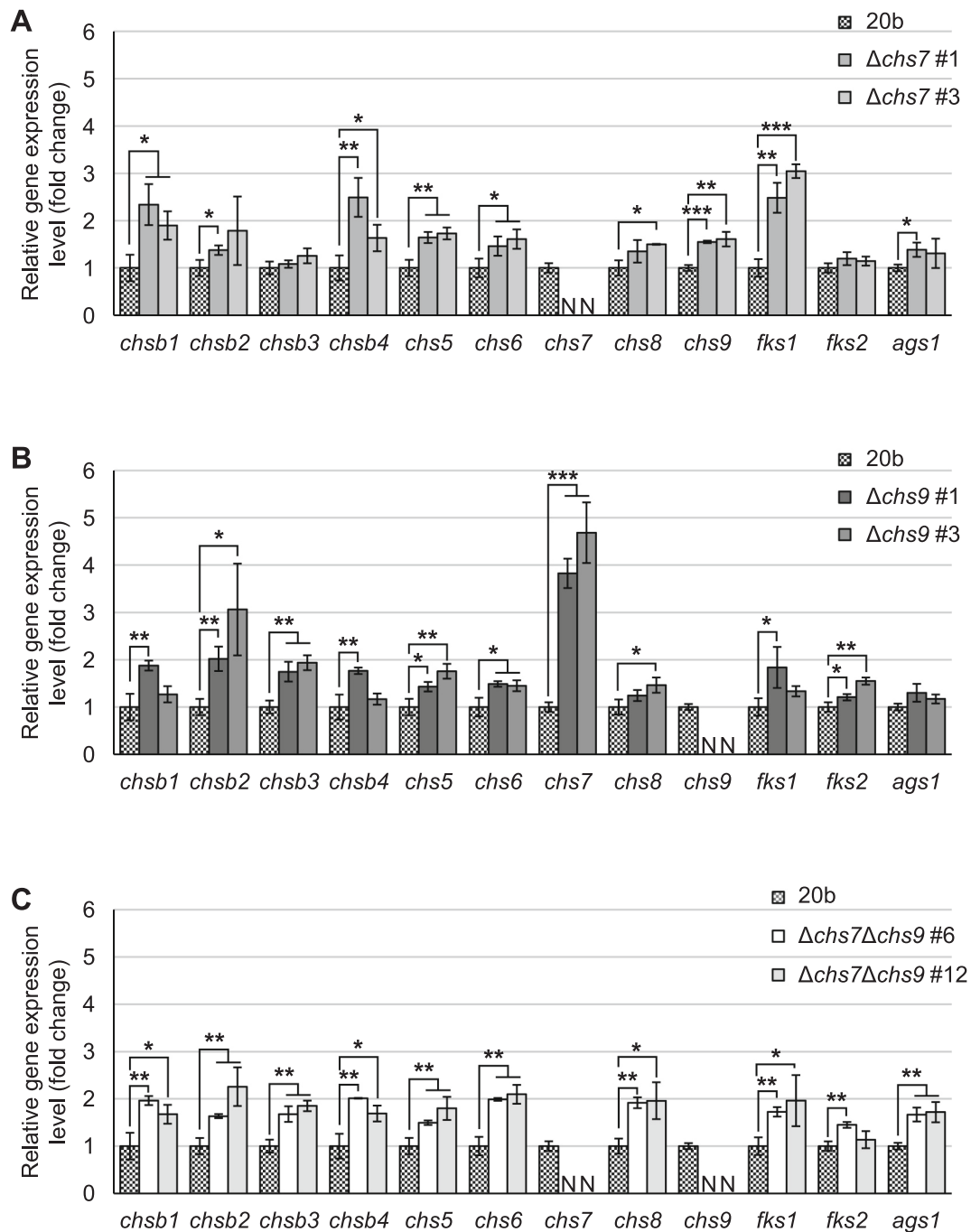


Fig. 4. Relative gene expression levels of various cell wall synthases in the class III *chs* disruption strains. (A) Δ chs7. (B) Δ chs9. (C) Δ chs7 Δ chs9. The bars indicate the standard deviations of three biological replicates ($n = 3$). Statistical significance was determined using a two-tailed equal variance *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N; Gene expression not detected. *chs*: chitin synthase, *fks*: β -glucan synthase, *ags*: α -glucan synthase.

2012). However, CFW staining shows no change in chitin distribution, and highlights the presence of chitin in hyphal tips and septa of all class III *chs* disruption strains (Fig. 2B). The relative proportions of chitin, β -glucan, and α -glucan also remained unchanged in all single and double class III *chs* disruption strains (Fig. 3). Although the overall cell wall component content relative to total mycelial dry weight remains unchanged, the thinner cell walls observed by TEM suggest the possibility of a reduction in the absolute amount of β -glucan, α -glucan, and chitin per individual cell wall (Fig. 6) (Schiphof et al., 2024; Schiphof et al., 2026). qRT-PCR results highlight transcriptional upregulation of other chitin and glucan synthase genes, including reciprocal upregulation of *chs7* and *chs9* in Δ chs9 and Δ chs7, respectively (Fig. 4). In Δ chs9 strains,

chs7 was upregulated 3.5–4.5 fold higher than in the 20b strain, whereas in Δ chs7 strains, *chs9* was upregulated only 1.5-fold suggesting that *chs9* is the main class III *chs* and *chs7* provides partial redundancy. Additionally, in our previous studies, *chs7* expression was upregulated in all *chs* disruption strains often 4-fold higher, and although many other *chs* genes were upregulated in Δ chs7 they were only 1.5–2-fold higher, supporting the hypothesis that *chs7* plays a more supportive role not only for *chs9* but also other *chs* genes (Schiphof et al., 2024; Schiphof et al., 2026). In the Δ chs7 Δ chs9 strains, all remaining *chs* genes were upregulated 1.5–2-fold (Fig. 4C), likely compensating for the gene disruption, and reducing phenotypic severity in the double disruption strains. Similarly, the lack of change in cell wall component ratios

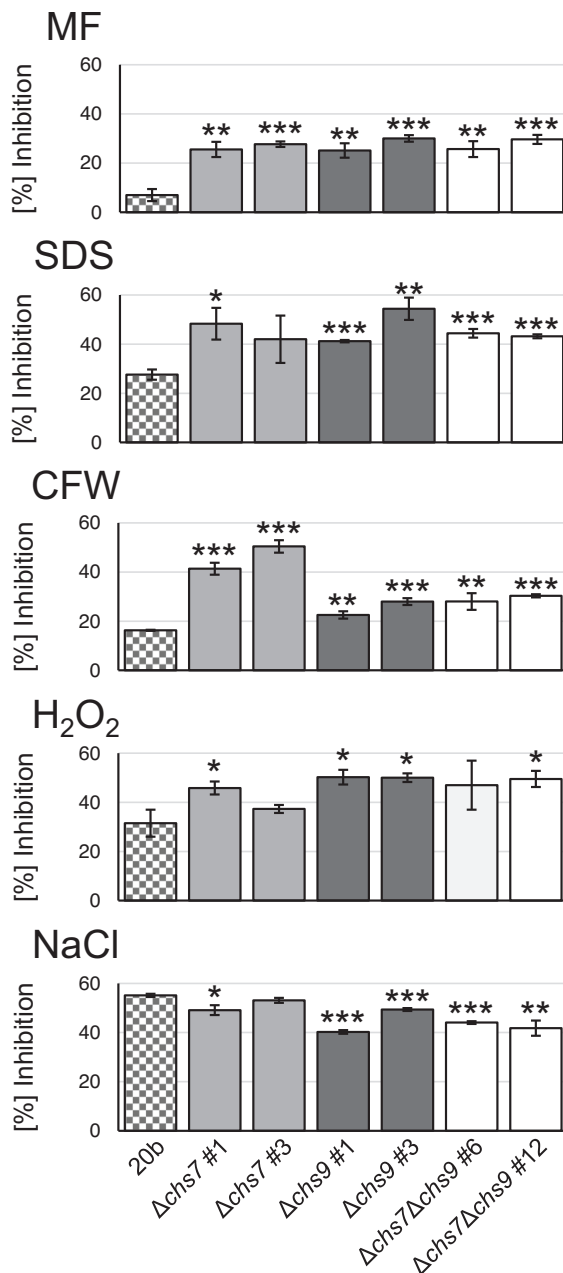


Fig. 5. Growth inhibition of the parent strain 20b and class III *chs* disruption strains. Growth on yeast and malt extract with glucose (YMG) agar medium supplemented with 500 µg/mL Calcofluor White (CFW), 100 µg/mL Micafungin (MF), 5 mM H₂O₂, 0.02% sodium dodecyl sulphate (SDS), and 0.3 M NaCl at 10 d. Bars indicate standard deviations of three biological replicates ($n = 3$). Statistical significance was determined using a two-tailed equal variance *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

(Fig. 3) may be attributed to the upregulation of various other cell wall synthase genes. The compensatory upregulation of multiple cell wall synthases illustrates functional redundancy and dynamic regulatory mechanisms that attempt to maintain cell wall homeostasis despite genetic disruption. However, given that some cell wall and growth defects were observed, the compensation is imperfect.

Fungal cell walls are important for environmental interactions and protection as well as structural integrity, and chitin plays important roles in maintaining cell wall functions (Brown et al., 2020; Gow et al., 2017; Gow et al., 2023). All strains had altered sensitivity to various cell walls, oxidative and osmotic stressors, indicating altered cell wall architecture and emphasizing the role of class III *chs* in maintaining cell

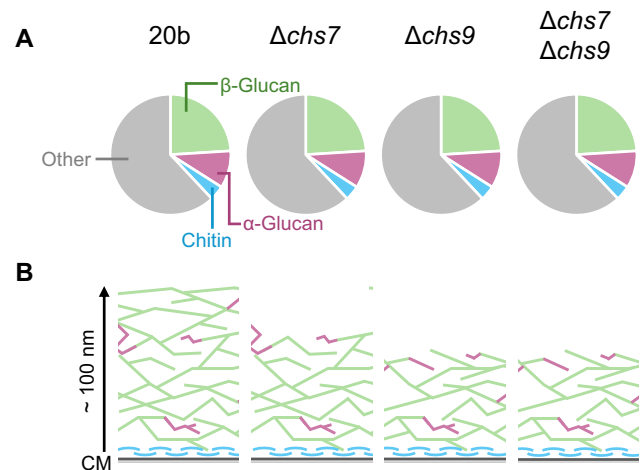


Fig. 6. Summary of cell wall impacts and schematic diagram of hypothesized inner cell wall remodeling in response to class III *chs* disruption in *P. ostreatus*. (A) Cell wall components as a percentage of total mycelial dry weight. (B) Cell wall thickness measured by transmission electron microscopy (TEM) alongside relative component data. Green: β -glucan, pink: α -glucan, blue: chitin. Despite unchanged β -glucan and chitin levels relative to total dry weight, TEM revealed thinner cell walls, suggesting a reduced absolute amount of these components per individual cell wall.

wall integrity under environmental challenges (Fig. 5). Notably, despite increased sensitivity to cell wall and oxidative stressors, almost all disruption strains were more resistant than 20b to the osmotic stressor NaCl. These trends are similar to those observed in the basidiomycete-specific *chsB* growth defect strains $\Delta chsB1$ and $\Delta chsB2\Delta chsB3$, where strains were also more resistant to NaCl than 20b, and may be due to cell wall remodeling or chitin conformation changes causing increased osmotic protection despite growth defects (Schiphof et al., 2026). Fungi have rigid but elastic cell walls and stress response pathways. In *Aspergillus niger* the *msnA* null mutants show increased sensitivity towards CFW but no change in NaCl, whereas single and double *rlmA* deletion strains show slightly increased tolerance to NaCl, suggesting that increasing the expression of cell wall genes might confer a tolerance towards salt stress (Fiedler et al., 2014). A study in *Candida albicans* revealed that the degree of cross-linking between cell wall polymers impacts osmotic tolerance, and decreases in cell wall elasticity can protect the plasma membrane during osmotic shock, leading to increased resistance to osmotic stress (Ene et al., 2015). Furthermore, inactivating the cross-linking related Crh family causes increased sensitivity to hyperosmotic shock, whereas overexpression reduces cell wall elasticity, causes structural realignment, and provides partial protection to osmotic shock (Ene et al., 2015). The observed increase or no change in resistance to osmotic stress in *P. ostreatus* could be due to a similar phenomenon, with disruption of the class III *chs* altering chitin binding or folding, which causes detrimental effects to oxidative and cell wall stress but decreases cell wall elasticity and provides increased osmotic tolerance. However, further experimentation to investigate cell wall elasticity and structural responses to osmotic stress are required to confirm this theory.

The results in this study are different from findings in ascomycetes such as *Aspergillus nidulans*, where disruption of the class III gene *chsB* results in severe growth defects, abnormal branching, multilayered cell walls, and intrahyphal hyphae (Borgia et al., 1996; Fukuda et al., 2009; Yanai et al., 1994). In *Aspergillus fumigatus*, disruption of *chsC* causes no notable phenotype differences, but disruption of *chsG* and double disruption of both class III *chs* genes, *chsCchsG* causes reduced radial growth and chitin synthase activity, poor conidiation, and highly branched hyphae, (Mellado et al., 1996a). In *Neurospora crassa*,

inactivation of the class III gene *chs-1* causes slow growth, altered hyphal morphology and increased sensitivity to chitin synthase inhibitors (Yarden et al., 1991). In plant pathogens such as *Colletotrichum graminicola* and *Ustilago maydis* class III *chs* disruption causes minor or no significant hyphal growth or pathogenicity defects (Weber et al., 2006; Werner et al., 2007). In *Botrytis cinerea*, disruption of the class III *chs*, *Bcchs3a* reduces chitin content, radial growth and virulence, but exhibits enhanced aerial hyphae growth likely due to compensatory hyphal ramification (Soulié et al., 2006). In *Fusarium graminearum*, disruption of the class III *chs* gene *FgCHS3b* is lethal (Cheng et al., 2015), whereas in the human pathogen *Wangiella dermatitidis*, disruption reduces chitin synthase activity without major morphological changes, and double disruption causes growth defects, altered hyphal morphology, and delayed pathogenicity (Wang et al., 2000). Together, these results highlight evolutionary divergence of class III *chs* functions between species.

Our previous study highlighted that within the *P. ostreatus chs* family, the functions of the basidiomycete-specific *chs* genes are clade specific, with clade BI *chs1* gene disruption having severe growth defects, clade BII *chs* gene single disruption having no impacts, but double disruption causing severe growth defects and abnormal septation, and clade BIII *chs4* disruption having only minor cell wall and aerial hyphae defects (Schiphof et al., 2024; Schiphof et al., 2026). The class III *chs* genes in this study had unique gene upregulation patterns, growth defects less severe than clade BI and BII disruptions strains, and double disruption did not severely exacerbate growth defects like in clade BII *chs* genes, suggesting specialized functions of class III *chs* in *P. ostreatus*, possibly related to hyphal orientation, extension or straightness.

5. Conclusion

In ascomycetes class III chitin synthases play diverse roles in fungal growth, hyphal organization, cell wall integrity, and pathogenicity, although their specific roles and importance vary widely across species. Our results demonstrate that both the class III *chs* genes in the basidiomycete *P. ostreatus* have roles in hyphal growth, with *chs9* being the main class III *chs* and *chs7* providing a more supportive role. Double *chs7chs9* disruptants additionally exhibited hyphae which extend more linearly, suggesting a unique role of *P. ostreatus* class III *chs* genes when compared to those characterized in other fungi. However, it remains unclear whether these functions are unique to this species or representative of a broader group within the basidiomycete phylum, as class III *chs* genes have not yet been studied in other non-yeast-like basidiomycetes. This study provides the first detailed insight into class III *chs* function in a non-yeast-like basidiomycete, revealing evolutionary divergence, specialized hyphal growth roles, functional redundancy, and regulatory complexity. These findings lay an important foundation for advancing our understanding of fungal cell wall biology in basidiomycetes.

CRediT authorship contribution statement

Kim Schiphof: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ayane Hitaka:** Visualization, Investigation, Formal analysis, Data curation. **Moriyuki Kawauchi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **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 competing financial interests or personal relationships that may have influenced the work reported in this study.

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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.2026.104081>.

Data availability

Data will be made available on request.

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