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BACTERIAL AND FUNGAL PATHOGENESIS - SHORT COMMUNICATION

Rapid screening method of *Saccharomyces cerevisiae* mutants using calcofluor white and aniline blue

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Abstract

Fungal cell walls are composed of polysaccharide scaffold that changes in response to environment. The structure and biosynthesis of the wall are unique to fungi, with plant and mammalian immune systems evolved to recognize wall components. Additionally, the enzymes that assemble fungal cell wall components are excellent targets for antifungal chemotherapies and fungicides. Understanding changes in the cell wall are important for fundamental understanding of cell wall dynamics and for drug development. Here we describe a screening technique to monitor the gross morphological changes of two key cell wall polysaccharides of chitin and β-1,3-glucan combined with polymerase chain reaction (PCR) genotyping. Changes in chitin and β-1,3-glucan were detected microscopically by using the dyes calcofluor white and aniline blue. Combining PCR and fluorescence microscopy, as a quick and easy screening technique, confirmed both the phenotype and genotype of the wild-type, h chitin synthase mutants (*chs1*Δ and *chs3*Δ) and one β-1,3-glucan synthase mutant *fks2*Δ from *Saccharomyces cerevisiae* knockout library. This combined screening method highlighted that the *fks1*Δ strain obtained commercially was in fact not *FKS1* deletion strain, and instead had both wild-type genotype and phenotype. A new β-1,3-glucan synthase knockout *fks1::URA3* strain was created. Fluorescence microscopy confirmed its phenotype revealing that the chitin and the new β-1,3-glucan profiles were elevated in the mother cells and in the emerging buds respectively in the *fks1*Δ cell walls. This combination of PCR with fluorescence microscopy is a quick and easy screening method to determine and verify morphological changes in the *S. cerevisiae* cell wall.

Keywords Yeast · Chitin · β-1,3-Glucan · Fluorescence

Introduction

Despite over one fifth of *Saccharomyces cerevisiae* genome being dedicated to cell wall biosynthesis, the wall consists mainly of three-core polysaccharides that are synthesized by a handful of genes. These are the inner layer of chitin (1–2%); β-1,3-glucan (50–55%); and β-1,6-glucan (10–15%) [1, 2]. However, the composition of the wall is dynamic, with changes in these polysaccharides due to environmental stress, and growth conditions. Deletion of cell wall biosynthesis genes can also lead to morphological changes in these cell wall polysaccharides. Fluorescent dyes have been used to examine these changes in the cell wall polysaccharides. In particular, calcofluor white (CFW) which interacts with β-1,4-glucans such as chitin, chitosan, and cellulose while aniline blue (AB) binds with β-1,3-glucans [3].

Chitin is largely confined to the bud scars and septa of *S. cerevisiae* and can be easily visualized using the fluorescent dye calcofluor white. Three chitin synthases are involved in

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its synthesis [2, 4], encoded by three genes *CHS1*, *CHS2*, and *CHS3*, respectively [1, 2]. Chs1p acts as a repair enzyme that adds chitin to the birth/bud scar at the end of cytokinesis [5]. Chs2p makes no more than 5% of the chitin in budding cells forming the central disk within the primary septum [6, 7]. Chs3p (also known as CSD2, CAL1, CAL4, CAL5, DIT101, or KTI2) synthesizes more than 90% of the chitin in *S. cerevisiae* and is responsible for depositing chitin as a ring at the base of an emerging bud [7–9]. Chs3p is retained by the mother cell (bud scar) after cell division and it is also involved in depositing chitin in the lateral wall during vegetative growth [7–9].

When stained with AB, β-1,3-glucans are uniformly detected in the entire cell wall of *S. cerevisiae* [10]. β-1,3-Glucans provide cell wall rigidity and are synthesized by β-1,3-glucan synthase, composed of a catalytic and regulatory subunit. They are the related catalytic subunits, *Fks1p* (FSK1) and *Fks2p* (FSK2), and a regulatory subunit of the small GTPase Rho1p [11–13]. Fks1p and Fks2p are expressed at different times, i.e., Fks1p during vegetative growth on glucose and Fks2p under starvation, stress, and stationary phase and in the absence of Fksp1p function [14]. *S. cerevisiae* also contains a third homologous subunit, Fksp3p, but little is known about its function. Evidence suggests that it plays a role in the ascospore wall assembly with the *FKS3* null mutant being defective in sporulation [15]. Both of the catalytic and regulatory components of Rho1p and Fsk1p are localized on the plasma membrane at the site of cell wall remodeling, i.e., the bud tip during bud growth and the bud neck during cytokinesis [13]. The aim of this paper was to use both fluorochromes, CFW, and AB, to validate the chitin and β-1,3-glucans cell wall profile of chitin synthase and β-1,3-glucan synthase knockouts of *S. cerevisiae*. This imaging test was a rapid complement test to PCR evaluations.

Material and methods

Strains and the chitin and β-glucan synthase mutants in *S. cerevisiae*

The wild-type *S. cerevisiae* strain was the haploid BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0). The β-1,3-glucan synthase knockouts *fks1*Δ and *fks2*Δ, and the chitin synthase knockouts *chs1* Δ and *chs3*Δ, were obtained from the non-essential *S. cerevisiae* knockout library [16, 17] purchased from Thermo Fisher (USA) (Table 1). PCR was used to confirm the knockout mutants (Table 1) were disrupted in the appropriate gene.

Genomic DNA (gDNA) was extracted from cells pelleted from 1 mL of overnight culture of each of the *S. cerevisiae* mutants, and was performed as outlined in the manufacturer's protocol for DNAzol® reagent (Life Technologies™) with some modifications. Briefly, the cell pellet was suspended in 500 µL of DNAzol® reagent followed by the addition of 100 µL of glass beads (acid washed, 180 µm, Sigma-Aldrich). Samples were vortexed for 2 min before insoluble material was removed by centrifugation in a microfuge at top speed for 5 min. The supernatant was transferred to a fresh tube and a half volume of 100% ethanol was added. The sample was mixed by inversion and allowed to stand for 10 min at room temperature before centrifugation at top speed for 5 min in a microcentrifuge. The pellet was washed twice in 75% ethanol before it was dried and dissolved in 50 µL of TE buffer (10 mM Tris-HCl containing 1 mM EDTA). This gDNA solution (1 µL) was used as the template for confirming gene disruption by PCR. The PCR was performed for 30 cycles with the following temperature profile: initial denaturing step of 94 °C; followed by 30 cycles of 94 °C, 30 s; 49 °C, 30 s; 72 °C, 1 min; with a final extension cycle of 72 °C for 10 min. The primer pairs used for the PCR were either the KanB or KanB1 reverse primer with the appropriate forward primer depending on the gDNA that was amplified (Table 2). PCR products were examined by electrophoresis on a 1% agarose Tris-acetate-EDTA (TAE) gel (90 v, 1 h).

Production of a knockout of the β-glucan synthase gene, FKS1, in *S. cerevisiae*

The *FKS1* gene in *S. cerevisiae* was disrupted by homologous recombination and insertion of the *URA3* gene for selection of transformed cells on media lacking uracil. The gene, *CCC1*, encoding the putative vacuolar Fe^{2+}/Mn^{2+} transporter was disrupted as a negative control.

Primers were designed to amplify the *URA3* gene with the addition of 40 bp from the 5′- and 3′-translated ends of the *FKS1* gene (Table 3). The pPS293 plasmid, containing the *URA3* gene, was used as the template DNA

Table 1 Cell wall polysaccharide synthesis genes

Gene	Systematic name	Protein
FKS1	YLR342W	Catalytic subunit of β-1,3-glucan synthase
FKS2	YGR032W	Catalytic subunit of β-1,3-glucan synthase
CHS1	YNL192W	Catalyzes the transfer of N-acetyl glucosamine to chitin
CHS3	YBR023C	Catalyzes the transfer of N-acetyl glucosamine to chitin

Table 2 Primer sequences for confirming gene disruptions

Name	Sequence	TM (°C)
KanB	5′ CTG CAG CGA GGA GCC GTA AT 3′	56
KanB1	5′ TGT ACG GGC GAC AGT CAC AT 3′	54
Fks1 A	5′ TCT TTC TAA GAA GGC CAG AAC ACT A 3′	54
Fks1 B	5′ TTT TAT AGT TAG CAG TAT CGC CAC C 3′	54
Fks2 A	5′ AGT GTG TGA CCA CGA AAT TCA AGA TA 3′	56
Chs1 A	5′ ACA GCC ATT AGT GTG AAA TTT GAT T 3′	51
Chs3 A	5′ ACA TAT TTG ACA TGT GCA TGA GAC T 3′	53

(kind gift from Dr. Ana Traven, Monash University) for a 50-µL PCR containing 2× Phusion® master mix and forward and reverse primers (0.4-µM final concentration). The reaction was performed with a denaturing step at 98 °C for 5 min followed by 35 cycles of the following temperature profile: 98 °C, 30 s; 64 °C, 30 s; and 72 °C, 1 min; and a final extension cycle of 72 °C for 10 min. The PCR product was confirmed to be the correct size by gel electrophoresis before it was excised from the gel and isolated using the Wizard® SV gel and PCR cleanup kit (Promega, WI, USA). This DNA fragment was transformed into *S. cerevisiae* using the method described in Wood and Komives [18]. In brief, cells from a log phase *S. cerevisiae* (BY4741) culture (2 mL) were pelleted and resuspended in 240 µL of PEG 4000 (50% *w*/*v*), 36 µL of lithium acetate (1 M), 50 µL of denatured salmon sperm carrier DNA (2 mg/mL), 34 µL of DNA fragment, and 29 µL of water. The mixture was incubated at 42 °C for 60 min before the cells were pelleted by centrifugation for 1 min at top speed and resuspended in 500 µL of sterile water. The cell suspension (250 µL) was spread onto synthetic dropout media lacking uracil (SD-URA, Sigma-Aldrich) agar plates and incubated at 30 °C for 4 days. Colonies were isolated and streaked onto SD-URA plates before genomic DNA was extracted. Disruption of the *FKS1* gene or the *CCC1* gene was confirmed by PCR using the same primer pair (Table 3).

Growth of wild type and β-1,3-glucan synthase and the chitin synthase knockouts

S. cerevisiae BY4741, *fks1*Δ, *fks2*Δ, *chs1*Δ, and *chs3*Δ mutants were grown on YPD agar plates (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose, 1.5% agar) at 30 °C. Freshly grown wild-type and mutant yeast cultures were used to inoculate 2 mL of YPD broth and shaken overnight at 30 °C. For testing conditions and screening with fluorochromes, strains grown overnight were diluted 1:100 into fresh half strength YPD broth (optical density at 600 nm [$OD_{600\,nm}$], 0.1) and the cultures grown overnight for chitin and β-1,3-glucan cell wall screening by microscopy.

Fluorescent dyes

Calcofluor white stain (CFW, Fluka) was freshly prepared prior to use according to manufacturer's protocol by mixing equal volume of the stain with 10% (*w*/*v*) potassium hydroxide (AnalaR, BDH). Aniline blue fluorochrome (ABF, Biosupplies Australia PTY Ltd.) was resuspended in sterile distilled water according to manufacturer's protocol. The stock solution (0.1 mg/mL) was stored at 4 °C in the dark. Prior to use, the stock solution was diluted 1:3 in sterile water. Samples of yeast cell suspensions were examined by mixing one drop of suspension with one drop of fluorescent solution on a slide at ambient room temperature.

Fluorescent microscopy

Yeast cells were examined with an Olympus IX81 inverted microscope with UPlanS Apo 100×/1.40 oil objective equipped with a filter cube U-MWU2 comprising an excitation filter 330–385 nm/emission filter LP 420 nm/dichroic mirror 400 nm. Images were acquired with F-View Soft Imaging System (SiS) Digital camera using AnalySIS

Table 3 Primer sequences used for the construction of *FKS1* knockout. Sequences in bold are the first 40 bp from the 5′ or 3′ end of the translated sequence of the *FKS1* or *CCC1* genes, while the underlined sequence is from the *URA3* gene

Name	Sequence
FKS1URA Fwd	5′ **ATGAACACTGATCAACAACCTTATCAGGGCCAAACGGACT**CTGTGCGGTATTTCACACCG 3′
FKS1URA Rv	5′ **TTATTTTATAGTTGACCAGGTCTTTAATGATGGCGTATGA**GATTGTACTGAGAGTGCAC 3′
CCC1URA Fwd	5′ **ATGTCCATTGTAGCACTAAAGAACGCAGTGGTGACCCTTA**CTGTGCGGTATTTCACACCG 3′
CCC1URA Rv	5′ **TTAACCCAGTAACTTAACAAAGAACCAAGCCGCACCTGCT**GATTGTACTGAGAGTGCAC 3′

software. Grayscale images were further colorized using Fiji software, i.e., cyan for CFW/chitin and yellow for ABF/β-1,3-glucan, respectively (Image J 1.47 h version) (Rasband WS, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, 1997–2012) and processed in Illustrator CS6 (Adobe).

Results

Confirmation of the genotype of the *S. cerevisiae* cell wall synthase mutants

The mutants were obtained from the yeast knockout collection [16, 17], and PCR was used to confirm they had the desired gene knockout (Fig. 1). All mutants had the appropriate deletion except for *FKS1* purchased knockout which had the FKS1 gene present (Fig. 2). Instead, we generated a *fks1*Δ strain in-house by insertion of the URA3 gene, outlined below.

Construction of the fks1Δ strain

As the purchased knockout of the *FKS1* returned a WT genotype by PCR, we instead constructed this mutant in-house. A construct was designed for the disruption of the *FKS1* gene in the *S. cerevisiae* BY4741 background, using homologous recombination and the selectable marker gene, *URA3*. This was performed by inserting the URA3 gene into the WT FKS1 gene, and two clones (F1 and F2) were evaluated (Fig. 3). Insertion of the *URA3* gene into gDNA of the *FKS1* in WT (BY4741) background was confirmed by PCR (Fig. 3). A construct for the disruption of the *CCC1* gene was also created, as a negative control, to ensure the presence of the *URA3* gene had no effect in further testing, as the addition of *URA3* improves the growth of the yeast in 0.5 times PDB (data not shown). Insertion of the *URA3* gene was confirmed by PCR along with the disruption of *CCC1* (Fig. 3). Two clones of the *fks1*Δ were evaluated.

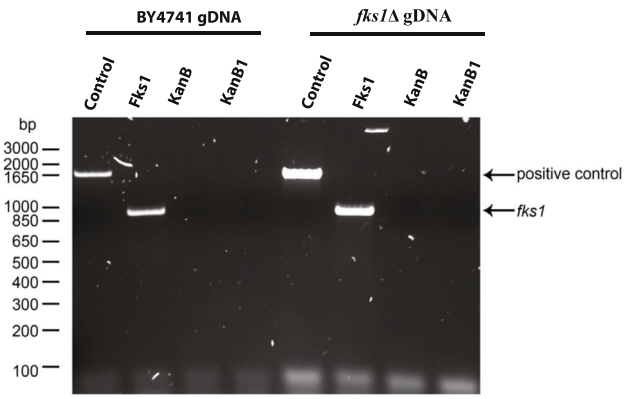

Fig. 2 Genotype of the putative *S. cerevisiae fks1*Δ mutant. Confirmation that the *FKS1* gene was not disrupted in the mutant supplied in the deletion set was obtained by amplification of gDNA from BY4741 (WT) and the putative β-glucan synthase gene mutant, *fks1*Δ, using a suite of primers: control primers that amplified the ubiquitous *SAM3* gene confirming the presence of gDNA; the WT *fks1* primers to amplify the non-disrupted *FKS1* gene; and the two primer pair combinations KanB and KanB1 to amplify different regions of the *FKS1*-disrupted gene

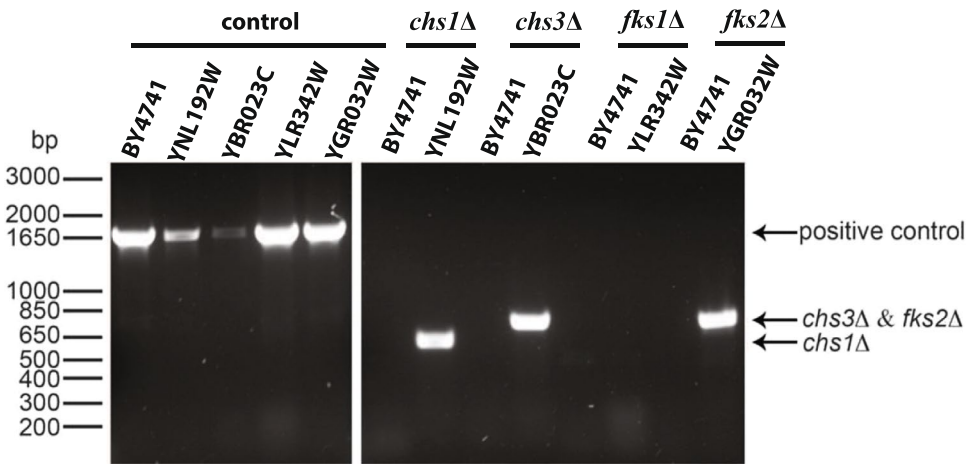

Fig. 1 Confirmation of the genotype of the *S. cerevisiae* cell wall synthase mutants. Genomic DNA from the cell wall synthase mutants, *chs1*Δ (YNL192W), *chs3*Δ (YBR023C), *fks1*Δ (YLR342W), and *fks2*Δ (YGR032W), was tested by PCR to confirm the appropriate gene knockout. Control primers for the ubiquitous gene *SAM3* were used to ensure the presence of gDNA (control). Wild-type (BY4741) and mutant gDNA was amplified with primers specific for each of the gene disruptions. Amplified DNA of the expected size was obtained for all the mutants (arrows) except for *fks1*Δ (YLR342W)

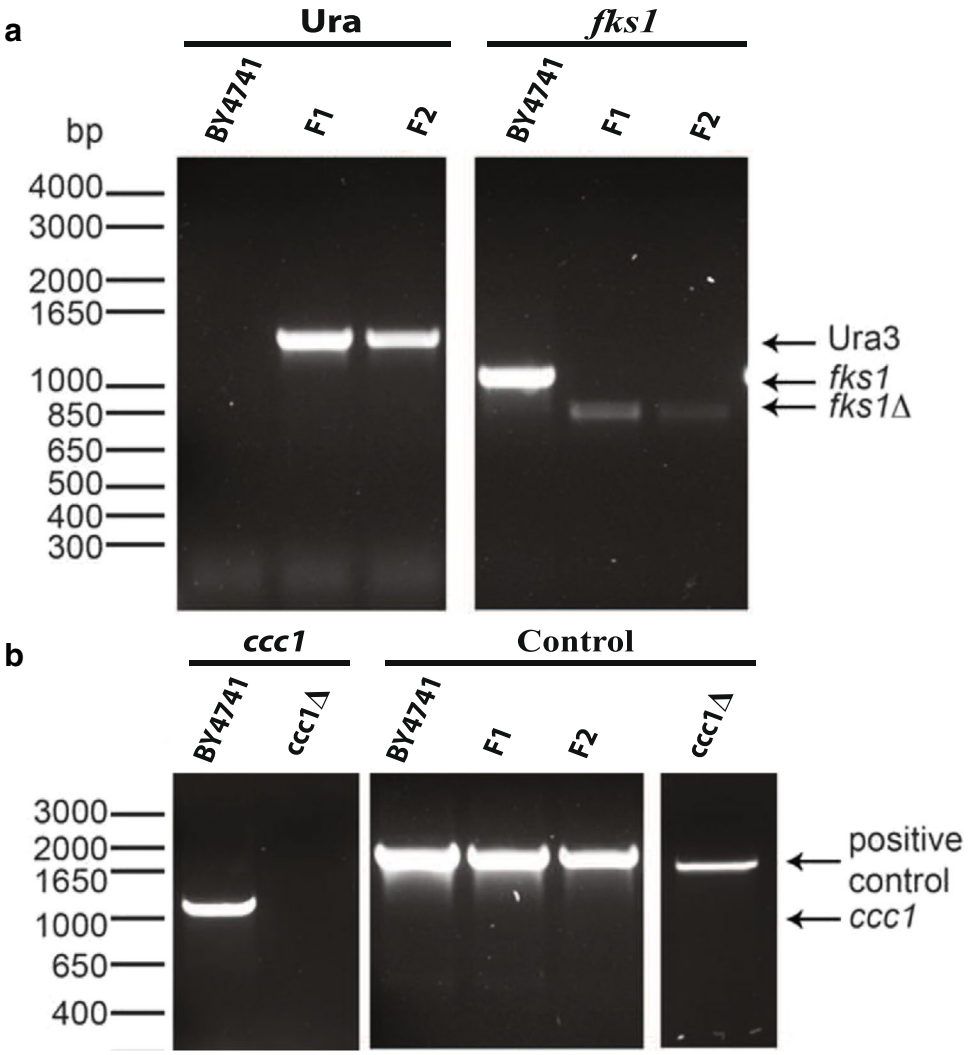

Fig. 3 Confirming the disruption of the *FKS1* gene in *S. cerevisiae*. Genomic DNA extracted from BY4741 (WT), and clones 1 and 2 (F1 and F2) of *fks1*Δ, was used as the template in a collection of PCRs. **a** The *URA3* primers (Ura) for amplification of the selectable marker *URA3* gene, and the *fks1* primers (*fks1*) for confirmation of disruption of *FKS1*. **b** The *ccc1* primers for confirmation of the disruption of the *CCC1* and control primer (control) for the amplification of the *SAM3* gene

Phenotypes of the chitin and β-glucan synthase mutants in *S. cerevisiae* by fluorescence microscopy

CFW and ABF were used to screen *S. cerevisiae* mutants to create a chitin and β-1,3-glucan profile (Figs. 4 and 5). In the *chs1*Δ mutant, chitin was localized to the septum by CFW staining and β-1,3-glucan to the entire cell wall by ABF staining respectively (Figs. 4b and 5b) similarly to that observed in the wild type (Figs. 4a and 5a).

In the *chs3*Δ mutants, chitin was detectable only at the septum (Fig. 4c and e) and no change was observed in ABF fluorescence in the *chs3*Δ mutant (Fig. 5c). There was a difference with CFW fluorescence observed in the *chs3*Δ mutant after a longer exposure time (466 ms) compared to the set time used for the wild type (120 ms). When exposed for a longer period, i.e., 466 ms, CFW staining did not only increase in fluorescence at the septum site (Fig. 4d) but also patches of fluorescence could be detected on or within *chs3*Δ mutant cells (Fig. 4d and f) and these were neither observed in the wild-type strain nor in the *chs1*Δ, *fks1*Δ, and *fks2*Δ mutants used in this study (Fig. 4a–g–i).

For β-glucan synthase in *S. cerevisiae fks1*Δ and *fks2*Δ mutants, PCR demonstrated that the *fks1*Δ mutant from the yeast knockout collection was not a deletion mutant for this gene due to the presence of the *fks*1 band (Figs. 1 and 2). Therefore, AB and CFW tests were done on the constructed *fks1Δ::URA3* strain (Fig. 3) and the *fks2*Δ mutant from the yeast knockout collection, respectively. In the new *fks1Δ::URA3* mutant, there was an increase in chitin in the mother yeast cells with CFW staining (Fig. 4i and j) and a strong AB fluorescence signal for β-1,3-glucan (Fig. 5f and g) in the emerging buds compared to the *ccc1Δ::URA3* as well as the WT (Figs. 4a, h and 5a, e). In the *fks2*Δ, there was a slight decrease in AB fluorescence (Fig. 5d) and no obvious change in CFW fluorescence (Fig. 4g).

Discussion

The patterns of calcofluor white and aniline blue staining observed confirmed the cell wall changes that have been reported in the literature for the deletion of these key cell

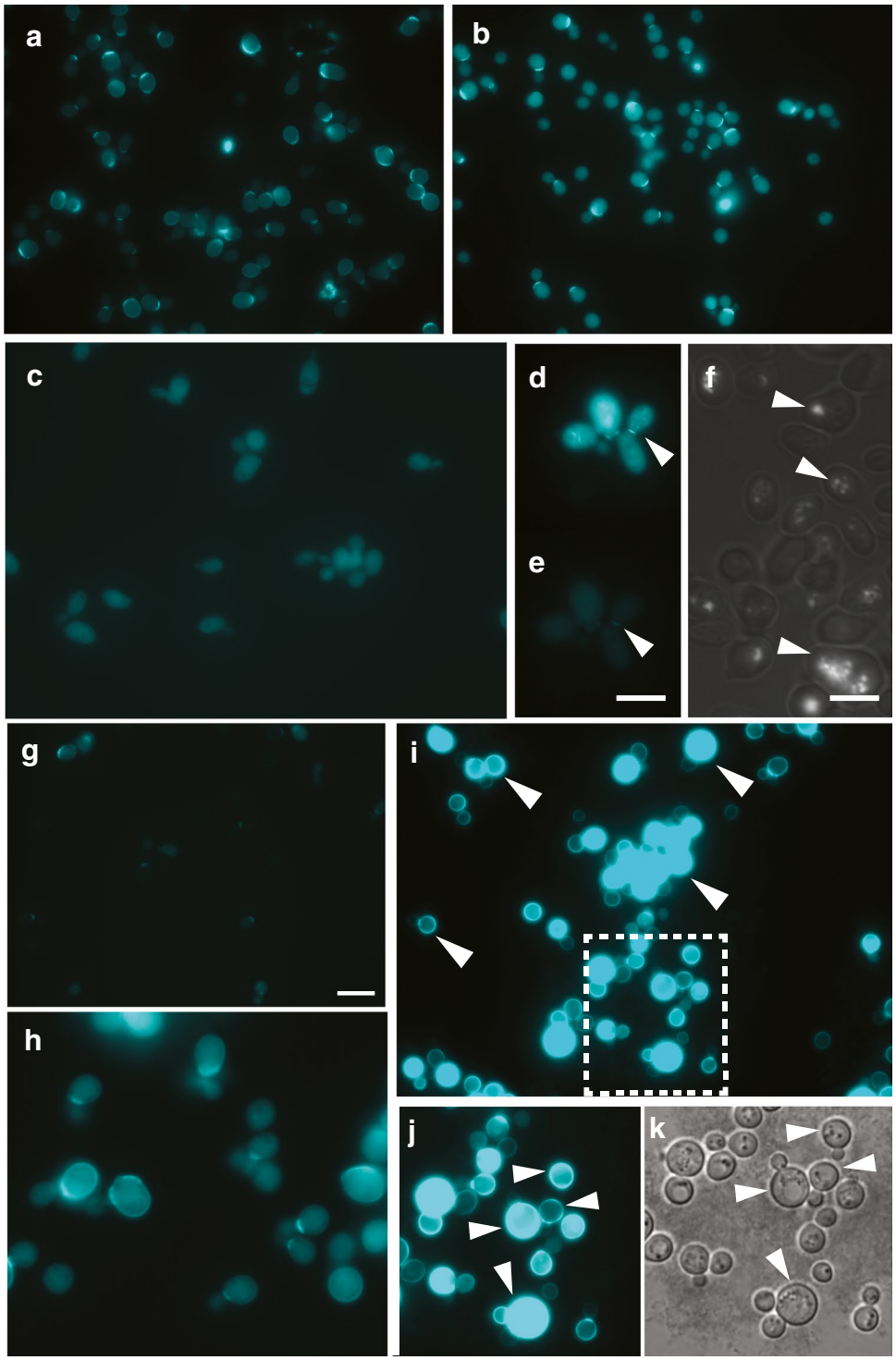

Fig. 4 Chitin wild-type *S. cerevisiae* and its mutants using calcofluor white (CFW). Cultures were grown overnight and samples were placed on microscope slides. CFW solution was used to detect chitin in wild-type yeast (BY4741) and various mutants. **a** BY4741 (WT). **b**YNL192W (*chs1Δ*). **c–f** YBR023C (*chs3Δ*). **g** YGR032W (*fsk2Δ*). **h** *ccc1Δ::URA3*. **i** *fks1Δ::URA3*. **j** close up of *fks1Δ::URA3* highlighted within the white dashed rectangle in (**i**) and (**k**) the same as (**j**) under light microscopy only. For micrographs (**a**), (**b**), (**c**), (**e**), and (**g**), exposure time was 120 ms and (**d**) and (**f**) was 466 ms with CFW. Light was used to highlight the cell structures and the location of fluorescence in (**f**). For *fks1Δ::URA3* mutant, exposure time was 40 ms for CFW (**i** and **j**) . Repeat experiment with the same exposure time in (**i**) displayed similar profiles with the wild-type and mutants observed in (**a**) to (**g**). In a separate experiment, the CFW exposure time was 74 ms (**h**) for WT (data not shown) and *ccc1Δ::URA3* mutant (**h**). Only one biological sample with three triplicate slides was done for this experiment. Micrographs (**a**) to (**g**) and (**i**) to (**k**) are representatives of at least two independent experiments where triplicate slides were done for each culture. Scale bars represent 10 μm

wall polysaccharide synthase genes. The *chs1*Δ chitin profile was a similar result to that previously described by Bulawa et al. [19]. This is unsurprising as Chs1p acts as a repair enzyme that adds chitin to the birth/bud scar at the end of cytokinesis [5], and therefore has little involvement in chitin deposition in vegetative growth. The growth environment plays a key role in the cell wall structure, if for example the *chs1*Δ had been cultured in more acidic medium, the lysed bud phenotype would have been more pronounced [5, 19, 20]. In addition, work by Lam et al. [21] demonstrated that *chs1* tagged with the green fluorescent protein (*chs1*-GFP) was localized around the emerging bud site.

In contrast, deletion of the main chitin synthase, Chs3p responsible for 90% of chitin synthesis resulted in chitin

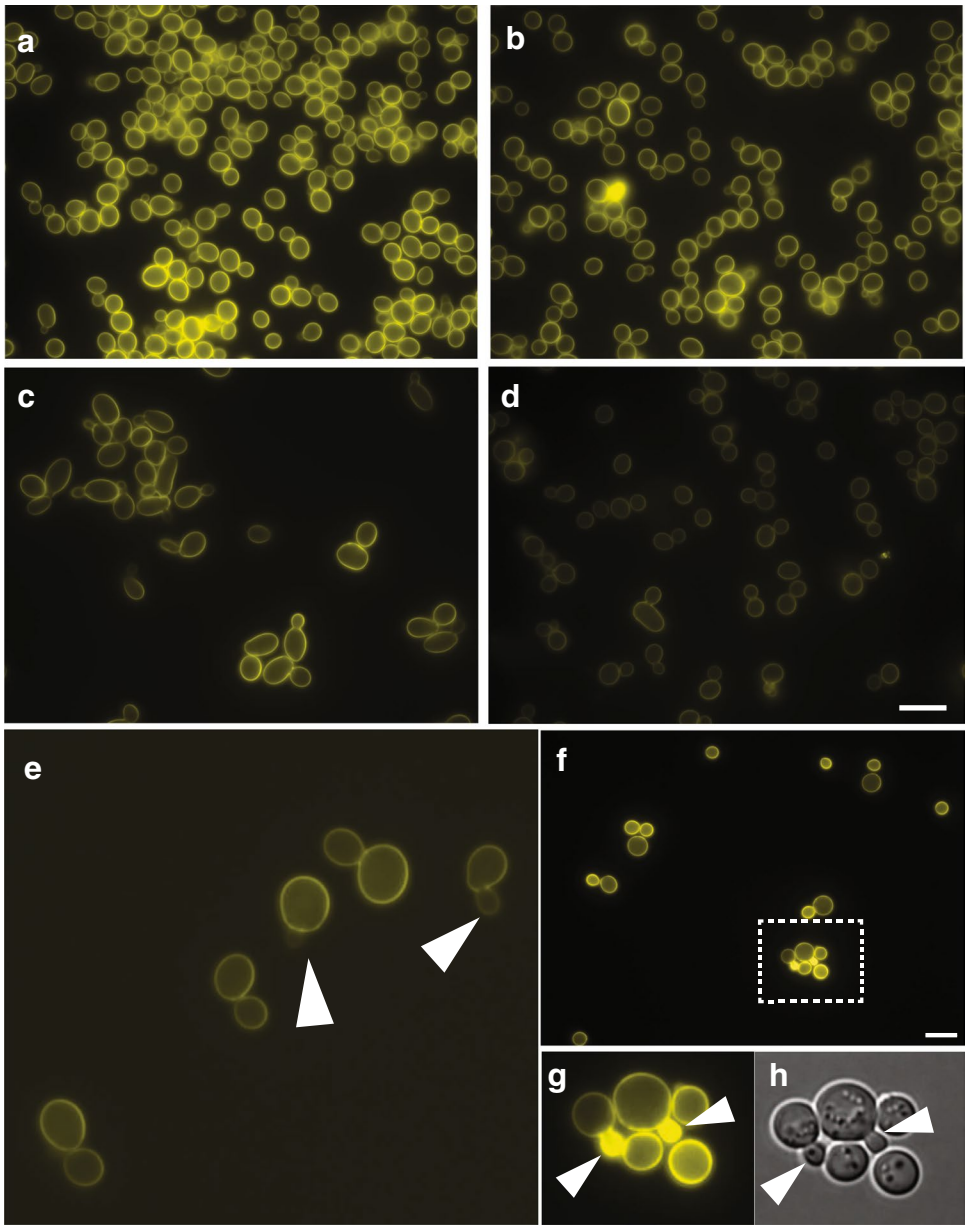

Fig. 5 β-1,3-Glucan profiles of wild-type *S. cerevisiae* and its mutants using aniline blue fluorochrome (AB). Cultures were grown overnight and samples were placed on microscope slides. AB solution was used to detect β-1,3-glucan in wild-type yeast (BY4741) and various mutants. **a** BY4741 (WT). **b** YNL192W (*chs1Δ*). **c** YBR023C (*chs3Δ*). **d** YGR032W (*fsk2Δ*). **e** *ccc1Δ::URA3*. **f** *fks1Δ::URA3*. **g** close-up of *fks1Δ::URA3* highlighted within white dashed rectangle in (**f**) and (**h**) the same as (**g**) under light microscopy only. For micrographs (**a**), (**b**), (**c**), and (**d**), exposure time was 166 ms with AB. For *fks1Δ::URA3* mutant, AB exposure time was 720 ms in (**f**) and (**g**). Repeat experiment with the same exposure time in (**f**) displayed similar profiles with the wild type and mutants observed in (**a**) to (**d**). In a separate experiment, the exposure time was 1000 ms in (**e**) for AB respectively for WT (data not shown) and *ccc1Δ::URA3* mutant. Only one biological sample with three triplicate slides was done for this experiment. Micrographs (**a**) to (**d**) and (**f**) to (**h**) are representatives of at least two independent experiments where triplicate slides were done for each culture. Scale bars represent 10 µm

being observed at the septum in addition to unique patches of staining across the wall at higher exposures. This is a characteristic of *chs3*Δ that has been described to lack chitin at the incipient bud site and in the lateral wall, with chitin detectable only at the septum [7]. In *chs3*Δ mutant cells, *chs1p* and *chs2p* are the only remaining functional chitin synthases [22]. The presence of chitin at the septum is due to the activity of Chs2p that would be synthesizing chitin in the central disk within the primary septum in budding yeast cells [6, 7] while Chs1p would be involved in synthesizing chitin for repair by replenishing chitin hydrolyzed by the excessive action of chitinase during cell separation/cytokinesis in the bud/birth scar [2, 5, 20] and no chitin has been detected in the birth scar of daughter cells [23]. Additionally, we observed enlarged and elongated *chs3*Δ cells. This abnormal cell morphology may be due to the previously observed *chs3*Δ forming septa of three layers with characteristics elongated neck regions between mother cell and bud [4, 7]. Molon et al. [24] demonstrated that *chs3*Δ mutants displayed abnormal bud scar morphology in the scanning electron microscope (SEM) micrographs. In addition, there was a decrease in the budding lifespan of *chs3*Δ mutant, as well as more than 50% of the *chs3*Δ population exploded during reproductive potential experiments with a decrease in final cell volume of the daughter cells compared to the WT [24]. The unusual CFW signal in the *chs3*Δ mutant used for this study may be related to the abnormal bud structures where *chs1p* may be recruited

at such abnormal budding sites to repair or to initiate budding within the *chs3*Δ mutant. It is still possible that *chs2p* could be playing a role at such sites. Work by Cabib et al. [25] proposed that when chitin is linked with β-1,3-glucan, the polysaccharide cannot be remodeled, thus regulating the localized growth at the *S. cerevisiae* mother-bud neck. This could explain the elongated neck structures in *chs3*Δ mutants due to the absence of a chitin ring to control the wall assembly via chitin/ β-1,3-glucan linkages at the septum site [25]. Interestingly, *chs1* is the only chitin synthase activity detectable in vitro from extracts of cells in stationary phase [26], but the biological significance of this finding still remains unclear. By compiling a genetic interaction network of genes synthetically interacting with CHS1, Lesage and colleagues [27] identified unique interactors in bud morphogenesis (*BEM4*, *BUD20*, *PEA2*) and in protein recycling through the endocytic pathway (*VPS5*, *VPS17*, *VPS29*, and *VPS35*) which modulated *chs2p* function. Future work may involve *chs1p* overexpression studies and the localization of *chs1p* tagged with fluorescent protein in such *S. cerevisiae* *chs3*Δ mutant backgrounds or a non-lethal *chs2* partial deletant *chs3*Δ mutant to determine *chs1p*'s modulating role.

The staining method also revealed the cell wall morphological and distribution changes that occur when β-1,3-glucan synthesis was disrupted. Two mutants were used, *fks2*Δ obtained commercially from the yeast knockout library along with *fks1Δ::URA3* mutant constructed in-house as the PCR screen revealed the commercially obtained mutant had the wild-type gene for *FKS1*. We expected little change in the cell walls of the *fks2*Δ cells, as Fks2p has only a minor role in β-1,3-glucan synthesis during vegetative growth and is instead activated under starvation, stress, and stationary phase and in the absence of Fks1p function. Hence, *fks1 fks2* double mutant is nonviable [28]. We did observe a slight decrease in aniline blue staining indicating a decrease in β-1,3-glucan levels. However, a *FKS2* causes no obvious cell wall defect [28]. Deletion of *fks1p* leads to a decrease in the level of β-1,3-glucan and an increase in the chitin and mannoprotein levels in the cell wall [26, 28]. This is also observed in the inhibition of Fks1p through the action of the antifungal echinocandins like caspofungin. Here we observed this increase in chitin through the increase in calcofluor white staining of the *fks1*Δ. Interestingly, one of the 1,3-β-D-glucan synthase inhibitors, echinocandins, can induce cell wall salvage mechanisms that result in the compensatory upregulation of chitin synthesis in *Candida albicans* [29–31]. In addition, Imtiaz et al. [32] demonstrated that echinocandin resistance caused by simultaneous FKS mutation increased cell wall chitin in a *C. albicans* bloodstream isolate following brief exposure to caspofungin.

Work by Dijkgraaf and colleagues [33] proposed that the deletion of *FKS1* produced an increase in chitin synthesis which was due to an activation of the Chs3p activity mediated by an unusual localization of the active complex. Previous studies have demonstrated that FSK2 increased in activity in *fks1Δ* mutants, under different growth conditions and in response to cell wall defects [14, 33, 34]. Lesage et al. [28] and Lagorce et al. [35] demonstrated that there is a complex interaction where *FKS1* and its mutation can affect not just Chs3p expression but also Fsk2p. Dague et al. [36] demonstrated that there was a change in chitin level in *fks1*Δ and *chs3*Δ mutants. Dijkgraaf et al. [33] demonstrated that GFP-Fks1p and GFP-Fks2p localized at the sites of polarized growth in wild type, but under conditions where Fsk1p was depolarized, Fks2p localized to polarized growth sites and significant Fks2p levels were found in the growing buds of β-1,6-glucan-deficient mutants [33]. Therefore, the strong AB fluorescence signal in the growing buds of *fks1Δ::URA3* mutants could be due to the *fks2* expression at such sites. Future study using such GFP-tagged constructs in double *chs3*Δ *fks1*Δ mutants or partial deletant of *fks1*Δ mutants could confirm these observations and monitoring the *FKS1* and CHS1/2/3 expression and localization in the *fks2*Δ mutant could help in understanding the CFW and AB profile.

The sub-cellular localization of chitin and β-1,3-glucan were evaluated by staining the yeast with calcofluor white and aniline blue respectively and observation by microscopy. This is a useful approach for an initial screening of mutants defective in cell wall synthesis, highlighting subcellular localization and morphology changes. The screening method can aide in understanding the importance of the components in the formation of a functional cell wall through mutational analyses. In addition, the imaging technique could be used to dynamically monitor yeast cell wall morphological changes in the presence of cell wall stressing agents such as antifungal drugs. Other dyes combined with confocal microscopy or flow cytometry could be useful in quantitative analysis of such mutants.

In conclusion, it is recommended that PCR should be used to verify genotype, and in combination with fluorescent dyes such as AB and CFW, to provide a cheap, quick, and easy way to determine gross morphological changes in the *S. cerevisiae* cell wall.

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Author contribution FP-W and JP conceived and designed the research. FP-W and JP conducted the experiments. JP designed the *fks1Δ::URA3* mutant and contributed to the PCR/molecular analyses,

and FP-W contributed to the microscopy and to image analyses in this study. FP-W wrote the first draft manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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