Deletion of YLR358C Contributes to Reduce Cell Wall Integrity in *Saccharomyces Cerevisiae*

Yu Zhang

Kunming Medical University

Mengyan Li

Kunming Medical University

Hanying Wang

Kunming Medical University

Juqing Deng

Kunming Medical University

Jianxing Liu

Kunming Medical University

Li Zhang

Kunming Medical University

Lechun Lyu (✉ minimillet@hotmail.com)

Kunming Medical University <https://orcid.org/0000-0003-0095-4050>

Short Report

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Abstract

The mechanism of fungal cell wall synthesis and assembly is still unclear. *Saccharomyces cerevisiae* (*S. cerevisiae*) and pathogenic fungi are conserved in cell wall construction and response to stress signals, and often respond to cell wall stress through activated cell wall integrity (CWI) pathways. Whether the YLR358C open reading frame regulates CWI remains unclear. This study found that the growth of *S. cerevisiae* with YLR358C knockout was significantly inhibited on the medium containing different concentrations of cell wall interfering agents Calcofluor White (CFW), Congo Red (CR) and sodium dodecyl sulfate (SDS). CFW staining showed that the cell wall chitin was down-regulated, and transmission electron microscopy also observed a decrease in cell wall thickness. Transcriptome sequencing and analysis showed that YLR358C gene may be involved in the regulation of CWI signaling pathway. It was found by qRT-PCR that WSC3, SWI4 and HSP12 were differentially expressed after YLR358C was knocked out. The above results suggest that YLR358C may regulate the integrity of the yeast cell walls and has some potential for application in fermentation.

Background

Saccharomyces cerevisiae (*S. cerevisiae*) is a single-celled fungus, as one of the commonly used strains in the fermentation industry[1, 2]. Yeast cells are facing the threat of rapid and extreme environmental changes. When the extracellular osmotic pressure is rapidly reduced, cells have to survive by limiting the influx of external fluid to avoid cell swelling and breakage to stabilize the intracellular environment[3]. Yeasts and other fungi solve this issue by using strong and relatively rigid cell walls to limit swelling[4, 5]. The application and development of fungi in industry is urgent to clarify the mechanism of synthesis and assembly of fungal cell walls. In addition, *S. cerevisiae* and pathogenic fungi are conserved in cell wall construction and stress signals. Different fungi, including various *Candida*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, are responsible for systemic infections in patients with immune disorders[6, 7]. *S. cerevisiae* is considered to be an ideal model for studying the biological reactions of fungal cell walls.

The cell wall is the cell structure required to maintain the shape of the cell and protect the internal environment. Its main components are β -1,3-glucan, β -1,6-glucan, chitin and mannoprotein polymers[8]. The polymers are covalently connected to form a macromolecular complex arranged into an inner layer and an outer layer. *S. cerevisiae* must continue to reshape the cell wall as the cell progresses in its life cycle and mitotic cycle stage and faces various environmental stressors. Cell wall remodeling stimulates response to cell wall stress through the activated cell wall integrity (CWI) pathway[6, 9].

When *S. cerevisiae* grows, develops or is disturbed by the external environment, the integrity of the cell wall is destroyed. At this time, the cell wall is remodeled in a highly regulated and polarized manner, and this process is mainly regulated by the CWI signaling pathway. The cell surface sensors of the CWI pathway (Wsc1p, Wsc2p, Wsc3p, Mid2p, and Mtl1p) detect cell wall damage and transmit cell wall stress signals to activate the guanine-nucleotide exchange factor (GEF) Rom2p, leading to the activation of the

small GTP enzyme Rho1p[10-12]. Activating Rho1p stimulates protein kinase C (Pkc1p) to trigger the MAPK cascade, which in turn causes MAPK to activate the transcription factor SBF (Swi4p-Swi6p) complex and MBF (Swi6p-Mbp1p) complex to induce the expression of specific cell wall genes[13-15].

YLR358C is an unknown open reading frame (ORF) of *S. cerevisiae*. Previous studies have found that YLR358C deletion strains are sensitive to cell wall interfering agents, suggesting that YLR358C may be involved in the regulation of cell wall integrity[16]. However, the role and mechanism of YLR358C in cell wall stress response are still unclear. In this study, we observed the effect of YLR358C deletion in *S. cerevisiae* on cell proliferation and cell morphological changes. Transcriptome analysis and PCR assay further clarified the possible mechanism of YLR358C gene in the CWI pathway.

Materials And Methods

Reagent

Yeast genomic DNA extraction kit, total RNA extraction reagent Trizol, Taq PCR premix and YPD medium components peptone, yeast extract, glucose, and agar were purchased from Sangon Biotech Co. Ltd. (Shanghai, China); cell wall interfering agent Calcofluor White (CFW), Congo Red (CR) and sodium dodecyl sulfate (SDS) were obtained from EN Chemical Technology Co. Ltd. (Shanghai, China), BioFroxx and Sangon Biotech Co. Ltd; FastKing one-step reverse transcription-fluorescence quantitative kit (SYBR Green) was purchased from Tiangen Biochemical Technology Co., Ltd(Beijing , China).

Strains and culture conditions

The wild-type *S. cerevisiae* (WT) has stored in our laboratory; the knock-out yeast library was purchased from Horizon Discovery Co., Ltd[17]. WT yeast can be stably propagated in YPD solid and liquid medium, but for genetic stability, knock-out yeast needs to add 200ug/ml G418 to YPD medium to stably propagate.

Verification of the YLR358C knockout strain

The YLR358C knockout yeast was cultured overnight at 10000rpm/min and centrifuged for 1min to collect the yeast, and the genomic DNA was extracted using the above kit. The obtained genome was used as a template, and A, KanB, KanC and D were used as primers (supplemental Table 1) to verify the YLR358C knockout yeast by PCR, and the successful knockout yeast were named *YLR358C*Δ.

Spot assay

The single clones of WT and *YLR358C*Δ yeasts were picked and inoculated into 10ml YPD liquid medium and YPD liquid medium containing 200μg/mL G418, respectively, and cultured overnight in a constant temperature shaker. After centrifugation of the yeast solution, resuspend the cells in fresh YPD medium to OD600=1.0. Then respectively dilute to 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, take 2.5 μL of diluent was spotted on

YPD plates containing different concentrations of CFW, SDS, and CR, and incubated in a constant temperature incubator at 28°C for 2 days to observe the growth.

CFW staining

The WT and *YLR358C*Δ yeast single clones were cultured to the logarithmic phase on a constant temperature shaker at 28°C and 200 rpm/min. After centrifugation, the cells were resuspended in 10 ml YPD medium containing 30 ug/ml CFW. After the yeast is stained with CFW, the cells are resuspended in an appropriate amount of PBS and transferred to the well plate, and the fluorescence is observed under an inverted fluorescence microscope after resting for 10-15 minutes.

Observation of microscopic morphology

The WT and *YLR358C* knock-out yeasts were cultured overnight at 28°C and 200 rpm/min constant temperature shaker. After the yeast is centrifuged, it is fixed with 1-2ml 3.5% glutaraldehyde, then fixed with 1% osmium acid, ethanol-acetone stepwise gradient dehydration, Epon-618 infiltration, embedding, semi-thin sectioning using Leica-R ultramicrotome (Germany), positioning by light microscope, double staining with lead citrate and uranyl acetate, observation by transmission electron microscope (Gatan Inc, USA).

Transcriptome sequencing and analysis

After centrifuging the yeast, the total RNA is extracted, the mRNA is used as a template to synthesize and purify cDNA, and finally the final cDNA library is obtained by PCR enrichment. The cDNA library is sequenced and the data is filtered to obtain the Clean Data, which is compared with the designated reference genome, and the Mapped Data obtained is subjected to the library quality evaluation such as the length check of the insert and the randomness check. Structural analysis of alternative splicing analysis, discovery of new genes and optimization of gene structure are carried out. According to the expression level of genes in different samples or different sample groups, differential expression analysis, functional annotation and functional enrichment of differentially expressed genes and other expression levels are analyzed.

Extraction of Yeast Total RNA and Real-time Quantitative PCR

After the yeast was collected by centrifugation, total RNA was extracted with Trizol, and real-time quantitative PCR was performed according to the instructions of the FastKing One-Step Reverse Transcription-Fluorescence Quantitative Kit. The primers used are as shown in the supplemental Table 2, TAF10 was used as the internal reference primer, and the results were quantified by the $2^{-\Delta\Delta CT}$ method.

Results

Knockout *YLR358C* interferes with cell wall of *S. cerevisiae*

To clarify that YLR358C ORF was knocked out in the *YLR358CΔ S. cerevisiae*, we used PCR to verify. Yeast Genomic DNA Rapid Extraction Kit was used to extract the yeast DNA, PCR amplification was carried out with primers A, KanB, KanC and D, and then the amplified products were verified by agarose gel electrophoresis, where A-KanB length is 565bp, and the length of KanC-D is 868bp (supplemental Figure 1). The agarose gel results are consistent with the above results, indicating that YLR358C was successfully knocked out.

To explore whether YLR358C is involved in the regulation of cell wall integrity of *S. cerevisiae*, we treated *YMR253CΔ* and WT yeast with different concentrations of cell wall interfering agents to detect the cell growth status of the two yeast strains. The results showed that there was almost no difference between the growth of yeast *YLR358CΔ* and WT yeast on YPD medium. On the solid medium containing 10ug/ml CFW, 20ug/ml CFW, and 30ug/ml CFW, the growth of *YLR358CΔ* and WT yeast was inhibited, but the growth of *YLR358CΔ* was slower (Figure 1A). The above consistent results were also observed on solid media containing 30ug/ml CR, 45ug/ml CR, 60ug/ml CR and 0.015%, 0.03%, and 0.06% SDS (Figure 1B, C). In summary, the deletion of YLR358C gene increases its sensitivity to cell wall interfering agents, indicating that YLR358C may be involved in the regulation of cell wall integrity.

Abnormal distribution of chitin in the cell wall of YLR358CΔ yeast

Chitin is a homopolymer of N-acetyl-β-D-glucosamine. Chitin in vegetative yeast cells is mainly deposited in the bud neck and bud scar. When there are defects in the cell wall, chitin synthase will synthesize additional chitin on the outside of the cell wall to make up for the defects of the damaged cell wall and make it as complete as possible. Cell wall mutants with elevated levels of chitin usually show sensitivity to CFW, so we stained yeast cells with CFW that specifically binds to chitin to observe the chitin difference between *YLR358CΔ* and WT yeast. In YPD medium, the chitin of WT yeast is mainly in the bud neck and bud scar, while in *YLR358CΔ* yeast, the chitin is distributed around the entire side wall in addition to the bud scar and bud neck. After treatment with cell wall interference agents CFW and SDS, it was found that compared with WT yeast, the chitin of *YLR358CΔ* knockout yeast accumulated more around the cell wall (Figure 2A).. Therefore, knocking out the YLR358C gene may participate in the regulation of cell wall stress by affecting the synthesis of chitin in the cell wall.

To observe the changes of *YLR358CΔ* cell wall intuitively, transmission electron microscopy observations of WT strain and *YLR358CΔ* strain were carried out in this study. The cell wall of yeast under the transmission electron microscope has a double-layer structure. The outer layer is the part with higher electron density, mainly composed of mannan and mannoprotein; the inner layer has lower electron density but larger thickness, mainly composed of β-1,3-glucan and chitin structure. Compared with WT yeast, the cell wall of *YLR358CΔ* yeast has more changes, and the inner structure of the cell wall of *YLR358CΔ* yeast mutant with lower electron density becomes thinner (Figure 2B). We speculate that the knockout of YLR358C gene firstly affected the synthesis of chitin, resulting in an increase in chitin content. As a compensation mechanism, to maintain the integrity of the cell wall, the amount of β-1, 3-

glucan synthesized by yeast cells was reduced. Finally, the *YLR358C* Δ mutant is sensitive to cell wall interfering agents.

YLR358C regulates multiple enrichment pathways

Transcriptome sequencing and analysis further explored the molecular mechanism of YLR358C regulating the biological effects of *S. cerevisiae*. First, the correlation coefficients of samples within and between groups are calculated based on the FPKM values of all genes in each sample, and the differences in samples between groups and the repetition of samples within groups are displayed. In this study, the minimum value of the correlation coefficient R^2 between the samples within the group was 0.947, and the maximum value of the correlation coefficient R^2 between the samples between the groups was 0.848 (Figure 3A). PCA analysis showed that the samples in the two groups of WT and *YLR358C* Δ were clustered and scattered among the groups (supplemental Figure 2A). Therefore, the sample selection in this study is reasonable and the result is reliable.

The genetic difference between *YLR358C* Δ and WT yeast was analyzed using DESeq2. In the analysis, $P \leq 0.05$, and the multiple of change ≥ 2.0 times were set as the threshold of significant differential expression. A total of 1659 differentially expressed genes were screened, of which 931 were down-regulated, accounting for 56.12% of the total differentially expressed genes, and 728 were up-regulated, accounting for 43.88% (Figure 3B, C).

GO enrichment analysis was performed on the above 1659 differentially expressed genes to clarify their biological processes, cellular components and molecular functions. Figure 3C shows the GO enrichment classification results of differential genes after YLR358C is knocked out in *S. cerevisiae*. Among them, biological processes are mainly annotated as DNA integration, DNA metabolism processes and carbohydrate metabolism processes; cell composition is mainly annotated as cytoplasm, ribosomes and non-membrane organelles; molecular functions focus on RNA binding, UDP-glycosyltransferase activity and microtubules protein binding (supplemental Figure 2B,C).

Differentially expressed genes may involve the most important biochemical metabolic pathways and signal transduction pathways. The bubble chart of the differential gene of *S. cerevisiae* after YLR358C is knocked out shows the result of KEGG enrichment analysis. The results show that ribosomes, carbon metabolism and TCA cycle pathways may be significantly enriched; secondly, they are also enriched in starch and sucrose metabolism, glyoxylic acid and dicarboxylic acid metabolism, glycolysis and gluconeogenesis pathways (Figure 3D).

Cell wall integrity pathway may be regulated by YLR358C

In the results of transcriptome analysis, we found that several key factors in the CWI signaling pathway were abnormally expressed, including WSC3, SWI4 and HSP12. In this study, the qRT-PCR assay was used to detect the effect of YLR358C on the above-mentioned mRNA expression at the transcription level. The results showed that the expression level of SWI4 mRNA in *YLR358C* Δ yeast was up-regulated, and the

increase in WSC3 was more significant. Compared with WT yeast, the expression of HSP12 was significantly decreased (Figure 4).

Discussion

S. cerevisiae is a single-celled eukaryotic organism wrapped in a multi-layered cell wall. When yeast cells face various environmental pressures, the cell wall must be constantly remodeled as the cell progresses during its life cycle and mitotic cell cycle stages[18]. This is achieved by the synergistic effects of glycoside hydrolase, glycosyltransferase and transglycosylase, as well as the incorporation or shedding of cell wall mannoprotein to modify the polysaccharide network[19, 20]. Yeast cell wall polysaccharides have been used as thickeners, emulsifiers, preservatives and anticoagulants in food additives to improve the flavor and stability of food[21–23]. In addition, yeast mannoprotein has several advantages in improving wine quality, it can prevent turbidity, reduce astringency, retain aroma components, and stimulate the growth of lactic acid bacteria[24]. Therefore, mastering the role of genes related to the cell wall of *S. cerevisiae* can modify the characteristics of yeast and optimize the fermentation process, which is of great significance to the food, medicine and fermentation industries[25]. This study found that the growth of *S. cerevisiae* with YLR358C gene deletion was inhibited on the medium containing cell wall interfering agents. The YLR358C gene knock-out strain was sensitive to cell wall interfering agents. CFW staining showed increased cell wall chitin synthesis and excessive accumulation on the side wall of the cell. Transcriptome sequencing and bioinformatics analysis indicated that YLR358C gene may be involved in the regulation of *S. cerevisiae* CWI signaling pathway.

YLR358C ORF is a gene with unknown function. It has been previously reported that YLR358C may be one of the genes affecting the rupture of the vacuolar membrane of *S. cerevisiae*, and it has also been found that the *YLR358CΔ* strain is sensitive to the cell wall interfering agent CFW[16]. In this study, after knocking out YLR358C, we found that yeast growth was inhibited in media containing different concentrations of cell wall interfering agents, especially under the stress of high concentrations of cell wall interfering agents, *YLR358CΔ* yeast cell wall chitin synthesis increased, it accumulates excessively around the sidewall of the cell. To maintain the stability of the cell wall, this may lead to a decrease in the synthesis of β -1, 3-glucan combined with it, and β -1, 3-glucan is the main component of the inner layer of the cell wall. This leads to the thinning of the chitin and glucan layers inside the cell walls.

In fact, there are many regulatory strategies to maintain cell wall integrity, such as transcriptional control, chromatin-based promoter structure control, mRNA stability and localization control, and proteolytic processes. In *S. cerevisiae*, the CWI signaling pathway is activated by mechanosensor proteins, such as Wsc1p/Slg1p and Mid2p, which sense damage in CWI signals through the sensor protein Rho1p[10, 26]. It culminates when protein kinase 1 (PKC) is activated, and PKC triggers a cascade of phosphorylation through MAP kinase. The last MAP kinase in this cascade is the Slt2p protein. Once phosphorylated, it is activated by phosphorylated transcription factors such as Rlm1p and Swi4/6p, which regulate the expression of CWI genes[27, 28]. This leads to a series of cellular responses that initiate cell wall repair and lead to remodeling. In this study, transcriptome analysis found that WSC3, SWI4 and HSP12 genes in

the CWI signaling pathway were abnormally expressed in *YLR358CΔ* yeast. PCR results showed that WSC3 and SWI4 were up-regulated in *YLR358CΔ* yeast, while HSP12 was down-regulated in *YLR358CΔ* yeast, which was consistent with the transcriptome results.

This study clarified that YLR358C gene deletion destroys the CWI of *S. cerevisiae*, but the specific mechanism is still unclear. First of all, this study did not clarify the details of YLR358C in the CWI signaling pathway. Whether YLR358C regulates CWI of *S. cerevisiae* through the CWI signaling pathway needs further molecular biology verification. Secondly, rescue assays need to confirm that YLR358C is involved in the regulation of CWI signaling pathway. Finally, other genes in the CWI signaling pathway that were found to be significantly down-regulated by the transcriptome should also be further verified by more RT-qPCR. These need to be further resolved by subsequent results.

In summary, this study found that YLR358C is involved in the regulation of cell wall integrity, and its effect may be mediated by regulating the CWI signaling pathway. In the future, it is necessary to conduct more in-depth functional and mechanism research on YLR358C, and hope to lay the foundation for the subsequent development of food, medicine and fermentation industries.

Declarations

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Authors Contribution

The authors declare that all data were generated in-house and that no paper mill was used.

Contributions

LM, LZ and JL conceived and designed the research. YZ and ML conducted the experiments and analyzed the data. JD and HW analyzed the data.

Ethics declarations

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

The YLR358C knockout increases the sensitivity of *S. cerevisiae* to cell wall interference reagents.

A 10-fold dilution of yeast was used for spotting assays on YPD plates with cell wall interference reagents (A. CFW, B. CR, C. SDS). The results showed that after YLR358C was knocked out, the growth of *S. cerevisiae* was significantly inhibited.

Figure 2

The chitin in the yeast cell wall is regulated by YLR358C.

(A) CFW was used to stain cell wall chitin of yeast. The chitin of WT yeast is distributed in the bud neck and the bud scar, and the *YLR358CΔ* mutant yeast is abnormally distributed; however, after treatment with cell wall interference agents at that time, due to the compensation mechanism, the chitin of the *YLR358CΔ* yeast was up-regulated;

(B) The results of transmission electron microscopy showed that the cell wall of the *YLR358CΔ* mutant yeast is thinner than that of the WT strain.

Figure 3

YLR358C knockout and transcriptome analysis

(A) Sample correlation analysis between YLR358C knockout and WT yeast;

(B) Volcano map of differentially expressed genes based on *YLR358CΔ* and WT yeast transcriptome analysis,

(C) Heat map of differentially expressed genes. Among them, 728 genes were up-regulated and 931 genes were down-regulated. A heat map of differentially expressed genes, where red indicates up-

regulated genes and green indicates down-regulated genes.

(D) KEGG analysis of differentially expressed genes between *YLR358CΔ* and WT yeast.

Figure 4

YLR358C regulates the expression levels of WSC3, SWI4, and HSP12 in the cell wall stress response pathway.

YLR358C knockdown and WT yeast were treated with YPD medium, with TAF10 as an internal control, for PCR of WSC3, SWI4, and HSP12, respectively. **, P<0.01; *, P<0.05.

Supplementary Files

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