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Interaction of host factors in response to yeast K2 toxin stress – attractiveness for plant protection

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Abstract

Killer toxin-producing yeasts are important in agriculture, as they may be used for the biological control of field and post-harvest bacterial and fungal diseases of plants. The foundation for the development and application of killer yeast as plant protection agents is understanding the mechanisms underlying killer toxin-conditioned biocontrol activity and the tolerance to toxin-driven stress. This study aimed to determine the interactions between genetic effectors of cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways under the stress conditions caused by *Saccharomyces cerevisiae* dsRNA-originated K2 toxin. Genetic interaction studies have used different measures of fitness including the relative growth rate and toxin susceptibility. During the research, 12 double deletion mutants were created by applying homologous recombination approach, and their growth parameters and response to toxin action were analysed. Most double gene deletion strains demonstrated insignificant (less than 30%) changes in growth rate compared to single gene mutants. Only elimination of *HOG1* gene in strains Δ*spt3* and Δ*spt8* resulted in significant increase of the growth rate reaching about 0.6 h^{-1}. K2 toxin sensitivity increased in most of the double mutants, independent of the involvement of gene products into the same or different signalling pathways thus pointing to weak interactions of gene products. *HOG1* mutation alters the phenotype (from resistant to sensitive) of mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* only and are epistatic to these CWI pathway effectors. In addition to the functional analysis, network of proteins involved in K2 toxin response was generated uncovering HOG and CWI players interconnected or acting through mediators.
This study deepens insight into the K2 toxin response-modulated genetic interactions and provides data important for practical application of killer yeasts.

Key words: yeast, toxin-driven stress, connections of signalling pathways, biocontrol mechanisms.

Introduction

Direct yield losses caused by plant pathogens significantly reduce global agricultural productivity. Therefore, the protection of crops against plant diseases caused by microbial pathogens is of primary importance (Pawlikowska et al., 2019). Chemicals are typically applied to prevent crop infections; however, nowadays such consensus has changed. It is widely recognized that the use of chemicals increases resistance of pathogens, negatively affects human health and causes environmental pollution (Abbey et al., 2019). Thus, biocontrol yeasts, producing volatile organic compounds, enzymes or antagonistic features possessing killer toxins, have been explored as a promising alternative to chemical fungicides (Contarino et al., 2019).

The widespread killer phenomenon in yeast is based on the secretion of killer toxins, lethal to the wide spectrum of fungi and bacteria (Belda et al., 2017). Killer toxins have been mainly studied with respect to the spoilage control in the food industry and treatment of clinical infections (Freimoser et al., 2019). Several toxins were shown to inhibit phytopathogens and proposed for plant protection (Perez et al., 2016). Nevertheless, further investigations are needed to evaluate toxin specificity and efficiency, to assess effects on beneficial microorganisms and mechanisms underlying the tolerance to toxin-driven stress.

Most extensively killer phenomenon has been studied in budding yeasts *Saccharomyces cerevisiae*, where dsRNR virus-originated killer toxins K1, K2, K28 and Klus have been described (Schmitt, Breinig, 2006; Rodríguez-Cousiño et al., 2011). Despite some similarities in their production, killer toxins have different primary sequences, biochemical properties and modes of action. *S. cerevisiae* K1 and K2 toxins act by disrupting

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the structural and/or functional integrity of the plasma membrane leading to the death of sensitive yeast strains (Breinig et al., 2002; Schmitt, Breinig, 2002; Lukša et al., 2015; Orentaite et al., 2016). K28 toxin kills the host cells by irreversibly blocking DNA synthesis and by triggering G1/S arrest and apoptosis (Eisfeld et al., 2000). The mode of the action of Klus mycotoxin has not been established yet (Rodríguez-Cousiño et al., 2011).

Many cellular factors play important roles in modulation of the response to toxins. By performing genome-wide screen, hundreds of such effectors, both unique and common for the most of *S. cerevisiae* produced toxins, were identified (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012). The susceptibility of *S. cerevisiae* yeast to K1 toxin was shown to be conferred by 268 gene products mainly related to the synthesis of cell wall components, secretion pathway, lipid and sterol biosynthesis and cell surface signal transduction (Pagé et al., 2003). To be implicated in K2 toxin susceptibility, 332 gene products were demonstrated. Genes involved in resistance were connected to cell wall and membrane structure and/or biogenesis, mitochondrial function, while genes involved in hypersensitivity encoded products active in osmosensory and cell wall stress signalling, ion transport and maintenance of homeostasis (Servienė et al., 2012). Genes, whose deletion caused hypersensitivity to K28 toxin, were related to stress-activated signalling and protein degradation, whereas resistant mutants were clustered to endocytic, lipid organization and cell wall biogenesis pathways (Carroll et al., 2009).

Multiple *S. cerevisiae* genome-wide screens revealed that numerous genetic factors related to high osmolarity glycerol (HOG) and cell wall integrity (CWI) stress response pathways are involved in susceptibility to all three killer toxins. HOG signalling pathway is modulated by Hog1 mitogen-activated protein kinase (MAPK) acting through cascades of MAPK and regulator proteins and inducing cytoplasmic and nuclear responses (Rodríguez-Peña et al., 2010; Saito, Posas, 2012). It was observed that inactivation either of Hog1 or its main partners Pbs2, Ssk1 and Ssk2 increased the sensitivity to K1, K2 and K28 toxins (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012). Hog1 kinase is tightly associated with the transcription machinery via numerous stress-mediating transcriptional activators or repressors; therefore, interruption of their functionality affects cellular response to the action of killer toxins.

Single mutants of RNA polymerase II transcription factor Δ*iwr1* or a subunit of polymerase mediator complex Δ*srb5* conditioned hypersensitivity to all three yeast killer toxins. Meanwhile, deletion of either transcription factors Δ*soh1* or Δ*sfp1* leads to the increased sensitivity to K2 and K28 toxins. Absence of Hog1 negative regulator Nbp2, recruiting the phosphatase Ptc1 to the Pbs2/Hog1 complex, caused resistance of the yeast cells to K1 and K2 toxins. The genome-wide screen of yeast knock-out mutants revealed numerous transcription regulators such as Med1, Snf2, Spt3, Spt7, Spt8, etc. to be important for the cellular response to K2 toxin action only (Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012).

The CWI pathway, responding to cell wall stress conditions, transmits signal from cell surface sensors to the Rho1 GTPase, which mobilizes a physiological response through MAPK cascade and a variety of effectors leading to substantial remodelling of the cell wall (Levin, 2011). Through the transcriptional reprogramming, yeasts modulate the expression of genes important for the cell wall biogenesis, metabolism, energy generation, signal transduction and stress (Sanz et al., 2017). It was demonstrated that inactivation of GTPase activating proteins Lrg1 and Bem2 resulted in the altered susceptibility to K2 toxin. Deletion of *BEM2* gene caused increased sensitivity to K2 toxin possibly because of the defects in the cytoskeleton organization involving the formin Bni1. Elimination of Bni1 and its activating protein kinase Fus3 also increased the sensitivity to K2 toxin. The inactivation of Rho1 effector Lrg1 led to the increased K2 toxin resistance, probably due to the decreased 1,3-beta-D-glucan synthase Fks1 activity and subsequent alterations in the cell wall structure. The elimination of transcription factor Rlm1, responding to Rho1 signals, resulted in the increased K2 toxin resistance (Servienė et al., 2012).

The HOG pathway is mainly involved in the adaptation of yeast cells to hyperosmotic stress, then the cell wall integrity pathway is activated under cell wall instability. However, several stressful conditions such as hyperosmotic stress, heat shock, low pH and oxidative stress activate both pathways suggesting that they can be positively coordinated (Rodríguez-Peña et al., 2010). HOG pathway plays a collaborative role with the CWI pathway in inducing cell wall remodelling via the upregulation of specific cell wall biosynthesis genes (Udom et al., 2019). To manage diverse stress conditions and coordinate adaptive responses, the MAPK pathways and their components crosstalk and enhance the signalling capabilities (Fuchs, Mylonakis, 2009; Saxena, Sitaraman, 2016). Given the limited number of components, crosstalk among signalling pathways could arise from the sharing particular members, e.g., Ste11 role in HOG and CWI pathways (Saxena, Sitaraman, 2016). However, for most of these situations, there is a lack of mechanistic insight and little information about the connections between these MAPK pathways could be found.

In this study, the data on interactions of genetic factors from CWI and HOG pathways under stressful conditions induced by *S. cerevisiae* K2 toxin were presented. To deepen insight into the role of genetic factors in the biocontrol activity of K2 toxin and cell resistance formation mechanisms, double mutants of HOG and CWI effectors were generated, their growth and toxin susceptibility features were analysed, and the profiles of interactions were uncovered. Understanding the mechanisms conferring toxin-driven biocontrol activity may be attractive for the application of killer yeast in plant disease control.

Materials and methods

The experiment was carried out in 2015–2020 at the Nature Research Centre, Lithuania.

Strains and culture media. Parental *Saccharomyces cerevisiae* strain BY4741 (*MAT*a, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*) and the non-essential haploid deletion strains (Δ*spt3*, Δ*spt8*, Δ*med1*, Δ*soh1*, Δ*ssk1*, Δ*ssk2*, Δ*fus3*, Δ*bem2*, Δ*lrg1*, Δ*slm4*, Δ*tax4*, Δ*rlm1*) derived from BY4741 by replacing single open reading frames by KanMX4 module were purchased from Thermo Fisher Scientific (USA). To isolate K2 toxin, *S. cerevisiae* M437 (*HM/HM [Kil-K2]*) was used (Naumov, Naumova, 1973).

Yeast *S. cerevisiae* strains were grown in standard yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% dextrose) and complete minimal (CM) medium (0.67% YNB (yeast nitrogen base without amino acids) and 2% dextrose). To test K2 toxin susceptibility, methylene blue agar (MBA) medium (0.5% yeast extract, 0.5% peptone, 2% dextrose and 2% agar), adjusted to pH 4 with 75 mM

phosphate-citrate buffer and supplemented with 0.002% methylene blue dye, was used. For the isolation of K2 toxin, synthetic complete (SC) medium (2% glucose, 6 mM K_2HPO_4, 8 mM $MgSO_4$ and 8 mM $(NH_4)_2SO_4$), adjusted to pH 4 with 75 mM phosphate-citrate buffer and containing 5% glycerol, was used.

Polymerase chain reaction (PCR)-cassette preparation. PCR product containing *HIS3* gene with flanking *HOG1* 3' and 5' sequences was amplified by forward F-HphN-delHog1 (5′-GGAACAAAGGGAAA ACAGGGAAAACTACAACTATCGTATATAATAATG CGTACGCTGCAGGTCGAC-3′) and reverse R-HphN-delHog1 (5′- CAAAAAGAAGTAAGAATGAGTGGTT AGGGACATTAAAAAAACACGTTTAATCGATGAA TTCGAGCTCG-3′) primers and using pYM15 plasmid DNA as a template (www.euroscarf.de). The PCR was performed in a total reaction of 100 µL consisting of 50 µL 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 3.2 µL of each primer (10 µmol L^{-1}), 100 ng of plasmid DNA and nuclease-free water. Amplification of PCR-cassette was carried out by PCR Thermal Cycler (ESCO) according to the following conditions: an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 2 min 40 s. The final extension was carried out at 72°C for 4 min. The PCR products were purified using GeneJet PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions and analysed by 1% agarose gel electrophoresis.

Yeast transformation. For homologous recombination-based PCR-cassette insertion, yeast transformation was performed according to the LiCl method (Guthrie, Fink, 1991) with several modifications. Yeast strain of interest was grown in liquid YPD medium until reaching 0.5 OD (optical density). Cells were collected by centrifugation at 4000 rpm for 5 min and washed by Tris-EDTA (TE) buffer. For competent cell preparation yeasts were incubated in 0.1 M LiCl/TE buffer at 30°C for 1 h, then followed by centrifugation at 800 rpm for 5 min and resuspension of the pellet in 0.1 M LiCl/TE buffer.

For transformation of the yeast cells, *HIS3* encoding PCR-cassette (5 µl) was mixed with competent cells (25 µl) and incubated at 30°C for 30 min. Then two volumes of 50% PEG-4000 in TE buffer were added into transformation mixture and followed incubation at 30°C for 1 h and at 42°C for 20 min. After transformation, cells were suspended in 1 mL of liquid YPD medium and incubated overnight at 30°C with 100 rpm agitation. For *HIS3* selection, cells were sedimented by centrifugation at 4000 rpm for 1 min, resuspended in 100 µl of remaining YPD medium and plated onto selective minimal dextrose (MD) agar plates supplemented with vitamins, ammonium sulphate, methionine, leucine and uracil and 200 µg mL^{-1} G418 (geneticin) but lacking histidine. Positive transformants with replaced *HOG1* gene with *HIS3* were analysed after incubation of plates at 30°C for 5 days.

Detection of Hog1 deletion in yeast cells. For detection of Hog1 deletion, overnight yeast cells of interest were collected by centrifugation at 10 000 rpm for 1 min and washed by SC medium (pH 4.0). Sedimented by centrifugation, yeast cells (5×10^7 CFU $sample^{-1}$) were resuspended in 1 ml of K2 toxin extract and incubated at 20°C for 1 h with 40 rpm agitation. Then, cells proceeded washing by Tris-HCl buffer (pH 7.5) and centrifugation at 10 000 rpm for 1 min. The collected yeast cells were mixed with 200 µL of 0.1 M NaOH (sodium hydroxide) solution and incubated at 20°C for 10 min. Cells were sedimented by centrifugation at 14 000 rpm for 2 min, suspended in 50 µL of 2x SDS-PAGE sample buffer and followed by heat denaturation at 85°C for 5 min. The supernatant was applied for SDS-PAGE by resolving proteins on 10% SDS-polyacrylamide gel and subsequent Western blotting. The gel was transferred onto polyvinylidene fluoride (PVDF) membrane, blocked for 1 h at room temperature with 5% of milk powder in TTBS containing 0.05% Tween 20 and washed three times with TTBS. Western blot analysis was carried out using primary anti-Hog1 antibody (1:3000) and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:5000). Membrane was visualised by colorimetric signal detection using NBT/BCIP system (Thermo Fisher Scientific) according to the manufacturer's recommendations.

Detection of yeast growth parameters. For growth rate measurements, overnight single and double yeast mutant cultures were inoculated into YPD medium starting from 0.1 OD_{600} and grown at 30°C with 100 rpm agitation. Culture growth was monitored by measuring absorbance at 600 nm every hour during a 24 h period. All the experiments were carried out in triplicate. Growth rate (h^{-1}) of mutants was calculated using software *GraphPad Prism* (Olivares-Marin et al., 2018).

K2 toxin preparation. The K2 toxin producing *S. cerevisiae* strain M437 was grown in SC medium for 4 days at 18°C with 40 rpm shaking. Cells were collected by centrifugation at 5000× *g* for 10 min. The supernatant was filtered through a 0.22 µm diameter sterile PVDF membrane and concentrated 100-fold by ultrafiltration through an Amicon PM-10 membrane. Such toxin isolate was used for yeast susceptibility assay.

Susceptibility to K2 toxin. The sensitivity of single and double mutants to K2 toxin was tested by depositing 100 µL of concentrated 100-fold toxin preparate into 10 mm diameter "punched wells" in the MBA medium plates overlaid with the yeast strain of interest (2×10^6 cells $plate^{-1}$). Plates were incubated for 2 days at 25°C. The diameter of the lysis zones was measured and compared with that formed on strain BY4741 overlay.

Protein network construction. Network diagrams were generated using web resource STRING v11 (https://string-db.org/) (Szklarczyk et al., 2011). The experiments-based active prediction method was used, and the medium confidence score (0.4) was utilized. Our created network uses the "confidence view" option of the program, where stronger associations are represented by thicker lines.

Statistical analysis. Detection of growth rate and susceptibility assay were carried out in triplicate, and the data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for the estimation of the statistical significance; the differences were considered significant at $p < 0.05$.

Results and discussion

Saccharomyces cerevisiae K2 toxin binds to the cell wall and interacts with the plasma membrane causing outflow of potassium ions and other cellular molecules (Lukša et al., 2015; Orentaite et al., 2016). Defects in high osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways results in altered susceptibility of yeast to the action of K2 toxin (Servienė et al., 2012). It was demonstrated that general cellular factors of both pathways are physically and functionally interconnected and coordinate their responses by managing stressful conditions (Rodríguez-Peña et al., 2010). Many genetic factors may be involved in conditions-dependent negative (when mutants are synergistically deleterious) or positive

(when the combination is less severe than is expected from independent effects) interactions. These factors may act directly or through mediators (Díaz-Mejía et al., 2018). The information on the nature of the connections between most of the gene products from HOG and CWI pathways, which are involved in formation of cellular susceptibility to K2 toxin, is scattered. Therefore, to deepen insight into the role of cellular factors in the biology of K2 toxin, double mutants of HOG and CWI genes were generated, and their growth and toxin susceptibility features were compared with single mutants as well as the profile of conditions-dependent interactions were uncovered.

Construction of yeast double mutants. For further analysis, 13 yeast single gene deletion strains related to the response of cell wall integrity and hyperosmotic stress pathways and having altered susceptibility to K2 toxin were selected (Table).

Table. Description of gene names, functions and susceptibility to *Saccharomyces cerevisiae* K1, K2 and K28 toxins as well as *S. paradoxus* K66 killer protein (according to Pagé et al., 2003; Carroll et al., 2009; Servienė et al., 2012; Vepštaitė-Monstavičė et al., 2018)

Gene ID	Description	*S. cerevisiae*			*S. paradoxus*
		K1	K2	K28	K66
		single mutant phenotype			
HOG1	Mitogen-activated protein (MAP) kinase involved in osmoregulation	S	S	S	S
SSK1	Cytoplasmic response regulator, part of a two-component signal transducer that mediates osmosensing	S	S	S	S
SSK2	MAP kinase of the HOG1 mitogen-activated signalling pathway	S	S	wt	S
SOH1	Subunit of the RNA polymerase II mediator complex	wt	S	S	wt
MED1	Subunit of the RNA polymerase II mediator complex	wt	S	wt	wt
SPT3	Subunit of the SAGA and SAGA-like transcriptional regulatory complexes	wt	S	wt	wt
SPT8	Subunit of the SAGA transcriptional regulatory complex; mutants sensitive to osmotic stress	wt	S	wt	wt
BEM2	Rho GTPase activating protein involved in signal transduction and cellular morphogenesis	wt	S	wt	wt
FUS3	Mitogen-activated serine/threonine protein kinase involved in pheromone-dependent signal transduction during mating	wt	S	wt	wt
TAX4	EH domain containing protein involved in cell wall organization, inositol lipid-mediated signalling	wt	S	wt	wt
RLM1	Component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity	wt	R	wt	R
LRG1	GTPase-activating protein (GAP) involved in the Pkc1p-mediated signalling pathway that controls cell wall integrity	wt	R	wt	R
SLM4	Subunit of EGO/GSE complex, phosphatidylinositol-3,4-bisphosphate binding	wt	R	R	wt

Note. Gene description was adapted from *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org); S – increased sensitivity to toxins is marked, R – resistant phenotype, wt – similar level as in parental strain BY4741.

Well-known Hog1 protein kinase-related regulators Ssk1 and Ssk2 were chosen. The abolishment of those genes determines hypersensitivity to *S. cerevisiae* K1, K2 and K28 toxins as well as to *S. paradoxus* K66 protein (Table). Among chosen strains, there are Δ*soh1* (gene encoding subunit of the RNA polymerase II mediator complex), which is sensitive to K2 and K28 toxins and strain Δ*slm4* (gene encoding subunit of EGO/GSE complex), which is resistant to K2 and K28 toxins. Mutations Δ*rlm1* and Δ*lrg1* are beneficial and confer higher resistance to *S. cerevisiae* K2 and *S. paradoxus* K66 toxins. Both gene products are involved in the maintenance of the cell wall integrity. Other selected strains, deficient in transcription regulators or signal transducers (Med1, Spt3, Spt8, Bem2, Fus3 or Tax4), modulating the expression of genes important for cell wall biogenesis, demonstrate hypersensitivity to K2 toxin only.

To obtain cells carrying two gene deletions, a homologous recombination approach was used (Janke et al., 2004). For *HOG1* gene replacement, the *HIS3* gene was PCR amplified from pYM15 plasmid by adding sequences flanking *HOG1* gene (Figure 1A). According to electrophoretic analysis, the purified PCR fragment size was about 1700 bp (Figure 1B). Amplified PCR cassette was transformed into single gene deletion yeast cells, and homologous recombination based *HOG1* gene deletion was generated (Figure 1C).

Transformed cells were selected by the growth on SC medium without histidine (Figure 2A). Afterwards, the viable colonies were tested for the absence of Hog1 protein (48.9 kDa) by Western blot hybridization using specific primary anti-HOG1 and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibodies (Figure 2B).

Determination of yeast growth rate. Yeast growth profile depends on growing conditions such as pH, temperature and medium type, but growth variability even more depends on yeast species and genetic background (Salari, Salari, 2017). To determine whether the removal of *HOG1* gene from yeast single mutants does affect yeast growth parameters and viability, the growth rate of single and double mutants incubated in rich YPD medium was evaluated and compared to the parental strain BY4741 (Figure 3).

It was observed that deletion of *HOG1* gene by itself does not significantly change the growth rate compared to the parental strain BY4741: growth rate of mutant Δ*hog1* was 0.4 ± 0.06 h^{-1} and strain BY4741 – 0.49 ± 0.05 h^{-1}. The comparison of parental strain growth rate *vs* tested single gene mutants also did not show remarkable differences. However, the growth

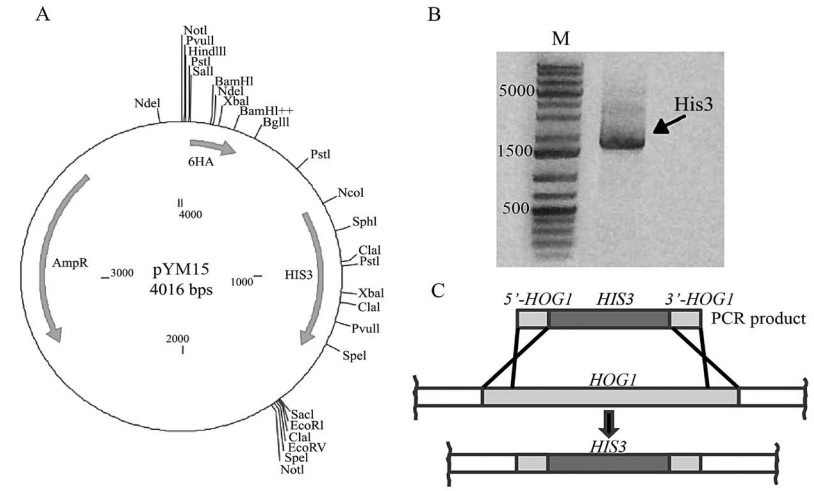


Figure 1. Principles of double mutants' construction: restriction map of pYM15 plasmid-encoding marker gene *HIS3* (www.euroscarf.de) (A), electrophoretic validation of PCR-amplified *HIS3* (B), scheme of *HOG1* replacement by *HIS3* using homologous recombination (C)

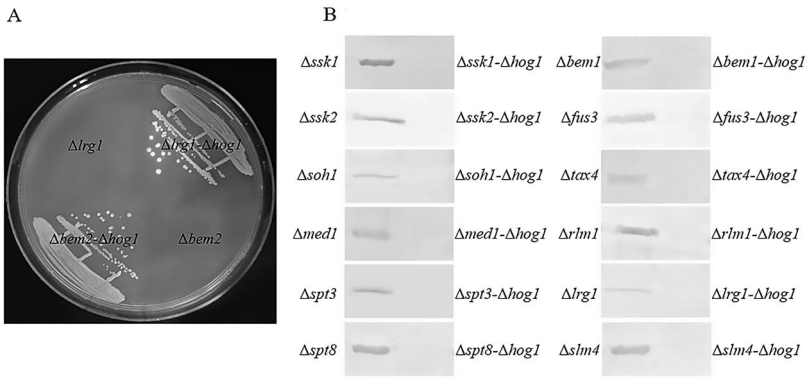
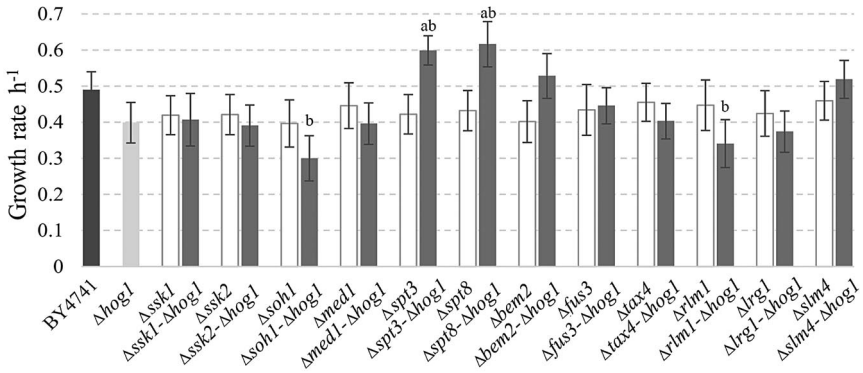


Figure 2. Validation of constructed *Saccharomyces cerevisiae* double mutants by growth on complete minimal medium without histidine (A) and by absence of Hog1 protein using Western blot hybridization (B)



Note. Growth rate of mutants were calculated from three experiments, and the data were expressed as mean ± SD; significant differences ($p < 0.05$) between the growth rate of the double *vs* single mutants and parental strain BY4741 are marked a and b, respectively; colour coding is as follows: black – parental strain BY4741, light grey – single mutant Δ*hog1* (control); white – single mutants, dark grey – double mutants.

Figure 3. Growth rate of *Saccharomyces cerevisiae* single and double mutants

rate of some double mutants was affected significantly in comparison with single deletions. The significant increase (about 40%) of the growth rate was observed in the case of strains Δ*spt8*-Δ*hog1* and Δ*spt3*-Δ*hog1* comparing to a single mutant and to *wt*. The growth rate of strains Δ*bem2*-Δ*hog1* increased up to 30% comparing to single *BEM*2 gene deletion.

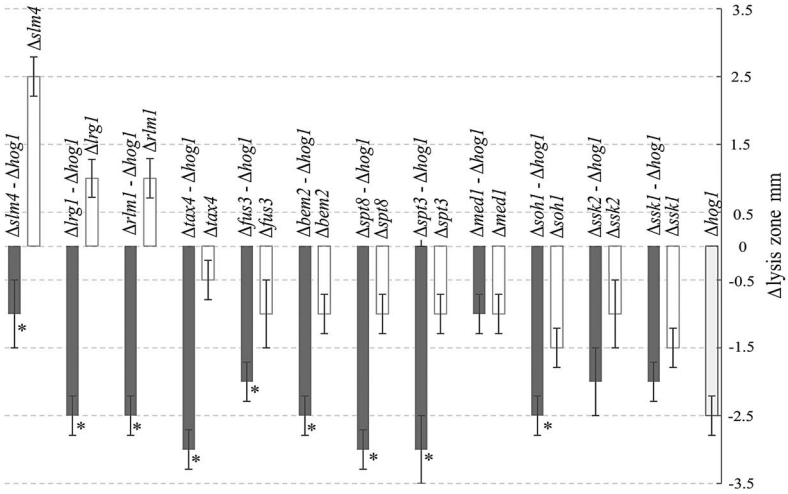
After elimination of *HOG1* gene, the doubling speed of yeast cells Δ*soh1* and Δ*rlm1* was reduced by 25%. No other significant differences between single and double gene mutants' growth rate were observed. The data on the rate of growth can be used when the goal is to find individual interactions or estimate the mean epistatic effect. It was reported that interactions leading to strong alterations of fitness and growth parameters of double mutants in relation to those of the respective single mutants are generally rare, and weak interactions are more abundant (Jakubowska, Korona, 2012). During

our experiment, also most double gene deletion strains demonstrated weak changes in growth rate *vs* individual gene deletion yeasts. Nevertheless, such changes in growth parameters may have an impact on yeast viability and response to various stressful factors, especially killer toxin action.

Response of double mutants to K2 toxin. For analysis of genetic interactions between the players of HOG and CWI pathways in response to K2 toxin action, a sensitivity-based approach was applied. The created K2 toxin-driven stress accomplishes activation of the HOG pathway, which regulates glycerol synthesis and affects cell wall integrity. Gene interactions in mutant cells were evaluated by comparing single and double mutant susceptibility to K2 toxin (Figure 4). Most tested single gene mutants (Δ*ssk1*, Δ*ssk2*, Δ*soh1*, Δ*spt3*, Δ*spt8*, Δ*bem2*, Δ*fus3*, Δ*tax4*, Δ*hog1*) demonstrated increased sensitivity to K2 toxin in relation to parental strain BY4741. The toxin susceptibility of most double mutants increased in comparison to single mutants but remained at a similar level to single mutant Δ*hog1*.

HOG1 gene deletion from cells Δ*ssk1* and Δ*ssk2*, which interact in the same signalling pathway, had no significant changes upon K2 toxin treatment (Figure 4). Soh1 and Med1 both are subunits of the RNA polymerase II mediator complex (Boube et al., 2002). The sensitivity of a double mutant Δ*soh1*-Δ*hog1* increased comparing to single gene deletion, while Δ*med1*-Δ*hog1* response to K2 toxin remained as Δ*med1*. Elimination of *HOG1* gene from defective in SAGA transcriptional regulation cells Δ*spt3* or Δ*spt8* K2 toxin sensitivity increased about 50% comparing to single mutants (Wu et al., 2004). Deletion of Hog1 protein kinase from defective in CWI pathway strains Δ*bem2* and Δ*tax4* augmented the killer toxin sensitivity phenotype for Δ*bem2*-Δ*hog1* about 35% and for Δ*tax4*-Δ*hog1* even higher. Thus, the K2 toxin response phenotype in the mentioned above double mutants, not depending on the involvement of gene products into the same or different signalling pathways, did not change, only the increase in sensitivity level was observed.

Our observations agree with those of other researchers indicating the abundance of weak genetic



Note. Toxin formed lysis zones were measured and compared to the ones obtained on parental strain BY4741; negative values mean that lysis zones were smaller than displayed on parental strain, positive – larger than wt; all data were expressed as mean ± SD; * – corresponds to significant difference ($p < 0.05$) in K2 toxin sensitivity of double and single mutants; colour coding is as follows: white – single mutants, dark grey – double mutants, light grey – single mutant Δ*hog1* (control).

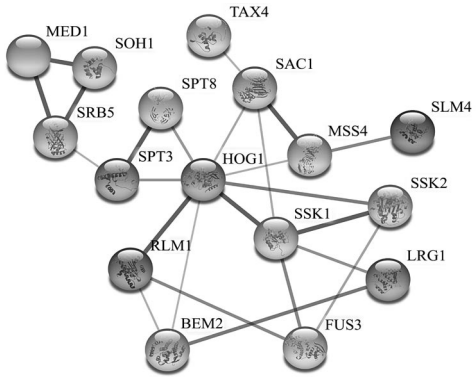
Figure 4. Sensitivity of yeast single and double mutants to *Saccharomyces cerevisiae* K2 toxin

interactions with an average effect close to zero or moderately positive (Jakubowska, Korona, 2012). In our research, only three single gene mutants, whose gene products are involved in CWI signalling, changed their response to K2 toxin, when additional deletion of *HOG1* gene was generated. Single mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* are more resistant to K2 killer protein than the parental strain BY4741, while the mutant Δ*hog1* is sensitive to K2 toxin. Importantly, that double mutants Δ*rlm1*-Δ*hog1*, Δ*lrg1*-Δ*hog1* and Δ*slm4*-Δ*hog1* are as sensitive to K2 toxin as the single gene deletion strain Δ*hog1*. The genes may be defined as epistatic to one another, when the phenotypic impact associated with a given mutation is altered by the presence of a second gene mutation (Batenchuk et al., 2010; Steidle et al., 2020). Thus, our results indicated that the mutation Δ*hog1* is epistatic to Δ*rlm1*, Δ*lrg1* and Δ*slm4*.

The sign and strength of interactions may change reflecting on the activation or inactivation of different pathways across environments. Thus, epistasis depends on both genetic and environmental context (Batenchuk et al., 2010). In silico study, He et al. (2010) found that negative interactions occur more frequently between genes with overlapping functions, whereas positive interactions are observed between functionally distinct metabolic pathways. Some studies have looked at the effect of environmental stress on the form of epistasis and pointed out that environmental conditions can have long-term effects on epistasis (de Visser et al., 2011).

Interconnections of gene products involved in K2 toxin response. To generate protein-protein interaction network, a STRING analysis was applied (Szklarczyk et al., 2011), which used high throughput datasets available in the *S. cerevisiae* Genome Database (https://www.yeastgenome.org). The network was drawn with medium confidence score and included overall 24 linkages. The line thickness represents the strength of data support and includes both functional and physical interactions (Figure 5). Hog1 has strong direct associations with HOG pathway regulator Ssk1 and CWI pathway mediator Rlm1.

The direct connections of Hog1 protein kinase with MAPK Ssk2 and transcription regulators Spt3 and Spt8 as well as Rho1 GTPase activating protein Bem2 were slightly weaker. In this experiment, the linkages of Hog1 with other players of CWI and HOG pathways analysed were indirect. They were mediated through protein kinases (for Fus3 and Lrg1), RNA polymerase



Note. Stronger associations are represented by thicker lines; confidence score = 0.4.

Figure 5. Interaction network of proteins from cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways involved in K2 toxin susceptibility

II complex and transcription regulators (for Soh1) and phosphatidylinositol signalling regulators (for Tax4 and Slm4). STRING analysis performed in this experiment suggests that there are still many interacting factors to be discovered between HOG and CWI pathways. More investigations are needed to detect small individual effects of epistasis.

Conclusions

1. The majority of created double gene deletion strains had minor changes in growth rate compared to individual gene mutants, and only strains Δ*spt3* and Δ*spt8* in combination with Δ*hog1* had a significant increase of growth rate.

2. The sensitivity towards K2 toxin increased in most generated double mutants thus suggesting weak interactions of all investigated gene products. Phenotype changes from resistant to sensitive in mutants Δ*rlm1*, Δ*lrg1* and Δ*slm4* caused by *HOG1* gene deletion showed epistatic effect on this cell wall integrity (CWI) pathway effectors.

3. The network of proteins involved in K2 toxin response uncovered interconnected or acting through mediators high osmolarity glycerol (HOG) and CWI players.

4. The obtained results deepen insight into mechanisms underlying the tolerance to *Saccharomyces cerevisiae* K2 toxin-caused cellular stress and toxin-driven biocontrol activity thus highlighting the potential of killer yeast in plant protection.

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Šeimininko veiksnių sąveika formuojant atsaką į mielių K2 toksino sukeltą stresą – pritaikomumas augalų apsaugai

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Santrauka

Toksinus sintetinančios mielės yra svarbios žemės ūkyje, nes gali būti panaudotos biologinei augalų apsaugai nuo bakterinių ir grybinių ligų. Biocidinio toksino veikimo ir ląstelių atsako toksino sukeltam stresui mechanizmų išaiškinimas yra itin svarbus, siekiant plėsti biocidinių mielių kaip augalų apsaugos priemonės pritaikomumą.

Tyrimo metu siekta nustatyti sąveiką tarp genetinių veiksnių iš ląstelės sienelės vientisumo (CWI, angl. *cell wall integrity*) ir atsako į hiperosmotinį stresą (HOG, angl. *high osmolarity glycerol*) signalinių kelių, paveikus *Saccharomyces cerevisiae* K2 toksinu. Tiriant genetinę sąveiką buvo naudoti du įverčiai – santykinis augimo greitis ir ląstelių jautrumas toksinui. Homologinės rekombinacijos būdu sukurta 12 mutantų su dviejų genų iškritomis ir išanalizuoti jų augimo parametrai bei atsakas į toksino sukeltą stresą. Dauguma dvigubų mutantų parodė nereikšmingus (mažiau nei 30 %) augimo greičio pokyčius, palyginti su vienetiniais mutantais. Tik Δ*spt3* ir Δ*spt8* kamienuose *HOG1* geno pašalinimas augimo greitį esmingai padidino iki 0,6 val^{-1}. Daugelio dvigubų mutantų jautrumas K2 toksinui padidėjo, nepriklausomai nuo signalinio kelio priklausomybės; tai rodo silpną genų produktų sąveiką. *HOG1* geno pašalinimas pakeitė tik mutantų Δ*rlm1*, Δ*lrg1* ir Δ*slm4* fenotipą iš atsparaus į jautrų, tuo patvirtindamas epistatinį poveikį šiems genetiniams efektoriams. Siekiant papildyti funkcinės analizės duomenis, sukurtas K2 toksino sukelto streso atsake dalyvaujančių HOG ir CWI baltymų tinklas ir atskleistos jų sąveikos. Nustatyta, kurie HOG ir CWI keliuose dalyvaujantys genų produktai sąveikauja tiesiogiai, o kurie per baltymus tarpininkus.

Tyrimo rezultatai pagilina žinias apie sąveikas genetinių veiksnių, moduliuojančių atsaką į K2 toksino sukeltą stresą, ir suteikia svarbios informacijos praktiniam biocidinių mielių pritaikymui.

Reikšminiai žodžiai: mielės, toksino sukeltas stresas, signalinių kelių ryšys, biokontrolės mechanizmas.