FERNANDA MATIAS ALBUINI

BIOTECHNOLOGICAL APPLICATIONS OF YEASTS: STRESS RESPONSES OF *Spathaspora passalidarum* AND MODE OF ACTION OF THE BIOCONTROL AGENT *Pseudozyma flocculosa*

Thesis submitted to the Applied Biochemistry Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Luciano Gomes Fietto

Co-adviser: Tiago Antônio de O. Mendes

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I dedicate this work to my family, for their unconditional love and support.

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ABSTRACT

ALBUINI, Fernanda Matias, D.Sc., Universidade Federal de Viçosa, April, 2023. **Biotechnological applications of yeasts: stress responses of *Spathaspora passalidarum* and mode of action of the biocontrol agent *Pseudozyma flocculosa***. Adviser: Luciano Gomes Fietto. Co-adviser: Tiago Antônio de Oliveira Mendes.

Yeasts are biodiverse microorganisms that present several biotechnological applications, such as in bioethanol production and biological control of plants against fungal pathogens. The first two chapters of this thesis focused on studying the mechanisms underlying the stress responses of *Spathaspora passalidarum*, a yeast capable of fermenting xylose into ethanol, and the third chapter aimed to investigate the mode of action of *Pseudozyma flocculosa*, a natural antagonistic yeast of powdery mildew fungi. In the first study, an RNA-seq experiment was performed with *S. passalidarum* cells exposed to 2 h of 4% (v/v) ethanol stress. The bioinformatics analyses supported that *S. passalidarum* activated responses regarding protein folding and antioxidant stress. However, the osmotic stress response of this yeast appeared impaired, and genes encoding proteins from lipid metabolism, transporters, and enzymes from glycolysis and fermentation pathways showed downregulation. Additionally, ethanol-treated cells presented a pseudo-hyphal morphology. Changes in fatty acid profile were only observed after 12 h of ethanol exposure, coinciding with the yeast recovery of its xylose consumption ability. Thus, the halt in nutrient acquisition and fermentation, the lack of fast membrane adjustments, and the limited osmotic stress response were the main aspects identified underlying the low ethanol tolerance of *S. passalidarum*. In the second study, comparative genomic analyses were performed with *Spathaspora passalidarum* and *Saccharomyces cerevisiae* searching for clues regarding their distinct robustness to stresses. The results showed major differences in transcription factor sequences while signaling pathways and responsive proteins appeared conserved between the yeasts. The evolutionary differences in transcription factors might contribute to distinct stress responses and, ultimately, different physiological profiles. Indeed, our results showed that *S. passalidarum* and *S. cerevisiae* present distinct tolerance profiles to ethanol, oxidative, and osmotic stresses. The overexpression of the *MSN-like* transcription factor of *S. passalidarum* in an *S. cerevisiae msn2 msn4* double-deleted strain indicated that the *MSN-like* only partially complemented the function of their orthologs in *S. cerevisiae* under stress, corroborating our hypothesis that transcription factors have diverged in *S. passalidarum* and it might have impacted the yeasts stress responses. In the third study, the role of the Pf2826 effector of *Pseudozyma flocculosa* was investigated in the yeast biocontrol activity against barley powdery mildew. This gene is highly expressed only during the *P. flocculosa*-barley-powdery mildew tripartite interaction. The knockout of this gene using the CRISPR-Cas9 mediated genome edition tool showed that the mutant line developed less aggressively and did not overtake the powdery mildew colonies, indicating that this gene is essential for the full biocontrol activity of *P. flocculosa*. The yeast-two-hybrid methodology was used to validate the interaction of the Pf2826 effector with seven putative interactors previously identified by a pull-down assay. The results confirmed the interaction of the Pf2826 effector with a barley pathogenesis-related protein that has putative roles in plant defense against biotic stress, and with a powdery mildew effector, which might be involved in the pathogen's aggressiveness. These interactions probably destabilize the effector-induced host immunity suppression and boost the plant defenses, resulting in the collapse of the powdery mildew colonies.

Keywords: Yeast biotechnology. Bioethanol production. Biological control.

RESUMO

ALBUINI, Fernanda Matias, D.Sc., Universidade Federal de Viçosa, abril de 2023 **Aplicações biotecnológicas de leveduras: respostas a estresses de *Spathaspora passalidarum* e modo de ação do agente de biocontrole *Pseudozyma flocculosa***. Orientador: Luciano Gomes Fietto. Coorientador: Tiago Antônio de Oliveira Mendes.

Leveduras são microrganismos biodiversos que apresentam várias aplicações biotecnológicas, como na produção de bioetanol e no controle biológico de patógenos em plantas. Os primeiros dois capítulos desta tese investigaram as respostas a estresse de *Spathaspora passalidarum*, uma levedura capaz de fermentar xilose a etanol, e o terceiro capítulo analisou o modo de ação de *Pseudozyma flocculosa*, uma levedura antagonista ao oídio. No primeiro estudo, um experimento de RNA-seq foi realizado com *S. passalidarum* sob 4% (v/v) de estresse por etanol durante 2 horas. As análises mostraram que *S. passalidarum* ativou respostas relacionadas ao enovelamento proteico e antioxidante. No entanto, a resposta ao estresse osmótico dessa levedura foi limitada, e os genes que codificam proteínas do metabolismo lipídico, transportadores e enzimas da glicólise e fermentação foram downregulados. As células tratadas com etanol apresentaram morfologia pseudo-hifal, e mudanças no perfil de ácidos graxos foram observadas apenas após 12 h sob estresse por etanol, coincidindo com o início do consumo de xilose pela levedura. Assim, a parada na absorção de nutrientes e na fermentação, a falta de ajustes rápidos na membrana e uma resposta limitada ao estresse osmótico foram os principais fatores relacionados à baixa tolerância de *S. passalidarum* ao etanol. No segundo estudo, análises de genômica comparativa entre *Spathaspora passalidarum* e *Saccharomyces cerevisiae* foram realizadas a fim de compreender seus distintos perfis de tolerância a estresses. Foram encontradas divergências nas sequências dos fatores de transcrição, e conservação nas vias de sinalização e proteínas responsivas a estresses. Diferenças evolutivas nos fatores de transcrição podem contribuir para distintas respostas a estresses e perfis fisiológicos. De fato, nossos resultados mostraram perfis distintos de tolerância das duas leveduras aos estresses etanol, oxidativo e osmótico. A superexpressão do fator de transcrição *MSN-like* de *S. passalidarum* em uma cepa de *S. cerevisiae* duplamente deletada para *msn2* e *msn4* indicou que o *MSN-like* complementou apenas parcialmente a função de seus ortólogos em *S. cerevisiae* sob estresse, corroborando com nossa hipótese de que os fatores de transcrição divergiram em *S. passalidarum*, e que isso pode ter impactado a resposta a estresse dessa levedura. No terceiro estudo, o papel do efetor Pf2826 de *Pseudozyma flocculosa* foi investigado na atividade de biocontrole dessa levedura contra o oídio da cevada. Este gene é altamente expresso apenas durante a interação tripartite involvendo *P. flocculosa*-cevada-oídio. O nocaute desse gene por CRISPR-Cas9 mostrou que a linhagem mutante foi menos agressiva e não causou o collapso do oídio, indicando que esse efetor é essencial para a atividade de biocontrole de *P. flocculosa*. Duplo híbrido de levedura foi utilizado para validar a interação do Pf2826 com sete possíveis interatores previamente identificados por pull-down. Os resultados confirmaram a interação entre Pf2826 e uma proteína relacionada à patogênese de cevada, possivelmente envolvida na defesa da planta contra estresse biótico, e um efetor do oídio, o qual pode estar envolvido com a agressividade do patógeno. Essas interações provavelmente desestabilizam a supressão da imunidade do hospedeiro induzida pelo efetor e favorecem as defesas da planta, resultando no colapso das colônias do oídio.

Palavras-chave: Biotecnologia de leveduras. Produção de bioetanol. Controle biológico.

SUMMARY

INTRODUCTION

Yeasts are unicellular fungi with remarkable biodiversity and enormous biotechnological importance. Their use by humans in food and alcoholic beverage processing dates from several millennia, although microorganisms were only discovered in the 19th century (Mattanovich et al. 2014). One of the main applications of yeasts is in fermentative processes, such as brewing, winemaking, baking, and bioethanol production (Maicas 2020). Yeasts are also an excellent scientific model due to their eukaryotic origin, simple life cycle, ease of cultivation, and vast knowledge about their metabolism and genome. In this regard, it is worth mentioning that the first eukaryotic genome to be completely sequenced belongs to the yeast *Saccharomyces cerevisiae* and was finished in 1996, boosting molecular studies and providing important information regarding genome structure and evolution (Botstein and Fink 2011). Additionally, yeasts are exploited as cell factories for heterologous protein production, for the study of diseases, for the *in vivo* analysis of protein-protein interactions, and as natural antagonists of other microorganisms with applications in fruit and crop protection against diseases (Brückner et al. 2009; Baghban et al. 2019; Nielsen 2019; Freimoser et al. 2019).

Undeniably, the conversion of sugars into ethanol and carbon dioxide is a pivotal step in several industrial bioprocesses. Yeasts can utilize renewable feedstocks to produce fuels or chemicals, such as bioethanol, organic acids, enzymes, and biopharmaceutical proteins (Nandy and Srivastava 2018). Bioethanol is a renewable biofuel produced from a variate of plant-based biomass, such as energy crops (first generation) and lignocellulosic biomass (second generation), which is considered an interesting alternative to assure energy security (Zabed et al. 2017). Brazil is the world’s second-biggest bioethanol producer. In 2021, the country produced 7,320 million gallons of ethanol, which corresponded to 27% of the world's production (Renewable Fuels Association). Bioethanol is produced in Brazil mainly from the microbial fermentation of sugarcane juice. The residual biomass from the juice extraction, such as sugarcane bagasse and straw, also contains fermentable sugars that can contribute to increasing ethanol production. The fermentation of the various components of plant biomass is of great industrial and economic interest as it allows increasing the productivity of the process without expanding the cultivable areas (Dos Santos et al. 2016).

Plant biomass is a complex substrate composed of cellulose (40 - 50%), hemicellulose (20 - 40%), and lignin (20 - 30%) (Pauly and Keegstra 2008). Pentoses, such as xylose and arabinose, can represent up to 20% of the total sugar in the biomass, with xylose being the second most abundant carbohydrate in these materials (Zabed et al. 2017). Efficient xylose fermentation is a bottleneck for the widespread production of ethanol from lignocellulosic materials. This is because the yeast *Saccharomyces cerevisiae*, the most relevant and widely used yeast species in industrial fermentative processes, does not naturally assimilate pentoses (Toivari et al. 2004). Despite the impressive advances regarding the engineering of xylose fermentation in *S. cerevisiae* strains, challenges remain in which concerns xylose assimilation rates and simultaneous co-utilization of xylose and other sugars in lignocellulosic hydrolysates, which demonstrates that there is room for improvement (Gao et al. 2019; Sun and Jin 2021). Thus, it is worth noticing that the domestication of xylose-fermenting yeasts is an interesting strategy for expanding the knowledge about the regulation of xylose metabolism in order to allow their direct use on industrial scales or improve other yeast strains.

Spathaspora passalidarum is a xylose-fermenting yeast isolated from the guts of woodboring beetles (Nguyen et al. 2006) that can ferment xylose, glucose, and cellobiose with high ethanol yields (0.48 $g.g^{-1}$) and productivities and has emerged as a candidate for domestication (Long et al. 2012; Cadete et al. 2016; Cadete and Rosa 2018). Several studies have characterized this yeast regarding the co-consumption of sugars, the best aeration and temperature conditions for ethanol production, resistance against contaminants and inhibitors, fermentation of hydrolysates, xylose metabolism through metabolome data, and others aspects (Hou 2012; Rodrussamee et al. 2018; Collograi et al. 2019; Veras et al. 2019; Ribeiro et al. 2021; Campos et al. 2022). Although *S. passalidarum* is currently among the best xylose-fermenting yeasts, it presents high sensitivity to mild ethanol concentrations and other chemicals (e.g. acetic acid) (Campos et al. 2022). The growth and fermentative metabolism of *S. passalidarum* are highly inhibited in ethanol concentrations equal to or greater than 4% (v/v), while the title of ethanol obtained in second-generation ethanol fermentations varies between 5% and 7%. Besides, industrial environments display other stressful conditions for yeasts, such as osmotic and oxidative stresses, changes in pH and temperature, and the presence of inhibitors, which reassures the importance of improving *S. passalidarum* tolerance to stresses (Deparis et al. 2017; Campos et al. 2022).

In the first two chapters of this thesis, the stress response and tolerance profile of *S. passalidarum* were evaluated under different stress conditions. In the first chapter, an RNA-seq analysis was performed to investigate *S. passalidarum* transcriptional changes in response to two hours of 4% (v/v) ethanol stress aiming to shed light on the mechanisms underlying the low ethanol tolerance profile of this yeast and search for clues to improve this phenotype. In the second chapter, divergences in transcription factor sequences of *S. passalidarum* and *S. cerevisiae* identified through comparative genomic analyses raised the hypothesis that these differences might influence the yeasts' stress response regulation and, ultimately, their stress adaptation abilities. Thus, the tolerance profiles of *S. passalidarum* and *S. cerevisiae* were compared under ethanol, oxidative, and osmotic stress conditions. In addition, the function of the *MSN-like* transcription factor of *S. passalidarum* was examined by performing phenotypic complementation assays with *S. cerevisiae msn2 msn4* double deleted strain overexpressing this gene under the mentioned stress conditions.

The third chapter of this thesis focused on studying another yeast species with a different but equally relevant biotechnological potential: *Pseudozyma flocculosa* and its biological control activity against plant-pathogenic fungi. Biocontrol agents are natural antagonists of phytopathogens that can control plant diseases by interfering with the pathogen’s growth, development, or reproduction (Zhang et al. 2020). The interest in biocontrol agents has emerged from the searches for more environmentally safe strategies to replace or reduce the use of synthetic fungicides while maintaining plant protection and low economic losses (Dukare et al. 2019). Yeasts present several properties desirable for biocontrol agents, such as single cell morphology - which is ideal for growth in fermenters, few nutritional requirements, genetic stability, effectivity in low concentrations, and low biosafety risks to human health and the environment (Teixidó et al. 2022). Some yeast species, such as *Candida oleophila,* strain O (NEXY®), has been successfully commercialized as a formulate for the postharvest control of fungi on fruits (Lahlali et al. 2011; Ballet et al.

2016). Deciphering the mechanisms involved in an organism's mode of action is crucial for efficiently controlling a given disease, besides understanding their potential risks for humans and the environment (Ghorbanpour et al., 2018).

Pseudozyma flocculosa is an epiphytic yeast-like fungus discovered in 1987 on powdery mildew-infected clover leaves (Traquair et al. 1988). This yeast presents a powerful antagonistic activity against a wide range of powdery mildew pathogens (Bélanger et al. 2012). Powdery mildew is a relevant plant disease distributed worldwide that causes significant crops yield losses. Powdery mildew species are obligate biotrophic pathogens that can infect and damage a vast spectrum of plants of economic interest, such as cereals, vegetables, and ornamental plants (Glawe 2008). In Brazil, some of the crops affected by this disease include barley (*Hordeum vulgaris L.*) (Agostinetto et al. 2014), wheat (*Triticum aestivum L.*) (Reis et al. 2013), eucalypt (*Eucalyptus spp. L'Hér.*) (Fonseca et al. 2017), and cashew (*Anacardium occidentale*) (Fonseca et al. 2019). *Blumeria graminis f. sp. hordei* is a powdery mildew species that causes severe yield losses in barley (Zhu et al. 2016), the fourth most produced grain in the world (Food and Agriculture Organization of the United Nations), and one of the main winter crops in Southern Brazil. In 2022, the national production of barley in Brazil achieved the highest number ever, with over 482 thousand tons produced (Conab, 2022).

The mode of action of *P. flocculosa* has been described so far as antibiosis, which would be related to a glycolipid molecule with antimicrobial activity secreted by this yeast termed flocculosin (Cheng et al. 2003). However, recent data has demonstrated that the deletion of a crucial gene involved in flocculosin synthesis resulted in a *P. flocculosa* strain unable to produce this glycolipid but still presenting its biocontrol activity against barley powdery mildew (Santhanam et al. 2021). The indication that flocculosin is not responsible for *P. flocculosa* antagonistic activity, along with the identification of *P. flocculosa* effector proteins highly expressed only during its interaction with powdery mildew infected plants (Laur et al. 2018), resulted in a new hypothesis about the mode of action of this biocontrol agent involving a new player, the Pf2826 effector protein.

In the third chapter of this thesis, the role of the Pf2826 effector protein in the mode of action of *P. flocculosa* against barley powdery mildew was evaluated by generating a knockout strain for this gene using the CRISPR Cas-9 system and by validating the effector interaction with proteins from barley and powdery mildew previously identified by a pulldown assay. The precise identification of how *P. flocculosa* kills its target can contribute to understanding the molecular dynamics that occur during the development of the plant-pathogen-biocontrol interaction and improve the exploitation of this microorganism as a biocontrol agent by enhancing its biocontrol properties.

References

Agostinetto L, Casa RT, Bogo A, Sachs C, Reis EM, Kuhnem PR (2014) Critical yield-point model to estimate damage caused by brown spot and powdery mildew in barley. Ciência Rural 44:957–963. https://doi.org/10.1590/s0103-84782014000600001

Baghban R, Farajnia S, Rajabibazl M, Ghasemi Y, Mafi AA, Hoseinpoor R, Rahbarnia L, Aria M (2019) Yeast expression systems: overview and recent advances. Mol Biotechnol 61:365–384. https://doi.org/10.1007/s12033-019-00164-8

Ballet N, Souche JL, Vandekerckove P (2016) Efficacy of *Candida Oleophila*, strain O, in preventing postharvest diseases of fruits. Acta Hortic 1144:105–111. https://doi.org/10.17660/ActaHortic.2016.1144.15

Bélanger RR, Labbé C, Lefebvre F, Teichmann B (2012) Mode of action of biocontrol agents: All that glitters is not gold. Can J Plant Pathol 34:469–478. https://doi.org/10.1080/07060661.2012.726649

Botstein D, Fink GR (2011) Yeast: an experimental organism for 21st century biology. Genetics 189:695–704. https://doi.org/10.1534/genetics.111.130765

Brückner A, Polge C, Lentze N, Auerbach D, Schlattner U (2009) Yeast two-hybrid, a powerful tool for systems biology. Int J Mol Sci 10:2763–2788. https://doi.org/10.3390/ijms10062763

Cadete RM, De Las Heras AM, Sandström AG, Ferreira C, Gírio F, Gorwa-Grauslund MF, Rosa CA, Fonseca C (2016) Exploring xylose metabolism in *Spathaspora species*: *XYL1.2* from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*. Biotechnol Biofuels 9:167. https://doi.org/10.1186/s13068-016-0570-6

Cadete RM, Rosa CA (2018) The yeasts of the genus *Spathaspora*: potential candidates for second-generation biofuel production. Yeast 35:191–199. https://doi.org/10.1002/yea.3279

Campos VJ, Ribeiro LE, Albuini FM, de Castro AG, Fontes PP, da Silveira WB, Rosa CA, Fietto LG (2022) Physiological comparisons among *Spathaspora passalidarum*, *Spathaspora arborariae*, and *Scheffersomyces stipitis* reveal the bottlenecks for their use in the production of second-generation ethanol. Brazilian J Microbiol 53:977–990. https://doi.org/10.1007/s42770-022-00693-6

Cheng Y, McNally DJ, Labbé C, Voyer N, Belzile F, Bélanger RR (2003) Insertional mutagenesis of a fungal biocontrol agent led to discovery of a rare cellobiose lipid with antifungal activity. Appl Environ Microbiol 69:2595–2602. https://doi.org/10.1128/AEM.69.5.2595-2602.2003

Collograi KC, da Costa AC, Ienczak JL (2019) Effect of contamination with *Lactobacillus fermentum* I2 on ethanol production by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 103:5039–5050. https://doi.org/10.1007/s00253-019-09779-y

Deparis Q, Claes A, Foulquié-Moreno MR, Thevelein JM (2017) Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS Yeast Res 17:fox036. https://doi.org/10.1093/femsyr/fox036

Dos Santos LV, De Barros Grassi MC, Gallardo JCM, Pirolla RAS, Calderón LL, De Carvalho-Netto OV, Parreiras LS, Camargo ELO, Drezza AL, Missawa SK, Teixeira GS, Lunardi I, Bressiani J, Pereira GAG (2016) Second-generation ethanol: the need is becoming a reality. Ind Biotechnol 12:40–57. https://doi.org/10.1089/ind.2015.0017

Dukare AS, Paul S, Nambi VE, Gupta RK, Sharma K, Vishwakarma RK (2019) Exploitation of microbial antagonists for the control of postharvest diseases of fruits: a review. Crit Rev Food Sci Nutr 59:1498–1513

Fonseca NR, Guimarães LMS, Pires RP, Klopfenstein NB, Alfenas AC (2017)

Eucalypt powdery mildew caused by *Podosphaera pannosa* in Brazil. Trop Plant Pathol 42:261–272. https://doi.org/10.1007/s40858-017-0143-7

Fonseca WL, Cardoso JE, Ootani MA, Brasil SOS, Assunção FS, Lima CS, Viana FMP, Muniz CR, Andrade IL, Martins MVV (2019) Morphological, molecular phylogenetic and pathogenic analyses of *Erysiphe* spp. causing powdery mildew on cashew plants in Brazil. Plant Pathol 68:1157–1164. https://doi.org/10.1111/ppa.13032

Freimoser FM, Rueda-Mejia MP, Tilocca B, Migheli Q (2019) Biocontrol yeasts: mechanisms and applications. World J Microbiol Biotechnol 35:154. https://doi.org/10.1007/s11274-019-2728-4

Gao M, Ploessl D, Shao Z (2019) Enhancing the co-utilization of biomass-derived mixed sugars by yeasts. Front Microbiol 9:3264. https://doi.org/10.3389/fmicb.2018.03264

Glawe DA (2008) The powdery mildews: A review of the world's most familiar (yet poorly known) plant pathogens. Annu Rev Phytopathol 46:27–51. https://doi.org/10.1146/annurev.phyto.46.081407.104740

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94:205–214. https://doi.org/10.1007/s00253-011-3694-4

Lahlali R, Raffaele B, Jijakli MH (2011) UV protectant for *Candida oleophila* (strain O) a biocontrol agent of postharvest fruit diseases. Plant Pathol 60:288–295

Laur J, Ramakrishnan GB, Labbé C, Lefebvre F, Spanu PD, Bélanger RR (2018) Effectors involved in fungal–fungal interaction lead to a rare phenomenon of hyperbiotrophy in the tritrophic system biocontrol agent–powdery mildew–plant. New Phytol 217:713–725. https://doi.org/10.1111/nph.14851

Long TM, Su YK, Headman J, Higbee A, Willis LB, Jeffries TW (2012) Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. Appl Environ Microbiol 78:5492–5500. https://doi.org/10.1128/AEM.00374-12

Maicas S (2020) The role of yeasts in fermentation processes. Microorganisms 8:1142. https://doi.org/10.3390/microorganisms8081142

Mattanovich D, Sauer M, Gasser B (2014) Yeast biotechnology: teaching the old dog new tricks. Microb Cell Fact 13:34. https://doi.org/10.1186/1475-2859-13-34

Nandy SK, Srivastava RK (2018) A review on sustainable yeast biotechnological processes and applications. Microbiol Res 207:83–90. https://doi.org/10.1016/j.micres.2017.11.013

Nguyen NH, Suh SO, Marshall CJ, Blackwell M (2006) Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. Mycol Res 110:1232–1241. https://doi.org/10.1016/j.mycres.2006.07.002

Nielsen J (2019) Yeast systems biology: model organism and cell factory. Biotechnol J 14:e1800421. https://doi.org/10.1002/biot.201800421

Pauly M, Keegstra K (2008) Cell-wall carbohydrates and their modification as a resource for biofuels. Plant J 54:559–568. https://doi.org/10.1111/j.1365-313X.2008.03463.x

Reis EM, Zanatta M, Brustolin F, Danelli ALD (2013) Sensitivity reduction in *Blumeria graminis f. sp. hordei* to triadimenol fungicide applied as barley seed treatment. Summa Phytopathol 38:55–57. https://doi.org/10.1590/s0100-54052013000400008

Ribeiro LE, Albuini FM, Castro AG, Campos VJ, de Souza GB, Mendonça JGP, Rosa CA, Mendes TAO, Santana MF, da Silveira WB, Fietto LG (2021) Influence of glucose on xylose metabolization by *Spathaspora passalidarum*. Fungal Genet Biol 157:103624. https://doi.org/10.1016/j.fgb.2021.103624

Rodrussamee N, Sattayawat P, Yamada M (2018) Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. BMC Microbiol 18:73. https://doi.org/10.1186/s12866-018-1218-4

Santhanam P, Labbé C, Fietto LG, Bélanger RR (2021) A reassessment of flocculosin-mediated biocontrol activity of *Pseudozyma flocculosa* through CRISPR/Cas9 gene editing. Fungal Genet Biol 153:103573

Sun L, Jin Y-S (2021) Xylose assimilation for the efficient production of biofuels and chemicals by engineered *Saccharomyces cerevisiae*. Biotechnol J 16:e2000142. https://doi.org/10.1002/biot.202000142

Teixidó N, Usall J, Torres R (2022) Insight into a successful development of biocontrol agents: production, formulation, packaging, and shelf life as key aspects. Horticulturae 8:305. https://doi.org/10.3390/horticulturae8040305

Toivari MH, Salusjarvi L, Ruohonen L, Penttila M (2004) Endogenous xylose pathway in *Saccharomyces cerevisiae*. Appl 70:3681–3686. https://doi.org/10.1128/AEM.70.6.3681

Traquair JA, Shaw LA, Jarvis WR (1988) New species of *Stephanoascus* with Sporothrix anamorphs . Can J Bot 66:926–933. https://doi.org/10.1139/b88-134

Veras HCT, Campos CG, Nascimento IF, Abdelnur P V., Almeida JRM, Parachin NS (2019) Metabolic flux analysis for metabolome data validation of naturally xylose-fermenting yeasts. BMC Biotechnol 19:58. https://doi.org/10.1186/s12896-019-0548-0

Zabed H, Sahu JN, Suely A, Boyce AN, Faruq G (2017) Bioethanol production from renewable sources: current perspectives and technological progress. Renew Sustain Energy Rev 71:475–501. https://doi.org/10.1016/j.rser.2016.12.076

Zhang X, Li B, Zhang Z, Chen Y, Tian S (2020) Antagonistic yeasts: A promising alternative to chemical fungicides for controlling postharvest decay of fruit. J Fungi 6:158. https://doi.org/10.3390/jof6030158

Zhu J, Zhou Y, Shang Y, Hua W, Wang J, Jia Q, Liu M, Yang J (2016) Genetic evidence of local adaption and long distance migration in *Blumeria graminis f. sp. hordei* populations from China. J Gen Plant Pathol 82:69–81. https://doi.org/10.1007/s10327-016-0643-1

Chapter 1

Transcriptome profiling brings new insights into the ethanol stress responses of *Spathaspora passalidarum*

1. INTRODUCTION

Microorganisms are cell factories to produce a large spectrum of value-added compounds. Among them, yeasts present many biotechnological properties that are of economic relevance. The model yeast *Saccharomyces cerevisiae* is one of the most studied microorganisms (Kavšček et al. 2015). This yeast became the preferred microbial factory for many industrial fermentative processes for several reasons, including the vast number of studies that gave rise to its robust physiologic and metabolic characterization, well-annotated genome, and the availability of various tools for genetic manipulations. Despite these advantages, *S. cerevisiae* lacks some desirable features that other non-conventional microorganisms, yet little exploited, might present (Fatma et al. 2020).

The ability to ferment xylose is a desirable trait shown by some groups of microorganisms (Jeffries 1983). Xylose is the second most abundant carbohydrate in lignocellulosic hydrolysates (Zabed et al. 2017). The fermentation of xylose, along with the other available sugars in vegetal biomass, is a crucial step in the optimization of second-generation (2G) ethanol production (Saha 2003). Despite the known advantages of *S. cerevisiae* utilization in the ethanol industry, this yeast does not naturally ferment xylose, and the genetically engineered strains for glucose and xylose fermentation still present relevant fermentative limitations (Gao et al. 2019; Sharma and Arora 2020).

Spathaspora passalidarum is a non-conventional yeast that has been receiving attention for biomass hydrolysate fermentation (Cadete and Rosa 2017). Described by Nguyen and colleagues (2006) (Nguyen et al. 2006), *Spathaspora passalidarum* NRRLY 27907 is a wood-boring beetle associated-yeast that efficiently ferments xylose and other sugars under different aeration conditions with high yields (close to the theoretical maximum) and productivities (Hou 2012; Long et al. 2012; Cadete and Rosa 2017; Ribeiro et al. 2021; Campos et al. 2022). In a comparative study performed by Veras and colleagues (2017) with four native xylose-fermenting yeasts, the highest ethanol yields under oxygen limitation were obtained for *S. passalidarum* and *Scheffersomyces stipitis*, while *S. passalidarum* showed the best xylose consumption and ethanol production rates compared to the other yeasts under anaerobic condition.

However, xylose-fermenting yeasts are usually sensitive to different stresses associated with industrial conditions, such as the accumulation of ethanol, the end product of fermentative metabolism (Campos et al. 2022). Although high levels of ethanol are required to reduce the distillation costs of the bioethanol production process, it severely damages the cells, impairs growth, and even stops fermentation (Deparis et al. 2017). Ethanol causes multi-aspect stress, which increases membrane permeability, promotes loss of proton motive force, causes cytosol acidification, interferes with transport systems, harms protein structure and generates protein aggregates, increases the production of reactive oxygen species (ROS), and others (Ma and Liu 2010; Auesukaree 2017).

Yeast cells must adapt their metabolism and many other aspects of their cell biology to ensure the necessary changes for stress adaptation. Among these changes, the translation of most proteins expressed under normal situations is repressed while stress-related proteins are induced. Relevant proteins for yeast environmental stress response include heat-shock proteins (HSPs), enzymes required for energy generation, proteins involved in cellular redox and antioxidant response, DNA repair, cell wall and membrane structures, transport systems, and cell signaling, for example (Gasch et al. 2000). Even though there are many other levels of gene regulation besides transcriptional control, one of the main ways of changing protein synthesis is by controlling the amount of mRNA produced. Therefore, changes in the transcriptional level constitute a significant component of stress responses (De Nadal et al. 2011).

Regarding the ethanol stress response of *S. passalidarum*, it was already demonstrated that some central genes activated in yeasts facing the effects of ethanol were downregulated in *S. passalidarum* submitted to 4% (v/v) ethanol (Campos et al. 2022). The study suggested that S. *passalidarum* might not have activated a proper response to deal with ethanol stress, which could have contributed to its low tolerance profile. The authors suggested a broader transcriptional approach to improve the understanding of this phenomenon.

Genome-wide transcriptional analyses have been used to elucidate transcriptional alterations and identify genes involved in ethanol stress response in yeasts (Alexandre et al. 2001; Chandler et al. 2004; Hirasawa et al. 2007; Dinh et al.

2009; Li et al. 2010; Kasavi et al. 2016; Diniz et al. 2017; Miao et al. 2018; Mo et al. 2019). Even though ethanol responses have been extensively studied in the yeast *S. cerevisiae*, ethanol tolerance is not yet fully understood for this model organism. On the other hand, very few studies have focused on changes in global gene expression of non-conventional yeasts under ethanol stress. In this work, the molecular responses of *Spathaspora passalidarum* subjected to ethanol stress were investigated by RNA-seq aiming to reveal important clues to improve this yeast tolerance to ethanol.

2. MATERIALS AND METHODS

2.1. Yeast strain and maintenance

This study used the *Spathaspora passalidarum* NRRLY 27907 strain. The yeast was stored in YPD medium (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose) with 20% (v/v) glycerol at -80°C. For routine use, cells were plated on YPX-agar medium (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ xylose, 20 $g.L^{-1}$ agar). Isolated colonies were used in cell growth assays in YPX liquid media conducted in three biological replicates. Growth measurements were performed by recording optical density readings at 600 nm (OD_{600}) using a spectrophotometer (Biospectro SP-220).

2.2. Tolerance assays and metabolites measurements

Overnight yeast cultures were grown at 32°C at 120 rpm. From the pre-inoculum, the cells were diluted to an initial OD_{600} of 0.50 in 25 mL of YPX fresh medium and incubated for growth until reaching the exponential phase. Subsequently, the OD_{600} was adjusted to 1.0 and serial dilutions were performed (10^{-1} – 10^{-3}). Five microliters of each dilution were spotted on YPX solid medium containing ethanol (0, 2, 4, and 8% v/v), and the plates were incubated at 30°C for 72 h. Ethanol tolerance tests were also performed in liquid YPX medium without ethanol or added with 1, 2, 3, 4, 5, 6, 7, and 8% ethanol (v/v). The overnight grown pre-inoculum was transferred into 25 mL fresh medium to an initial OD_{600} of 0.50. The flasks were incubated for 48 h at 32°C at 120 rpm before OD_{600} measurements.

For the fermentation assays, *S. passalidarum* cells were inoculated into 125 mL Erlenmeyer flasks containing 25 mL of fresh YPX medium added of ethanol (0, 4, 5, and 6% v/v) and incubated at 32°C, 120 rpm. The initial OD_{600} was adjusted to 1.0. Samples were taken during the incubation to evaluate cell viability and quantify xylose and ethanol concentrations. Cell viability assays were performed by adjusting the OD_{600} to 1.0 and then spotting serial dilutions of the cultures on solid media (YPX-agar), followed by incubation for 36 h. Colonies were counted, and the number of viable cells was expressed as $CFU.mL^{-1}$. Xylose and ethanol were quantified by high-performance liquid chromatography (HPLC) using a refractive index detector (Shimadzu RID-10A) and Bio-Rad Aminex HPX-87H column at 45°C. The mobile phase consisted of sulfuric acid (5 mM) at a flow rate of 0.7 $ml.min^{-1}$. The samples were previously filtered using a 0.22 µm membrane filter. Metabolites quantification was performed using xylose and ethanol standard curves.

2.3. Experimental design, RNA isolation and sequencing

Cells used for RNA isolation were grown in YPX medium overnight and then transferred to 125 mL Erlenmeyer flasks containing 20 mL of fresh medium (initial OD_{600} ~ 0.20). The flasks were incubated at 32°C and stirred at 120 rpm until the cells reached the mid-exponential phase of growth (OD_{600} 0.8 ~ 1.0). Then, the cultures volumes were divided into two new flasks: one with fresh YPX (control) medium and the other containing YPX added of ethanol to a final concentration of 4% (v/v) (treatment). The flasks were incubated in the conditions described above, and cell growth was recorded during the experiment. After 2 hours of stress exposure, cells from control and treatment conditions were collected and treated with Lyticase from *Arthrobacter luteus* (Sigma-Aldrich). Total RNA was extracted using the RNeasy Mini kit (Qiagen) and quantified with Qubit 3.0 Fluorometer (Invitrogen). The sequencing was performed with the Illumina NovaSeq 6000 (100bp PE (pair-end)) sequencing platform (Macrogen Inc., Seoul, South Korea). Three biological replicates were performed for each condition.

2.4. RNA sequencing analysis

The reports of FastQC (version 0.11.5, Andrews, 2010) assessed the quality of mRNA-Seq sequencing data, and Trimmomatic (version 0.36, (Bolger et al. 2014)) trimmed and filtered the low-quality (Phred score <15, MINLEN: 50) data. The bowtie2-build tool of Bowtie2 (version 2.2.8, (Langmead and Salzberg 2012)) indexed the *S. passalidarum* reference genome (NCBI Accession Number: GCF_000223485.1), and the reads were mapped to the indexed genome using TopHat (version 2.1.1, (Trapnell et al. 2009)). The Cufflinks (version 2.2.1, (Trapnell et al. 2010)) assembled the transcripts sequences and estimated their expression in Fragments Per Kilobase per Million mapped reads (FPKM). The Fold Change (FC) calculation was performed by dividing the FPKM values of the genes expressed in the treatment by the genes expressed in the control condition. The selection of differentially expressed genes (DEGs) considered combined criteria of $|Log2FC| \geq 1.75$, P value <0.05, and FDR (false discovery rate) <0.001.

2.5. Functional annotation

The proteins encoded by *Spathaspora passalidarum* differentially expressed genes were aligned to the reviewed sequences of the Swiss-Prot database and to the reference proteomes of *S. passalidarum* (Proteome ID UP000000709) and *S. cerevisiae* (Proteome ID UP000002311) available in the UniProt database (Consortium 2023), using the BLASTp tool of BLAST version 2.13.0 (Altschul et al. 1990). The proteins were also aligned with the Position-Specific Scoring Matrices (PSSMs) of the Pfam database (Mistry et al. 2021), using the Reverse Position Specific BLAST (RPS-BLAST) tool of BLAST. The E-value score threshold $\leq 1^{-10}$ filtered the significant alignments, and the gene ontology (GO) terms assigned to the proteins were those from the best-hit sequences.

2.6. GO and KEGG enrichment analysis for differentially expressed genes

The GO enrichment analysis performed by YeastEnricher (Kuleshov et al. 2019) (https://maayanlab.cloud/YeastEnrichr/) determined the statistically (P-value < 0.0001) overrepresented terms of biological process, molecular function, and cellular component categories among the DEGs. The pathways enrichment analysis performed using KOBAS (Xie et al. 2011) (http://kobas.cbi.pku.edu.cn) identified significantly enriched pathways (adjusted P-value < 0.01) among those from the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.7. RT-qPCR analysis

The experimental design described for RNA isolation was repeated for harvesting samples for quantitative PCR analysis at 2 and 12 h of incubation of *S. passalidarum* cells under ethanol stress (0% and 4% (v/v)). The experiments were conducted in biological triplicates. Total RNA was quantified using Qubit 3.0 Fluorometer (Invitrogen), run in agarose gel to assess the quality, and treated with RNase-free DNase I (Sigma-Aldrich). cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's instructions. Primers for the target genes were designed using the GenScript Real-time PCR tool (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool), and the nucleotide specificity was evaluated using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye et al. 2012). The Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOne™ Real-Time PCR System (Applied Biosystems) were used for the qPCR analysis. The assays were carried out in technical duplicate using the following conditions: 10 min at 95ºC and 40 cycles of 15 s at 95ºC, 1 min at 60ºC. Relative mRNA quantification was performed by the standard curve method. As such, a standard curve was obtained for each gene by plotting the average Ct from the technical duplicate versus the log10 of different concentrations of *S. passalidarum* genomic DNA (1.56 – 100 $ng.\mu L^{-1}$). The results were normalized using actin (ACT1). An unpaired two-tailed t-test (GraphPad Prism 5) was used for the statistical analysis. Thirteen genes were selected for the qPCR analyses. Primers sequences and gene accession numbers are available in Supplemental Table 1 (Appendix A).

2.8. Fatty acid profile determination

Single yeast colonies were inoculated in YPX media in triplicate and incubated for approximately 16 h at 32°C at 120 rpm. From the pre-inoculum, cells were re-activated to the mid-exponential phase of growth (OD_{600} 0.8 ~ 1.0) as described in the experimental design for the RNA isolation. Each culture was divided into two new Erlenmeyer flasks, and ethanol was added to one of them to a final concentration of 4% (v/v). For the determination of the fatty acid content of *S. passalidarum*, the total volume of the cells was harvested (10.000 rpm, 4°C, 10 min) at 2 and 12 h of incubation. The three pellets from the same condition were pulled together and immediately used for fatty acid extraction according to the Sherlock Instant Fame™ User's Guide (Newark). Agilent 7890A gas chromatograph with a flame ionization detector (Agilent Technologies) was used to separate the mixture and identify the fatty acids using the MIDI microbial identification system (Sherlock 6.0 Microbial Identification System, Newark, USA).

2.9. Scanning electron microscopy (SEM) analysis

The cells were cultured as described in the previous item. Samples of *S. passalidarum* cells from the control (0% (v/v) ethanol) and stress (4% (v/v) ethanol) conditions were collected at 2 and 12 h of incubation at 32ºC and 120 rpm. Pellets were washed twice (10,000 rpm, 10 min) with potassium phosphate buffer (100 mM, pH 7.2) and resuspended in 500 µl of the same buffer. Cells were visualized under a light microscope, and dilutions were performed accordingly. Thirty µl of 0.1% (w/v) poly-L-lysine (Sigma-Aldrich) were mixed with 10 µl of each sample. The total volume was pipetted on the surface of a parafilm, covered with a coverslip, and maintained at room temperature for 40 min. The coverslip was washed twice with potassium phosphate buffer (100 mM, pH 7.2) for 10 min, then fixed overnight in 2.5% glutaraldehyde. Subsequently, three washes with potassium phosphate buffer (100 mM, pH 7.2) were performed, and the samples were sequentially dehydrated with alcohol 30, 50, 70, 80, and 90% (v/v) at room temperature for 15 min each time. A further dehydration step was performed by incubating the samples in 100% alcohol three times. Afterward, the coverslips were dried to the critical point (Critical Point Dryer - CPD®, Bal-tec, model 030), mounted on the specimen holders (stubs), and covered with gold using the metallizer Sputter coater (Balzers, model FDU 010). The images were captured using a scanning electron microscope (LEO, model 1430 VP) operating at 20 kV.

3. RESULTS

3.1. Physiological characterization of *S. passalidarum* under ethanol stress

Ethanol tolerance assays showed that *S. passalidarum* was unable to grow on solid media with ethanol concentrations higher than 6% (v/v) (Fig. 1a). The presence of ethanol severely limited *S. passalidarum* growth in the liquid medium, reducing its relative growth by 50% in 4% ethanol (v/v) and by more than 90% in 6% ethanol (v/v) after 48 h of incubation (Fig. 1b). Overall, the addition of ethanol at the start of the fermentation slowed sugar consumption and yeast growth. No ethanol production was observed under the evaluated stress conditions. Cell viability was also not reduced. In 6% ethanol (v/v), the cells remained latent for the initial 48 h. During this period, there was a constant decrease in ethanol concentration. When approximately 31 $g.L^{-1}$ of ethanol was reached (~ 4% v/v), growth and xylose consumption began, indicating that higher concentrations inhibit *S. passalidarum* metabolism in liquid media and that ethanol impaired growth without causing cell death (Fig. 1c).

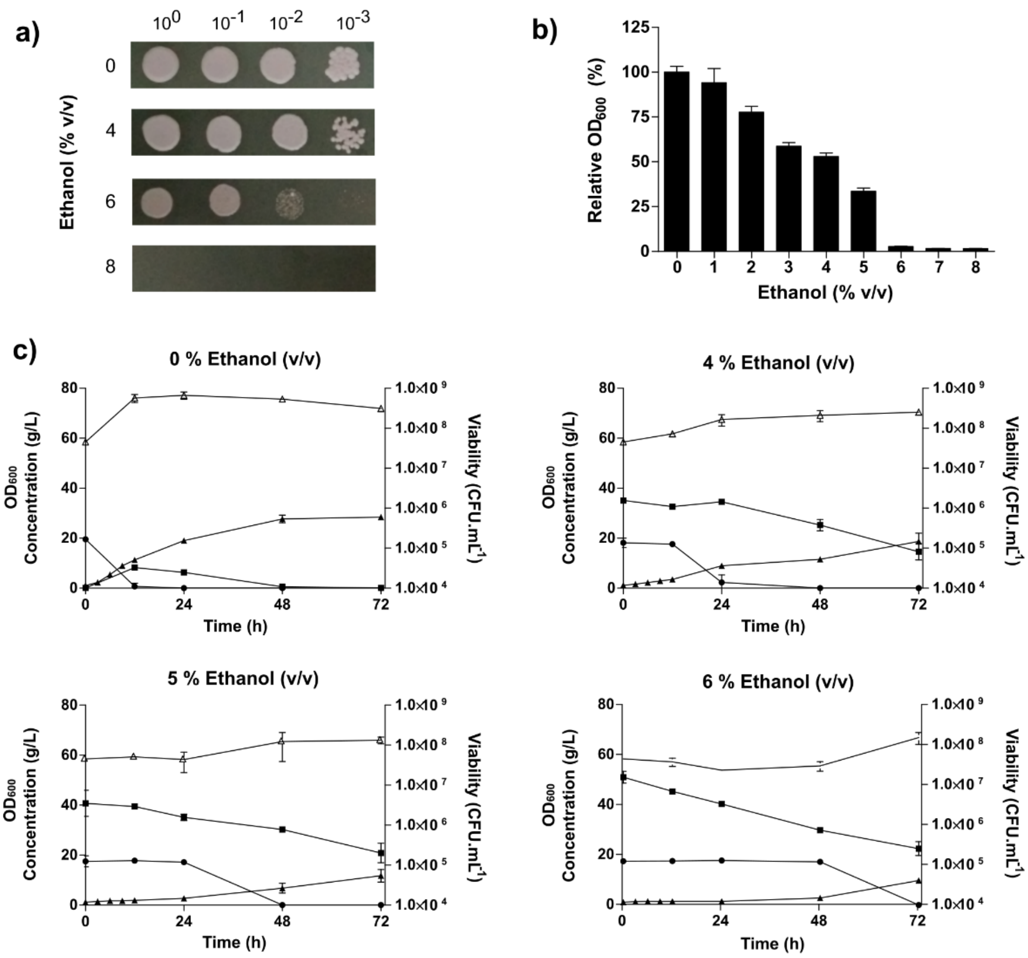


Figure. 1 Ethanol tolerance and production in *Spathaspora passalidarum*. **a)** Spotting assay in YPX media containing different ethanol concentrations. Petri dishes were incubated for 3 days at 30ºC before pictures were taken. **b)** Ethanol tolerance in liquid medium. OD_{600} values were measured after 48 h of growth and were expressed relative to the control (0% ethanol). **c)** Fermentation profile of *S. passalidarum* in YPX medium in the absence and presence of ethanol (v/v). Samples

taken at 12, 24, 48, and 72 h were used for xylose (●) and ethanol (■) quantifications, to perform OD_{600} measurements (▲), and to evaluate cell viability (△).

3.2. Overall differential gene expression in response to ethanol

An RNA-seq experiment comparing the expression profiles of *S. passalidarum* growing without ethanol exposure (control) and under 2 hours of 4% (v/v) ethanol stress (treatment) was performed to understand the mechanisms underlying this yeast ethanol response (Fig. 2a). This stress condition was selected because it promoted a considerable reduction in the yeast specific growth rate (0.38 h^{-1} (control) to 0.22 h^{-1} (treatment)) without causing cell death (Fig. 2b). Principal component analysis (PCA) demonstrated that there are no confounding effects between the treatments, with the first principal component (PC1) separation corresponding to 96.73% of the existing differences in the transcriptional profile (Fig. 2c). The transcriptome analysis mapped 5,982 from the 6,013 genes predicted in the *S. passalidarum* genome (Wohlbach et al. 2011). The analysis revealed that 36 genes were only expressed in the control condition, and 65 genes were exclusively expressed on cells exposed to ethanol stress (Supplemental File S1 - T6 – Appendix A). Moreover, a total of 663 genes met the criteria of the differential expression analysis (Supplemental File S1 - T1 - Appendix A). Of these, 448 were more abundant and 215 less abundant in ethanol-stressed cells. Among the DEGs, only 5.58% of them had a gene name assigned. In addition, 38.91% are uncharacterized proteins, 24.73% are unspecific domain-containing proteins, and 1.05% are putative proteins. Thus, the functional annotation analysis predicted the functions of the proteins encoded by the DEGs. Among them, 540 aligned with proteins of the Swiss-Prot database, 499 with proteins of *S. cerevisiae* reference proteome, and 456 with profiles of the Pfam database. The annotation of up and downregulated DEGs of *S. passalidarum* under ethanol stress is available in Supplementary file S2 - T2 - Appendix A.

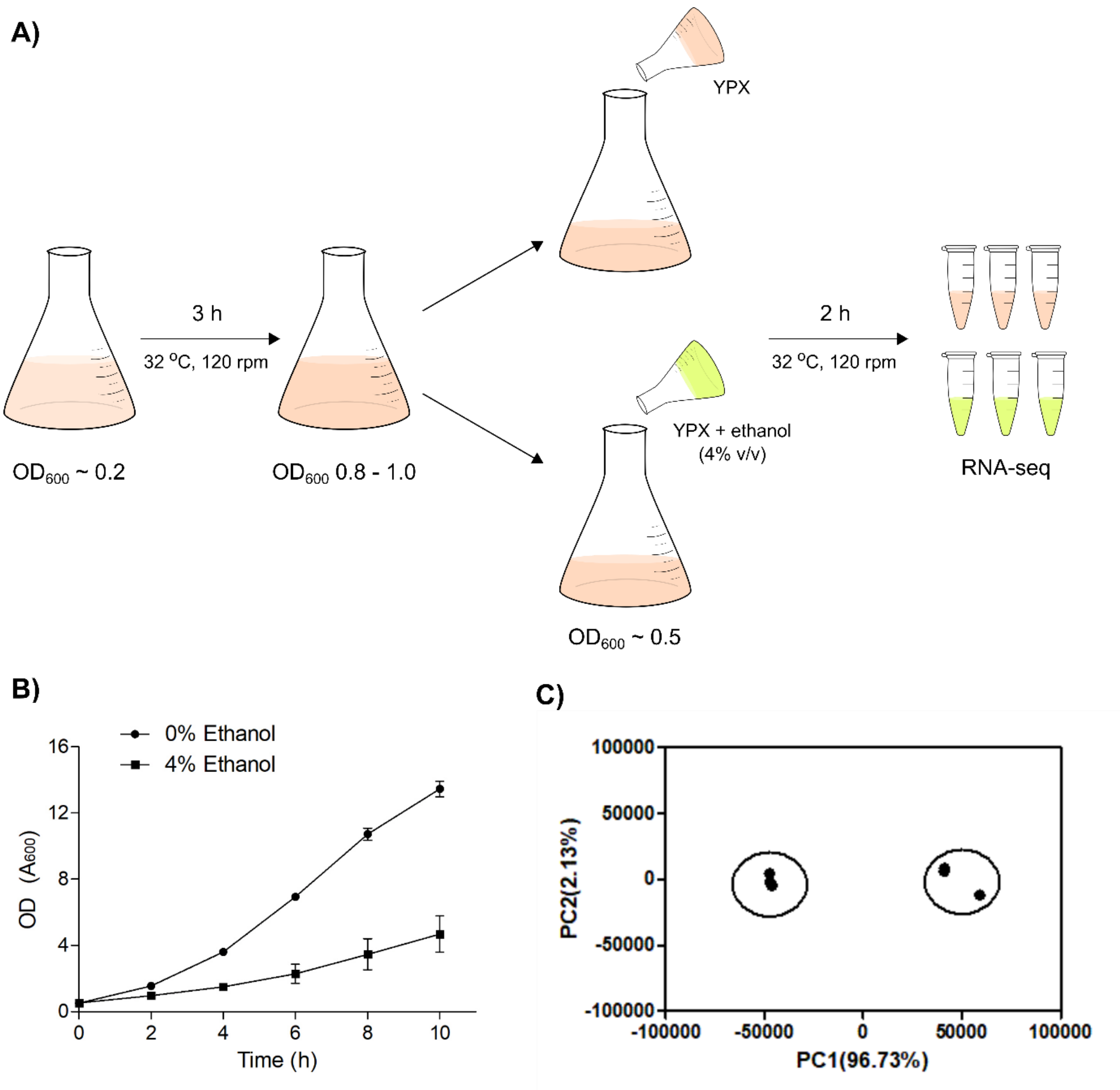


Figure. 2 RNA-seq experimental design of *Spathaspora passalidarum* under ethanol stress. **a)** Samples were collected for RNA isolation after 2 h of cultivation of *S. passalidarum* cells at 120 rpm in the absence and presence of ethanol stress. **b)** Growth curves of control (0% (v/v) ethanol) and ethanol-treated cells (4% (v/v) during 10 h of incubation. Values are represented as mean ± S.D. **c)** Principal component analysis performed using the FPKM data from three biological replicates of each condition indicating that the analyzed samples clustered distinctively.

3.3. GO and KEGG enrichment analysis

The GO enrichment analysis of biological process, molecular function, and cellular component used the DEGs annotated as orthologs of the *S. cerevisiae* genes. The set of downregulated genes was enriched in the biological processes “transcription from RNA polymerase II promoter” and in “transmembrane transport”, and the cellular component terms “nucleus”, “integral component of the plasma membrane”, and “vacuole”. The upregulated genes were mainly enriched in translation-related biological processes and in the categories of “cytosol” and “ribosome-related structures” for cellular components. All GO classifications are available in Supplemental File S1 - T3 - Appendix A.

The data were also analyzed using KOBAS to search for KEGG pathways statistically enriched (q-value < 0.01) with the DEGs. In this analysis, only the set of upregulated genes resulted in significantly enriched pathways, as shown in Supplemental File S1 - T4 - Appendix A. The enriched pathways retrieved were related to “ribosome” (spaa03010, q < 0.01) and “ribosome biogenesis in eukaryotes” (spaa03008, q < 0.01).

3.4. Ethanol compromises the expression of membrane components and might affect other membrane-related processes

The enrichment analysis demonstrated that the cellular membrane was a cellular component negatively affected by ethanol stress. Genes involved in ergosterol biosynthesis (*ERG3* and *ERG25*), as well as genes related to inositol biosynthesis and metabolism, such as *INO1*, which encodes inositol-3-phosphate synthase, were found to be downregulated (Supplementary File S2 - T5 - Appendix A). Moreover, genes related to the biosynthesis of unsaturated fatty acids were not differentially expressed between the conditions evaluated in our study.

The expression of genes encoding putative proteins that localize within or across the membranes was also negatively affected, such as the ones involved in the transport of nutrients (GO:0055085; GO:0015758; GO:0006812). In this regard, we observed the downregulation of many putative sugar transporters after ethanol stress, including members of the *HXT* family and an ortholog of *SLT1*, which encodes a glycerol proton symporter (Tab. 1). Genes encoding an ammonium transporter (*MPE3*) and a plasma membrane iron permease (*FTR1*) were also less expressed in the stress condition. Similarly, downregulation was observed for the genes encoding the general amino acid permeases *GAP1* and *AGP1*, and the specific proline and arginine permeases *PUT4* and *CAN1*, respectively (Tab. 1). Consistent with these results, the *STP2* gene, which encodes a transcription factor that activates the transcription of amino acid permease genes, also showed downregulation.

The expression of genes involved in amino acid metabolism was also reorganized. The amino acid biosynthetic enzymes *ARG7* and *CPA2* (arginine biosynthesis), and *TPR2* (tryptophan biosynthesis) had their expressions upregulated. Moreover, the *CTR86* gene, which is related to the threonine biosynthetic process, was expressed only under the stress condition. The *GLY2* gene, involved in glycine biosynthesis by converting threonine to glycine, was downregulated. The *GCV2* gene, which encodes a decarboxylase that catabolizes glycine into CO2 and NH3, was upregulated. *PUT1*, involved in proline oxidation to be used as a nitrogen source, and *GLN1*, which synthesizes glutamine from glutamate and NH3, showed both downregulations (Supplemental File S1 - T5 - Appendix A).

It is worth noticing that some genes encoding putative membrane transporters were upregulated. The *MDR1* gene of *S. passalidarum*, which encodes a multidrug resistance protein, appears as the fourth most upregulated gene of the transcriptome. Besides this gene, another two genes encoding drug resistance proteins (*FLR1* and *PDR5*) were upregulated under the stress condition (Tab. 1).

Table 1. Differentially expressed genes encoding membrane transporters/sensors/permeases of *S. passalidarum* cultivated in the presence of 4% (v/v) ethanol.

ID	Gene name (Sp)	Gene name (Sc)	Protein annotation (Sp)	Protein annotation (Sc)	Log_2FC
SPAPADRAFT_59608	XUT1	RGT2	Putative sugar trans-porter, high affinity	High glucose sensor RGT2	-2,70487
SPAPADRAFT_57693	HXT2.4	HXT2	Uncharacterized protein HXT2.4	High-affinity glucose trans-porter HXT2	-3,35831
SPAPADRAFT_55953	HGT1	HXT11	High affinity glucose transporter	Hexose transporter HXT11	-2,71576
SPAPADRAFT_64051	HGT1.2	HXT13	High affinity glucose transporter	Hexose transporter HXT13	-2,34583
SPAPADRAFT_145342	HGT2	RGT2	Uncharacterized protein HGT2	High glucose sensor RGT2	-1,81759
SPAPADRAFT_59267	Not annotated	STL1	MFS domain-containing protein	Sugar transporter STL1	-2,93901
SPAPADRAFT_61698	FTR1	FTR1	Plasma membrane iron permease	Plasma membrane iron permease	-3,6575
SPAPADRAFT_62045	Not annotated	MEP3	Ammonium transporter	Ammonium transporter MEP3	-2,90631
SPAPADRAFT_61775	Not annotated	ITR1	MFS domain-containing protein	Myo-inositol transporter 1	-2,01319
SPAPADRAFT_60662	Not annotated	ITR2	MFS domain-containing protein	Myo-inositol transporter 2	-2,56663
SPAPADRAFT_61231	Not annotated	GAP1	General amino acid permease	General amino-acid permease GAP1	-1,87303

SPAPADRAFT_56131	GAP2.1	GAP1	Uncharacterized protein GAP2.1	General amino-acid permease GAP1	-4,75696
SPAPADRAFT_143839	AGP1	AGP1	High-affinity glutamine permease	General amino acid permease AGP1	-2,78465
SPAPADRAFT_51185	Not annotated	PUT4	Putative proline permease	Proline-specific permease	-3,59016
SPAPADRAFT_63185	Not annotated	CAN1	Arginine, lysine, histidine permease Can1p	Arginine permease CAN1	-2,12722
SPAPADRAFT_154329	Not annotated	PDR5	Drug resistance protein 1	Pleiotropic ABC efflux transporter of multiple drugs	1,93855
SPAPADRAFT_142204	Not annotated	FLR1	Multidrug resistance protein 7	Fluconazole resistance protein 1	4,53128
SPAPADRAFT_61521	MDR1	FLR1	Benomyl/methotrexate resistance protein	Fluconazole resistance protein 1	5,41267

Sp, *Spathaspora passalidarum*; Sc, *Saccharomyces cerevisiae*

3.5. Metabolic reorganization upon ethanol exposure

The presence of ethanol altered the expression of some genes that constitute the main carbon pathways (Fig. 3). Genes encoding putative enzymes involved in glycolysis and ethanol fermentation, such as glyceraldehyde-3-phosphate dehydrogenase (*TDH3*), glycerate phosphomutase (*GMP1-like*), and alcohol dehydrogenase (*ADH5*), were downregulated under the stress condition. Likewise, the xylose metabolism also appears to have been affected by ethanol. The gene that encodes xylose reductase (*XYL1.1*), the first enzyme of the xylose assimilation pathway, was upregulated, and the gene encoding the enzyme involved in the second reaction, xylitol dehydrogenase (*XYL2.1*), was downregulated. This expression profile can result in xylitol accumulation, which would prevent the carbon source from being directed to glycolysis and ethanol production.

The expression data suggest that part of the acetyl-CoA generated under the stress condition was directed to the glyoxylate cycle due to the upregulation of the genes encoding the enzymes isocitrate lyase (*ICL1*) and malate synthase (*MLS1* and *DAL7*) (Fig. 3). The gene encoding the enzyme malate dehydrogenase (*MDH3*), which oxidates malate into oxaloacetate using NAD+, showed downregulation. Thus, it is possible that the malate produced by this cycle was used for the biosynthesis of precursors. Besides turning into malate, another possible alternative for the glyoxylate produced under this condition was oxidation to oxalate and final conversion into formate. The expression of the gene encoding formate dehydrogenase, *FDH1*, was upregulated. This enzyme catalyzes the NAD^+-dependent oxidation of formate to carbon dioxide and H2O, which provides redox equivalents for respiratory metabolism (Fig. 3).

Fatty acid metabolism also appears to have been enhanced under ethanol stress, with both fatty acid biosynthetic and degradation pathways showing upregulation. For the fatty acid biosynthesis, the upregulated genes encoded the enzymes fatty acid synthetase (*FAS1*), enoyl-[acyl-carrier-protein] reductase (*ETR1*), and long-chain-fatty-acid-CoA ligase (*FAA24*). For the beta-oxidation pathway, the genes with increased expression encoded the enzymes acyl coenzyme A oxidase (*POX1*), 3-ketoacyl-CoA thiolase (*POT1*), carnitine O-acetyltransferase (mitochondrial) (*CAT2*), and peroxisomal acyl-coenzyme A thioester hydrolase 1 (*TES1*) (Supplementary File S2 - T5 - Appendix A).

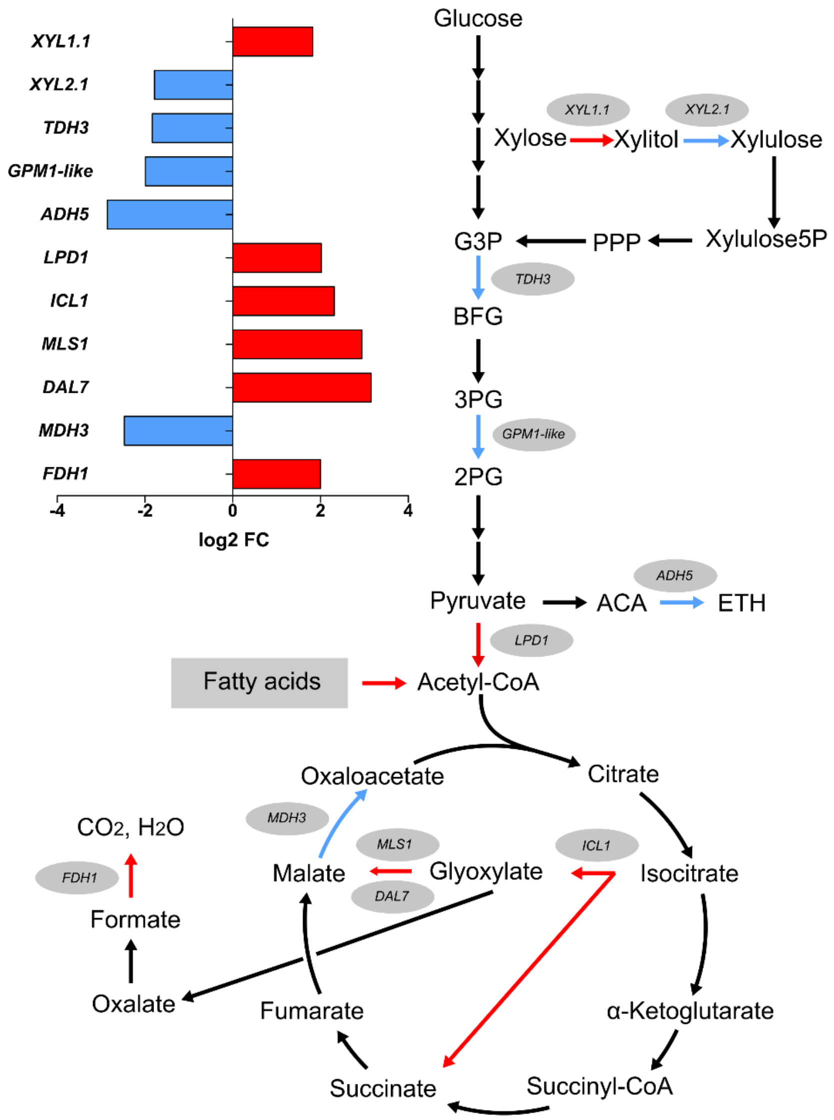


Figure. 3 Ethanol exposure alters the expression of genes encoding enzymes involved in the central metabolic pathways. The enzymes are represented by arrows. Blue arrows account for enzymes whose gene expression was downregulated. Red arrows represent enzymes whose gene expression was upregulated. The expression profile of the genes is shown in the graph containing the Log2(FC) values. Blue columns indicate that the expression was downregulated. Red columns indicate that the expression was upregulated. *THD3*, glyceraldehyde-3-phosphate dehydrogenase 3; *GPM1-like*, glycerate phosphomutase; *ADH5*, alcohol dehydrogenase 5; *LPD1*, dihydrolipoyl dehydrogenase; *XYL1.1*, xylose reductase; *XYL2.1*, xylitol

dehydrogenase; *ICL1*, isocitrate lyase; *MLS1*, malate synthase; *DAL7*, malate synthase; *MDH3*, malate dehydrogenase; *FDH1*, formate dehydrogenase 1.

3.6. Expression of stress responsive genes in *S. passalidarum* under ethanol stress

In response to the stress, it was observed the upregulation of putative chaperone-related genes as *SSB1* and *SSZ1* (members of the HSP70 family), and some heat shock proteins (*HSPs*) genes as *HSP60* and *HSP31*, suggesting that ethanol exposure induces genes involved in preventing protein aggregation and promoting protein refolding (Fig. 4a). Additionally, a putative J domain-containing protein and the chaperone *HGH1* were only expressed under the stressed condition (Supplemental File S1 - T6 - Appendix A). On the other hand, the *HSP30* gene, which encodes a negative regulator of the H^+-ATPase Pma1p, showed downregulation, and genes involved in trehalose biosynthesis were not differentially expressed in our study (Fig. 4a).

Ethanol exposure also triggers the development of oxidative stress. A glutaredoxin domain-containing protein ortholog of the *GRX8* gene of *S. cerevisiae* was present among the genes only expressed in the ethanol treatment condition. Moreover, oxidative stress-responsive genes showing putative superoxide dismutase activity (SPAPADRAFT_62004), NAD(P)H oxidoreductase activity (*OYE32*, *OYE3*, *QOR2*), peroxisome functions (*PEX25*), and other oxidative stress responses (*MHR1*, *LTV1*, *HSP31*) were upregulated under ethanol stress (Fig. 4b). On the other hand, other classical genes involved in the cellular response to oxidative stress (*FAP7*, *POS5*) were downregulated, including two transcription factors (*SKN7* and *HCM1*) (Fig. 4b).

Changes in osmotic pressure are another consequence of ethanol accumulation. The yeast osmoadaptation response involves the activation of the high-osmolarity glycerol (HOG) pathway, which promotes a part of the transcriptional response. The expression of *CLA4*, a gene encoding a putative serine/threonine-protein kinase that shares some essential functions with the HOG pathway kinase member *STE20*, showed upregulation. *PTC1*, another gene from this pathway, which encodes a putative phosphatase that de-activates Hog1p, was downregulated. The expression of the osmo-adaptative genes *ENA1* and *SLT1* were also downregulated (Fig. 4c, d). Additionally, other well-characterized osmo-responsive genes were not observed among the DEGs, especially the ones involved in the biosynthesis of the osmoprotectant molecule glycerol.

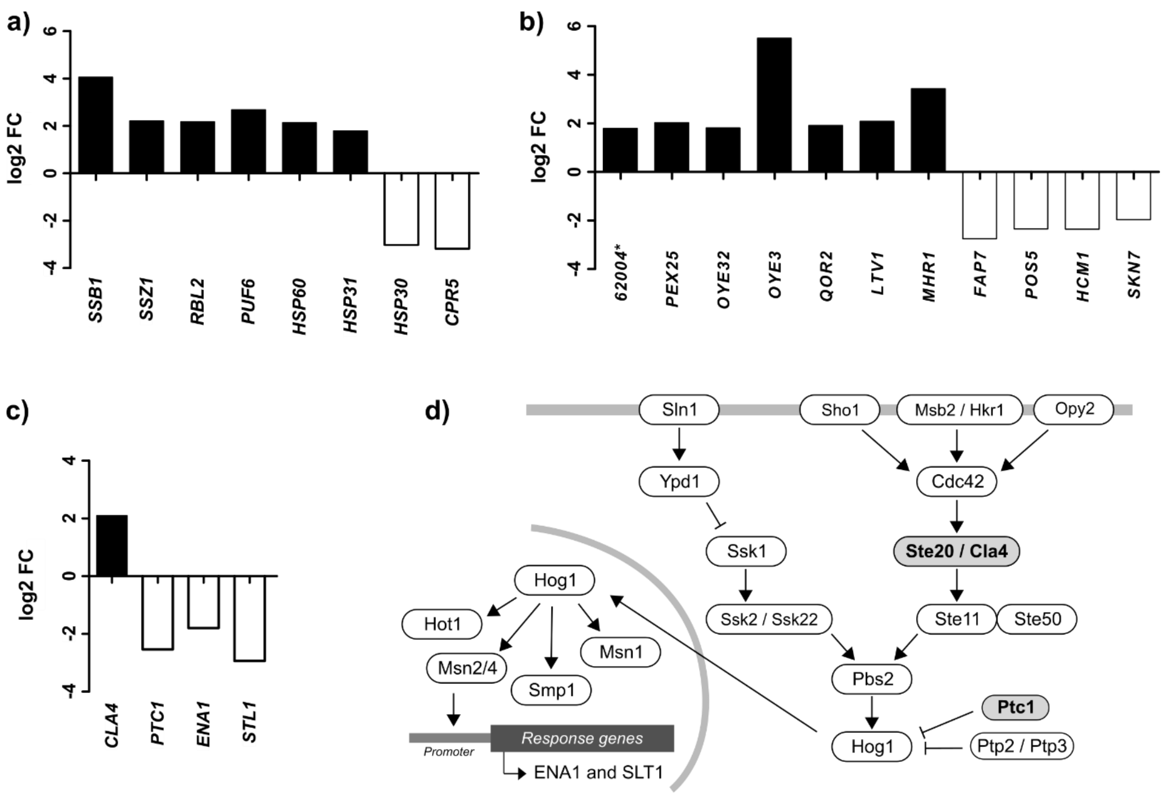


Figure. 4 Expression levels (Log2(FC)) of differentially expressed genes related to **a)** protein folding, **b)** oxidative and **c)** osmotic stress responses. **d)** Representation of the HOG signaling pathway.

3.7. Expression analysis of selected DEGs by quantitative PCR in two time points

Gene expression analysis by qPCR used *S. passalidarum* cells at 2 and 12 hours of incubation under 4% (v/v) ethanol and without the stress (Fig. 5). As expected, all thirteen genes selected for the analysis showed the same expression profile of the transcriptome (2 h), validating our RNA-seq results. At 12 h, a different profile was observed. According to the expression results, xylose metabolism seems to have been activated by the upregulation of the two genes from the xylose assimilation pathway and the *TDH3* gene from the glycolytic pathway. On the contrary, production of ethanol was kept hindered, as shown by the downregulation of *GPM1-like* and *ADH5* genes. Genes from the glyoxylate cycle were either downregulated or not significantly differentially expressed (*ICL1*, *MSL1*, and *DAL7*), and the repression on the sugar transporters started to be released, as shown by the upregulation of the *HGT2* gene.

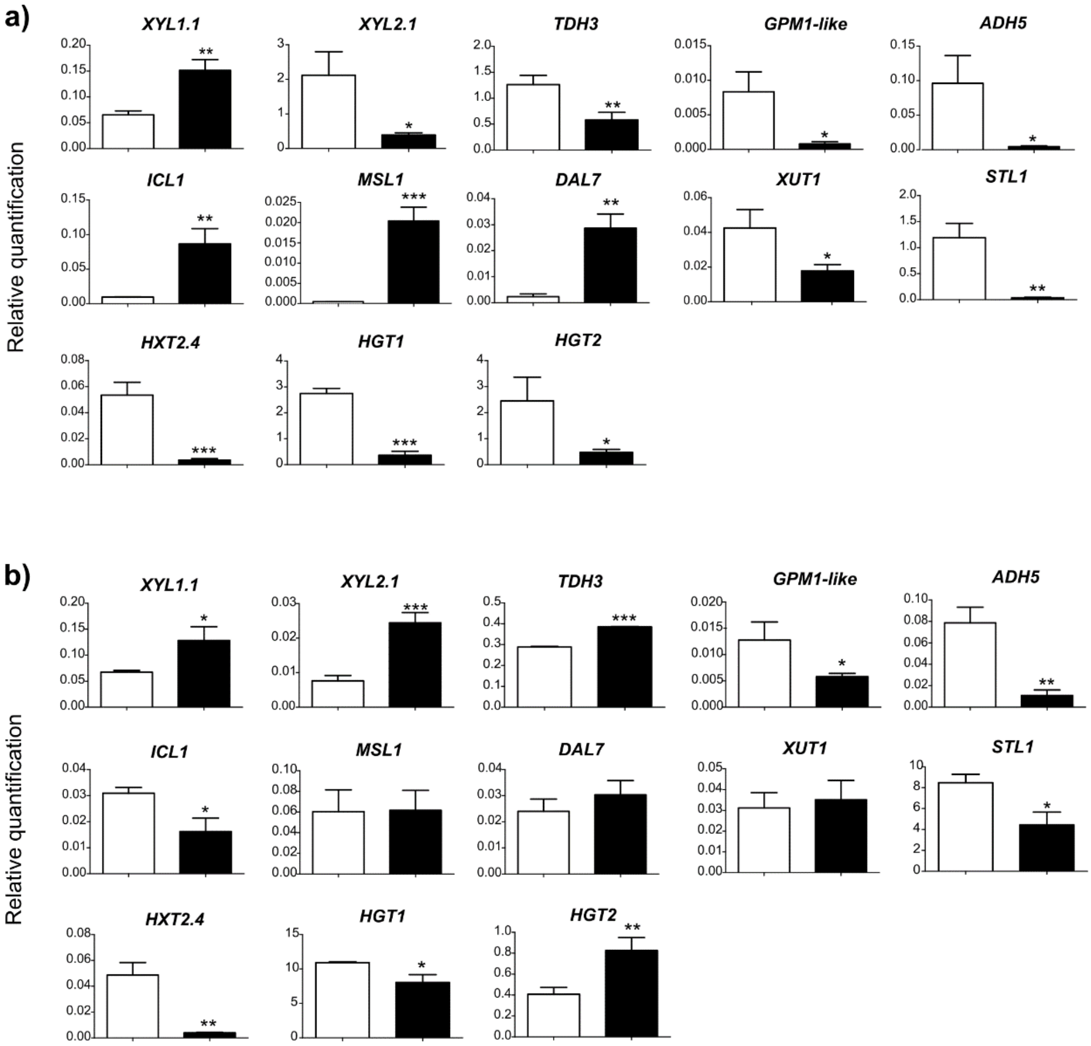


Figure. 5 Expression analysis by qPCR of *S. passalidarum* DEGs after 2 **(a)** and 12 h **(b)** of incubation in the control condition (white column) and 4% (v/v) ethanol stress (black column). The graphs represent the estimated mean and standard deviation values. Asterisks denote statistical significance by the t test (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001). *XYL1.1*, xylose reductase; *XYL2.1*, xylitol dehydrogenase; *THD3*, glyceraldehyde-3-phosphate dehydrogenase 3; *GPM1-like*, glycerate phosphomutase; *ADH5*, alcohol dehydrogenase 5; *ICL1*, isocitrate lyase; *MLS1*, malate synthase; *DAL7*, malate synthase; *XUT1*, putative sugar transporter,

high affinity; *SLT1*, MFS domain-containing protein; *HXT2.4*, uncharacterized protein; *HGT1*, high affinity glucose transporter; *HGT2*, uncharacterized protein.

3.8. Effect of ethanol on *S. passalidarum* fatty acid profile and cellular ultrastructure

The fatty acid profile analysis showed that the ethanol stress changed the lipid composition of *S. passalidarum* cells. The most abundant fatty acids, which correspond to around 80% of the total, are shown in Fig. 6a. At 2 hours, the content of oleic acid (18:1 w9c) showed a slight increase, while the abundance of vaccenic acid (18:1 w7c/ 18:1 w6c) decreased in the stress condition compared to the control. This profile was more evident at 12 hours, in which the oleic acid content was three times higher in the presence of ethanol than in the absence of stress. The total amount of saturated and unsaturated fatty acids did not change over time or between ethanol and control conditions. Microscopy analysis also showed that ethanol altered the shape of the yeast cells since the first couple hours of stress (Fig. 6b). More elongated cells were observed in both analyzed times, but especially at 12 h of incubation under ethanol stress, while rounded cells were found in the control condition.

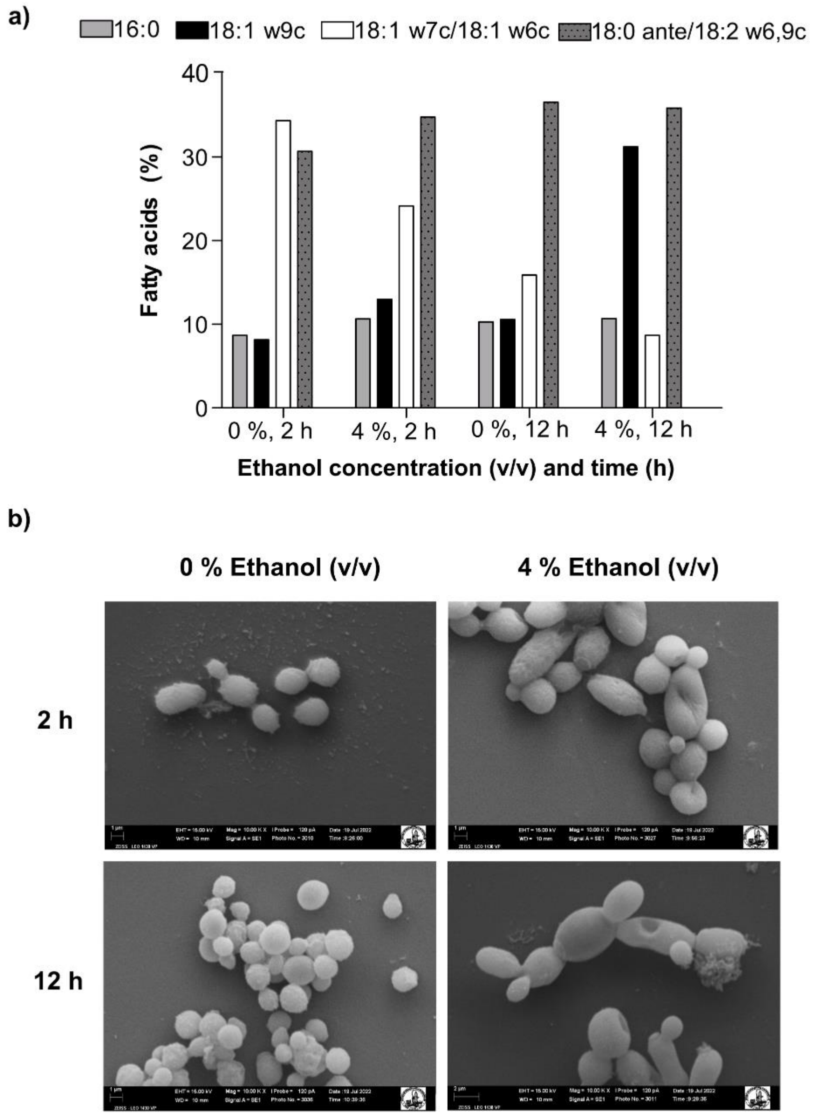


Figure. 6 a) Fatty acid profile, and **b)** scanning electron microscopy analysis of *S. passalidarum* cells after 2 and 12 hours of incubation in the control condition (without ethanol) and 4% (v/v) ethanol stress.

4. DISCUSSION

Spathaspora passalidarum, a non-conventional yeast that can efficiently ferment pentoses and hexoses into ethanol, is a potential candidate for 2G ethanol production (Cadete and Rosa 2017). However, this yeast is sensitive to ethanol stress, given that its growth is markedly impaired by mild ethanol concentrations (Fig. 1). Our analyses showed that *S. passalidarum* metabolism was severely affected when the initial ethanol concentration was close to 6% (v/v), compromising cell growth, xylose consumption, and alcoholic fermentation. The concentration of 4%

ethanol (v/v) also caused significant stress in *S. passalidarum* cells. However, the yeast cells grew since the beginning of the experiment and rescued some metabolism features throughout time, such as the ability to consume sugar after 12 hours of incubation.

As with most non-conventional yeasts, the knowledge about the molecular mechanisms underlying *S. passalidarum* phenotypes is still very scarce, especially in which concerns the basis of its stress responses. The phenotype of low ethanol tolerance, for example, is poorly understood. In this study, an RNA-seq methodology analyzed the transcriptional reorganization of *S. passalidarum* cells in response to ethanol stress (4% (v/v)) exposure (2 h), aiming to investigate possible molecular aspects that differ from other yeasts with higher ethanol tolerance. The results revealed that only 5.58% of the DEGs had a gene name assigned, and around 65% of the total DEGs had poor protein annotation, which hindered gene ontology analysis. Thus, a functional annotation analysis identified and assigned orthologs from reference genomes to *S. passalidarum* DEGs, improving the knowledge about the biological processes altered by ethanol in this yeast. The significantly enriched GOs indicated the downregulation of genes associated with transcription regulation, which included genes encoding putative proteins involved in the recruitment of RNA polymerase II to the promoters; in controlling mRNA initiation, elongation, and degradation; in histone acetylation and deacetylation; in regulation of transcription from RNA polymerase II promoter, and others.

On the other hand, protein synthesis appears induced in *S. passalidarum* cells under ethanol stress, which might have compensated for the downregulation of transcription. For instance, translation, ribosome biogenesis, ribosome assembly, rRNA processing, and ribosomal large/small subunit biogenesis were some GO terms enriched within the upregulated genes. The analysis of the KEGG pathways enriched with the DEGs also returned metabolic pathways related to the translation. Previous investigations on the transcriptional response of yeasts to ethanol stress showed that protein synthesis is transiently downregulated in an initial stress stage, probably to conserve energy, which leads to growth arrest. The *S. cerevisiae* (30 min, 7% (v/v)) and *Kluyveromyces marxianus* (1 and 4 h, 6% (v/v)) yeast species showed this profile after their exposure to short-term ethanol stresses (Alexandre et al. 2001; Diniz et al. 2017). Otherwise, our results suggest that the induction of genes related to translation was important for *S. passalidarum* survival and growth under the stress condition. Our experimental design demonstrated that the ethanol concentration assessed reduced to 57.89% the yeast growth rate compared to the control condition but was not sufficiently high to prevent the cells from growing since the beginning of the experiment. High ethanol concentrations appear to trigger the entry of yeast cells into the stationary phase, which is associated with a decrease in the expression of genes related to translation and protein synthesis (Dinh et al. 2009). Thus, it is possible that the ethanol concentration evaluated in our study was not stressful enough to stop protein translation or failed in activating signaling networks crucial for triggering precise stress adaptative responses to adverse environmental conditions.

It is vastly documented that ethanol stress severely affects many biological processes and cellular structures (Auesukaree 2017). Among these, yeasts' membranes have been considered one of the main targets of ethanol in the cells. Ethanol exposure increases cellular membrane fluidity and can result in loss of membrane integrity (Jones and Greenfield 1987; Piper 1995). Changes in membrane composition are a well-known strategy employed by yeasts to overcome the damage caused by ethanol (Henderson and Block 2014). In this regard, the increased amounts of unsaturated fatty acid, especially palmitoleic and oleic acids, and ergosterol content are correlated with ethanol tolerance (Agudo 1992; You et al. 2003). Our data showed that genes related to unsaturated fatty acids biosynthesis, such as *OLE1*, were not among the DEGs, and genes involved in ergosterol biosynthesis showed downregulation, suggesting the reduction of ergosterol content under ethanol stress. Diniz and colleagues (2017) (Diniz et al. 2017) showed for *K. marxianus* that the expression of unsaturated fatty acid and ergosterol biosynthetic genes were downregulated under ethanol stress, and additional measurements of these compounds demonstrated that their contents did not change in the conditions analyzed. The authors suggested that the absence of changes in the membrane composition of *K. marxianus* can contribute to the low ethanol tolerance of this yeast. The assessment of the fatty acid profile of *S. passalidarum* in the present study evaluated the effect of ethanol on their contents at two distinct moments of the growth curve: at the beginning of the experiment (2 h) and when the cells started to consume xylose (12 h). The recovery of sugar consumption ability at 12 h indicates that the cells had somehow altered their physiology to adapt to the stress condition. Our results demonstrated that the most expressive changes in the fatty acid profile were at the latest point, in which there was a three-fold increase in oleic acid content in the presence of ethanol, a change likely involved in the recovery of the yeast cell membrane integrity. Interestingly, the upregulation of genes related to the biosynthesis of fatty acids was reported by Neitzel and colleagues (2022) as one of the main adaptation mechanisms of *S. passalidarum* cells submitted to fed-batch fermentations with cell recycles, a condition in which the cells were exposed to long-termed and increasing ethanol concentrations. These results agree with the relevance of cellular fatty acid composition in improving ethanol tolerance in yeast.

Still in the context of cell membranes, our results showed several downregulated DEGs enriched in GO terms associated with transport systems, including sugar, ammonium, and amino acid transporters. Consistent with that, our metabolite measurements showed the postponing of the xylose uptake in the presence of 4% (v/v) ethanol. In the first hours of fermentation, xylose concentration barely reduced, which is in line with the decreased expression of transporters and genes encoding enzymes from carbon metabolism and fermentation shown by the RNA-seq and qPCR (2 h) expression analyses. It has been proposed that ethanol stress causes a pseudo-starvation state, in which the nutrients are not accessible to the cells even though abundant amounts are present in the medium (Chandler et al. 2004). Thus, the induction of transporter genes possibly contributes to protecting the cells against ethanol damage by enhancing nutrient uptake. The upregulation of transporters has been demonstrated in studies with *S. cerevisiae* under short-term stress and different ethanol concentrations (Chandler et al. 2004; Kasavi et al. 2016). A transcriptome profiling of the yeast *Issatchenkia orientalis* also showed the statistical overrepresentation of GO categories related to transmembrane transporters under ethanol stress (4 h, 10% (v/v) (Miao et al. 2018). Interestingly, our expression analysis performed by qPCR at the time of 12 h showed that from the five genes encoding putative sugar transporters/sensors that were initially downregulated (2 h), three remained less expressed, one was non-significant, and the ortholog gene of the “high glucose sensor RGT2” was upregulated (*HGT2*). This new expression profile suggests a release of the transporters’ repression profile and agrees with the start of xylose consumption demonstrated by the HPLC measurements.

Changes in amino acid metabolism (e.g. the upregulation of arginine and tryptophan biosynthetic genes) possibly compensated for the apparent reduction in amino acid transportation in *S. passalidarum* under ethanol stress. Amino acids are the building blocks of protein synthesis and a source of nitrogen. Nutritional starvation, especially nitrogen starvation, can result in yeast pseudohyphal growth, meiosis, and sporulation (Schröder et al. 2000). Genes involved in pseudohyphal growth are among the only expressed under the stress condition and those of the set of upregulated DEGs. In addition, the electron micrographs from SEM observations showed *S. passalidarum* cells presenting an elongated shape under ethanol treatment at both evaluated moments (2 and 12 h), but most notably at the latest, contrasting with the rounded-shaped cells seen in the control condition. The observed pseudohyphal morphology is a clear response of *S. passalidarum* cells to ethanol stress. According to Lorenz and colleagues (2000), ethanol induces filamentation in *S. cerevisiae* in a manner that bypasses the nutrient-sensing machinery. The authors then suggested that the yeast might have co-opted its fermentative metabolic by-product (ethanol) to act as a signal molecule to estimate nutrient availability and regulate its development under poor nutritional conditions. In this sense, pseudohyphal differentiation might contribute to nutrient scavenging because of a higher surface area (Gimeno et al. 1992).

The diminished transportation of nutrients in *S. passalidarum* cells under ethanol stress might have also affected the flux through some of the major metabolic pathways in the first hours of stress exposure. Our results showed less expression of genes encoding enzymes from glycolysis, fermentation, and xylose assimilation under ethanol stress, a strategy likely adopted to avoid increasing the stress condition in an organism that is very sensitive to ethanol. Indeed, there is no ethanol production when its added 4% ethanol (v/v) in the beginning of the fermentation. On the contrary, ethanol concentration decreased throughout the monitoring of the experiment, which can be due to ethanol evaporation or consumption by the yeast. In another study, it was also found genes from glycolysis less expressed in *S. passalidarum* under ethanol stress (2h, 4% (v/v)) (Campos et al. 2022). The flow decrease throughout these pathways might have led to diminished energy production under the stress condition. The lipid metabolism appeared enhanced in *S. passalidarum* subjected to ethanol stress, probably to cope with the energy demand.

Both fatty acid biosynthetic and degradation pathways were upregulated in the presence of ethanol. In addition to that, part of the acetyl-CoA molecules generated from the central carbon metabolism and beta-oxidation pathway was most likely directed to the glyoxylate cycle since the enzymes from this cycle showed upregulation. The glyoxylate cycle is activated according to the carbon source available in the medium, allowing cells to utilize fatty acids and two-carbon compounds, such as ethanol and acetate. Also, the glyoxylate cycle is an important source of precursors for the biosynthesis of amino acids and carbohydrates since it does not include the two decarboxylation reactions of the tricarboxylic acid (TCA) cycle, resulting in the production of essential organic compounds from two-carbon substrates (Kunze et al. 2006). It is interesting to notice that the expression profile of the genes from metabolism showed some changes at the time of 12 h. Overall, the upregulation of the two genes encoding enzymes from the xylose assimilation pathway and the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase encoded by the gene *TDH3* indicated the induction of xylose metabolism as well as glycolysis. Ethanol fermentation was kept halted as suggested by the maintenance of the downregulation of *ADH5*, and the glyoxylate cycle was deactivated as two enzymes from this cycle showed non-significant expression levels between the evaluated condition, and the third gene showed downregulated under stress. The repression of the glyoxylate cycle agrees with the utilization of preferable carbon sources by the yeast, such as xylose.

The complex stress generated by ethanol also triggers the expression of stress-responsive genes to minimize cellular dysfunctions and promote adaptation to the stress condition. Several studies have pointed out that ethanol disrupts protein conformation, generates ROS, and promotes changes in osmotic pressure, resulting in other ethanol-associated stress (Ma and Liu 2010; Auesukaree 2017). In the present study, we observed the upregulation of genes encoding putative chaperones and HSPs, consistent with results from studies with other yeast species under ethanol stress (Alexandre et al. 2001; Chandler et al. 2004; Fujita et al. 2004; Miao et al. 2018; Mo et al. 2019). Chaperones and heat-shock proteins are crucial to assist the folding of newly synthesized proteins, refolding misfolded proteins, and disassembling protein aggregates, which prevent the loss of protein functions (Verghese et al. 2012). However, some classical HSPs usually induced by ethanol were found downregulated (*HSP30* and *CRP5*) or were not present among the DEGs (e.g., *HSP104*, *HSP12*, *HSP26*, *HSP78, SSA4*) (Ma and Liu 2010; Auesukaree 2017). In addition, genes encoding enzymes involved in the biosynthesis of trehalose were not present among the DEGs. This molecule is considered a stress protectant because it stabilizes protein structures and preserves the integrity of the plasma membrane (Elbein et al. 2003). Thus, the lack of induction of some classical HSP genes and genes from trehalose biosynthesis suggests that *S. passalidarum*'s response to misfolded proteins might not be adequate to counteract the effects of more stressful conditions, such as higher ethanol concentrations.

The *S. passalidarum* cells also showed changes in the transcription of antioxidant-encoding genes under ethanol stress. Oxidative stress arises mainly from ROS generation under aerobic conditions due to incomplete reduction of O_2 in respiration. ROS accumulation can cause serious harm to the cells by promoting protein oxidation, lipid peroxidation, and damage to DNA within the nucleus and mitochondria (Morano et al. 2012). The presence of ethanol also induces ROS formation (Du and Takagi 2007). *S. passalidarum* response to oxidative stress included the expression of a gene encoding a glutaredoxin domain-containing protein, expressed only in the treatment condition. Genes involved in mitochondrial DNA replication in response to oxidative stress (*MDH1*) and some oxidoreductases (*OYE32*, *OYE3,* and *QOR2*) with potential roles in oxidative stress responses showed upregulation. *YOE32* and *QOR2* also have been pointed out by transcriptome analysis of *Pichia pastoris* as potential targets to improve this yeast tolerance to lignocellulose-derived inhibitors (Paes et al. 2021). *OYE3* was the third most upregulated gene in the present transcriptome. This gene also showed upregulation in all three *S. cerevisiae* strains investigated by Kasavi and colleagues (2016) (Kasavi et al. 2016) under ethanol stress. Additionally, we observed the upregulation of a gene encoding a putative protein with superoxide dismutase activity under the stress condition in the present study. The *HSP31* gene also showed increased expression under stress. This gene encodes a glutathione-independent glyoxalase involved in the regulation of redox homeostasis through reducing ROS levels while maintaining cellular glutathione and NADPH levels (Bankapalli et al. 2015). On the other hand, two genes encoding transcription factors associated with the oxidative stress response of *S. cerevisiae* showed downregulation in the present study (*SKN7* and *HCM1*). *SKN7* is one of the major transcription factors that regulate the transcriptional response to oxidative stress along with *YAP1* (Morano et al. 2012). *SKN7* is associated with the optimal induction of genes encoding HSPs under oxidative stress (Raitt et al. 2000). *HCM1* is a forkhead transcription factor involved with NAD synthesis and oxidative stress resistance during nutrient deficiency (Rodríguez-Colman et al. 2013). The decreased expression of these genes could have impacted the transcriptional activation of their targets and the yeast oxidative response in case post-transcriptional mechanisms did not offset it.

The transcription data showed very few differentially expressed genes related to osmotic stress response in *S. passalidarum* under ethanol stress. High ethanol concentrations have been reported to cause water stress and activate the HOG pathway in *S. cerevisiae* (Alexandre et al. 2001; Hohmann 2002; Udom et al. 2019). Hyperosmotic stress induces water efflux, which might cause cell shrinkage and the accumulation of ions (e. g. Na^+), which can arrest cellular activities and impair growth (Hohmann 2002; Saito and Posas 2012). The HOG pathway is responsible for sensing and transducing osmotic stress. It plays a relevant role in regulating the transmembrane transport of ions and the accumulation of the compatible solute glycerol by enhancing the expression of genes encoding glycerol biosynthetic enzymes and promoting its uptake from the medium (Saito and Posas 2012). Despite specifically responding to osmotic stress, the HOG pathway is also related to ethanol stress because Hog1p activates the transcription factors Msn2p/Msn4p, which are involved in general stress response (Rep et al. 2000). The transcription factors Msn2p/Msn4p are key proteins in ethanol response because they are associated with the upregulation of several ethanol-responsive genes (Ma and Liu 2010). Our expression results also showed the upregulation of *CLA4* and the downregulation of *PTC1*, which suggests the enhancement of the HOG pathway. However, the HOG pathway-responsive gene *GPD1*, which encodes the enzyme NAD-dependent glycerol 3-phosphate dehydrogenase, was not differently expressed in our study, while other reports pointed out the induction of this gene in *S. cerevisiae* under short-term ethanol stress (30 min) (Alexandre et al. 2001; Udom et al. 2019). Our data also showed the downregulation of the osmo-responsive genes *SLT1*, which encodes a symport that actively transports glycerol inside the cell, and *ENA1*, which encodes a sodium pump involved in Na^+ and Li^+ efflux. The work developed by Mo and colleagues (2019) with the yeast *K. marxianus* also showed the induction of genes from the HOG pathway in an evolved strain under ethanol stress. However, the authors observed the downregulation of most genes involved in glycerol biosynthesis, suggesting that glycerol production might not be the strategy adopted by the yeast to cope with the stress. Based on our transcription data, the osmotic stress response of *S. passalidarum* under ethanol stress appears to be very limited, suggesting that it could also be one of the reasons to explain the low ethanol tolerance profile of this yeast.

5. CONCLUSION

It is worth noticing that *S. passalidarum* low tolerance to ethanol is a complex phenotype that is likely not explained by a single reason. Overall, our RNA-seq data suggest that *S. passalidarum* can activate responses regarding protein folding and antioxidant stress. On the other hand, *S. passalidarum* appeared to have failed to adjust its membrane lipid composition rapidly in response to ethanol. Additionally, genes encoding transporters and enzymes from glycolysis and fermentation showed downregulation at 2 hours of stress, which might have led to a lack of nutrients and energy for the cells to cope with the stress condition. The release of repression began only after 12 h of incubation under ethanol, preceding the start of xylose consumption. Another physiological adaptation observed was the pseudohyphal morphology of *S. passalidarum* colonies in response to ethanol, probably due to ethanol-induced nutritional starvation. Finally, the *S. passalidarum* limited osmotic stress response suggests that the low ethanol tolerance of this yeast can also be related to a lack of ability to cope with ethanol-induced osmotic changes.

6. REFERENCES

Agudo L del C (1992) Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance. Appl Microbiol Biotechnol 37:647–651

Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B (2001) Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Lett 498:98–103. https://doi.org/10.1016/S0014-5793(01)02503-0

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Auesukaree C (2017) Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng 124:133–142. https://doi.org/10.1016/j.jbiosc.2017.03.009

Bankapalli K, Saladi SD, Awadia SS, Goswami AV, Samaddar M, D’Silva P (2015) Robust Glyoxalase activity of Hsp31, a ThiJ/DJ-1/PfpI Family Member Protein, Is Critical for Oxidative Stress Resistance in *Saccharomyces cerevisiae*. J Biol Chem 290:26491–26507. https://doi.org/10.1074/jbc.M115.673624

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Cadete RM, Rosa CA (2017) The yeasts of the genus *Spathaspora*: potential candidates for second-generation biofuel production. Yeast 191–199. https://doi.org/10.1002/yea

Campos VJ, Ribeiro LE, Albuini FM, de Castro AG, Fontes PP, da Silveira WB, Rosa CA, Fietto LG (2022) Physiological comparisons among *Spathaspora passalidarum*, *Spathaspora arborariae*, and *Scheffersomyces stipitis* reveal the bottlenecks for their use in the production of second-generation ethanol. Brazilian J Microbiol 53:977–990. https://doi.org/10.1007/s42770-022-00693-6

Chandler M, Stanley GA, Rogers P, Chambers P (2004) A genomic approach to defining the ethanol stress response in the yeast *Saccharomyces cerevisiae*. Ann Microbiol 54:427–454

Consortium TU (2023) UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res 51:D523–D531. https://doi.org/10.1093/nar/gkac1052

De Nadal E, Ammerer G, Posas F (2011) Controlling gene expression in response to stress. Nat Rev Genet 12:833–845. https://doi.org/10.1038/nrg3055

Deparis Q, Claes A, Foulquié-Moreno MR, Thevelein JM (2017) Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS Yeast Res 17:1–17. https://doi.org/10.1093/femsyr/fox036

Dinh TN, Nagahisa K, Yoshikawa K, Hirasawa T, Furusawa C, Shimizu H (2009) Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioprocess Biosyst Eng 32:681–688. https://doi.org/10.1007/s00449-008-0292-7

Diniz RHS, Villada JC, Alvim MCT, Vidigal PMP, Vieira NM, Lamas-Maceiras M, Cerdán ME, González-Siso MI, Lahtvee PJ, da Silveira WB (2017) Transcriptome analysis of the thermotolerant yeast *Kluyveromyces marxianus* CCT 7735 under ethanol stress. Appl Microbiol Biotechnol 101:6969–6980. https://doi.org/10.1007/s00253-017-8432-0

Du X, Takagi H (2007) N-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. Appl Microbiol Biotechnol 75:1343–1351. https://doi.org/10.1007/s00253-007-0940-x

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: A multifunctional molecule. Glycobiology 13:17–27. https://doi.org/10.1093/glycob/cwg047

Fatma Z, Schultz JC, Zhao H (2020) Recent advances in domesticating non-model microorganisms. Biotechnol Prog 36. https://doi.org/10.1002/btpr.3008

Fujita K, Matsuyama A, Kobayashi Y, Iwahashi H (2004) Comprehensive gene expression analysis of the response to straight-chain alcohols in *Saccharomyces cerevisiae* using cDNA microarray. J Appl Microbiol 97:57–67. https://doi.org/10.1111/j.1365-2672.2004.02290.x

Gao M, Ploessl D, Shao Z (2019) Enhancing the co-utilization of biomass-derived mixed sugars by yeasts. Front Microbiol 10:1–21. https://doi.org/10.3389/fmicb.2018.03264

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. Mol Biol Cell 11:4241–4257

Gimeno CJ, Styles CA, Fink GR (1992) Unipolar Cell Divisions in the Yeast S. *cerevisiae* Lead to Filamentous Growth: Regulation by Starvation and RAS

Henderson CM, Block DE (2014) Examining the role of membrane lipid composition in determining the ethanol tolerance of *Saccharomyces cerevisiae*. Appl Environ Microbiol 80:2966–2972. https://doi.org/10.1128/AEM.04151-13

Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2007) Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. J Biotechnol 131:34–44. https://doi.org/10.1016/j.jbiotec.2007.05.010

Hohmann S (2002) Osmotic Stress Signaling and Osmoadaptation in Yeasts. Microbiol Mol Biol Rev 66:300–372. https://doi.org/10.1128/MMBR.66.2.300–372.2002

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94:205–214. https://doi.org/10.1007/s00253-011-3694-4

Jeffries TW (1983) Utilization of xylose by bacteria, yeasts, and fungi. Adv Biochem Eng Biotechnol 27:1–32. https://doi.org/10.1007/bfb0009101

Jones RP, Greenfield PF (1987) Ethanol and the fluidity of the yeast plasma membrane. Yeast 3:223–232. https://doi.org/10.1002/yea.320030403

Kasavi C, Eraslan S, Oner ET, Kirdar B (2016) An integrative analysis of transcriptomic response of ethanol tolerant strains to ethanol in *Saccharomyces cerevisiae*. Mol Biosyst 12:464–476. https://doi.org/10.1039/C5MB00622H

Kavšček M, Stražar M, Curk T, Natter K, Petrovič U (2015) Yeast as a cell factory: Current state and perspectives. Microb Cell Fact 14:1–10. https://doi.org/10.1186/s12934-015-0281-x

Kuleshov M V., Diaz JEL, Flamholz ZN, Keenan AB, Lachmann A, Wojciechowicz ML, Cagan RL, Ma'ayan A (2019) ModEnrichr: A suite of gene set enrichment analysis tools for model organisms. Nucleic Acids Res 47:W183–W190. https://doi.org/10.1093/nar/gkz347

Kunze M, Pracharoenwattana I, Smith SM, Hartig A (2006) A central role for the peroxisomal membrane in glyoxylate cycle function. Biochim Biophys Acta - Mol Cell Res 1763:1441–1452. https://doi.org/10.1016/j.bbamcr.2006.09.009

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. https://doi.org/10.1038/nmeth.1923

Li BZ, Cheng JS, Ding MZ, Yuan YJ (2010) Transcriptome analysis of differential responses of diploid and haploid yeast to ethanol stress. J Biotechnol 148:194–203. https://doi.org/10.1016/j.jbiotec.2010.06.013

Long TM, Su YK, Headman J, Higbee A, Willis LB, Jeffries TW (2012) Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. Appl Environ Microbiol 78:5492–5500. https://doi.org/10.1128/AEM.00374-12

Lorenz MC, Cutler NS, Heitman J (2000) Characterization of Alcohol-induced Filamentous Growth in *Saccharomyces cerevisiae*. Mol Bil Cel 11(1): 183-199. https://doi.org/10.1091/mbc.11.1.183

Ma M, Liu ZL (2010) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 87:829–845. https://doi.org/10.1007/s00253-010-2594-3

Miao Y, Xiong G, Li R, Wu Z, Zhang X, Weng P (2018) Transcriptome profiling of *Issatchenkia orientalis* under ethanol stress. AMB Express 8. https://doi.org/10.1186/s13568-018-0568-5

Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, Tosatto SCE, Paladin L, Raj S, Richardson LJ, Finn RD, Bateman A (2021) Pfam: The protein families database in 2021. Nucleic Acids Res 49:D412–D419. https://doi.org/10.1093/nar/gkaa913

Mo W, Wang M, Zhan R, Yu Y, He Y, Lu H (2019) *Kluyveromyces marxianus* developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways. Biotechnol Biofuels 12:1–15. https://doi.org/10.1186/s13068-019-1393-z

Morano KA, Grant CM, Moye-Rowley WS (2012) The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190:1157–1195. https://doi.org/10.1534/genetics.111.128033

Neitzel T, Lima CS, Hafemann E, Paixão DAA, Junior JM, Persinoti GF, dos Santos LV, Ienczak JL (2022) RNA-seq based transcriptomic analysis of the non-conventional yeast Spathaspora passalidarum during Melle-boinot cell recycle in xylose-glucose mixtures. Renew Energy 201:486-498. https://doi.org/10.1016/j.renene.2022.10.108

Nguyen NH, Suh SO, Marshall CJ, Blackwell M (2006) Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. Mycol Res 110:1232–1241. https://doi.org/10.1016/j.mycres.2006.07.002

Paes BG, Steindorff AS, Formighieri EF, Pereira IS, Almeida JRM (2021) Physiological characterization and transcriptome analysis of *Pichia pastoris* reveals its response to lignocellulose-derived inhibitors. AMB Express 11. https://doi.org/10.1186/s13568-020-01170-9

Piper PW (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol Lett 134:121–127. https://doi.org/10.1016/0378-1097(95)00431-9

Raitt DC, Johnson AL, Erkine AM, Makino K, Morgan B, Gross DS, Johnston LH (2000) The Skn7 Response Regulator of *Saccharomyces cerevisiae* Interacts with Hsf1 In Vivo and Is Required for the Induction of Heat Shock Genes by Oxidative Stress. Mol Biol Cell 11:2335–2347

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem 275:8290–8300. https://doi.org/10.1074/jbc.275.12.8290

Ribeiro LE, Albuini FM, Castro AG, Campos VJ, de Souza GB, Mendonça JGP, Rosa CA, Mendes TAO, Santana MF, da Silveira WB, Fietto LG (2021) Influence of glucose on xylose metabolization by *Spathaspora passalidarum*. Fungal Genet Biol 157. https://doi.org/10.1016/j.fgb.2021.103624

Rodríguez-Colman MJ, Sorolla MA, Vall-llaura N, Tamarit J, Ros J, Cabiscol E (2013) The FOX transcription factor Hcm1 regulates oxidative metabolism in response to early nutrient limitation in yeast. Role of Snf1 and Tor1/Sch9 kinases. Biochim Biophys Acta - Mol Cell Res 1833:2004–2015. https://doi.org/10.1016/j.bbamcr.2013.02.015

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291. https://doi.org/10.1007/s10295-003-0049-x

Saito H, Posas F (2012) Response to hyperosmotic stress. Genetics 192:289–318. https://doi.org/10.1534/genetics.112.140863

Schröder M, Chang JS, Kaufman RJ (2000) The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast. Genes Dev 14:2962–2975. https://doi.org/10.1101/gad.852300

Sharma S, Arora A (2020) Tracking strategic developments for conferring xylose utilization/fermentation by *Saccharomyces cerevisiae*. Ann Microbiol 70. https://doi.org/10.1186/s13213-020-01590-9

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: Discovering splice junctions with RNA-Seq. Bioinformatics 25:1105–1111. https://doi.org/10.1093/bioinformatics/btp120

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Van Baren MJ, Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515. https://doi.org/10.1038/nbt.1621

Udom N, Chansongkrow P, Charoensawan V, Auesukaree C (2019) Coordination of the cell wall integrity and highosmolarity glycerol pathways in response to ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 85:1–16. https://doi.org/10.1128/AEM.00551-19

Veras HCT, Parachin NS, Almeida JRM (2017) Comparative assessment of fermentative capacity of different xylose-consuming yeasts. Microb Cell Fact 16:1–8. https://doi.org/10.1186/s12934-017-0766-x

Verghese J, Abrams J, Wang Y, Morano KA (2012) Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (*Saccharomyces cerevisiae*) as a Model System. Microbiol Mol Biol Rev 76:115–158. https://doi.org/10.1128/mmbr.05018-11

Wohlbach DJ, Kuo A, Sato TK, Potts KM, Salamov AA, LaButti KM, Sun H, Clum A, Pangilinan JL, Lindquist EA, Lucas S, Lapidus A, Jin M, Gunawan C, Balan V, Dale BE, Jeffries TW, Zinkel R, Barry KW, Grigoriev I V., Gasch AP (2011) Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. Proc Natl Acad Sci U S A 108:13212–13217. https://doi.org/10.1073/pnas.1103039108

Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L (2011) KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 39:316–322. https://doi.org/10.1093/nar/gkr483

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction

You KM, Rosenfield C, Knipple DC (2003) Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. Appl Environ Microbiol 69:1499–1503. https://doi.org/10.1128/AEM.69.3.1499

Zabed H, Sahu JN, Suely A, Boyce AN, Faruq G (2017) Bioethanol production from renewable sources: current perspectives and technological progress. Renew Sustain Energy Rev 71:475–501. https://doi.org/10.1016/j.rser.2016.12.076

Chapter 2

Comparisons between *Spathaspora passalidarum* and *Saccharomyces cerevisiae* stress signaling pathways point to transcription factors as hot spots of divergence

This chapter comprises a manuscript derived from the work developed by Fernanda Matias Albuini and Alex Gazolla de Castro (Castro, 2019) in which they share the co-authorship.

1. INTRODUCTION

Fermentative environments display multiple simultaneous stressful conditions that can impair the economically viable performance of fermenting microorganisms (Deparis et al. 2017). Fluctuation in temperature and pH, presence of inhibitory compounds, high concentration of substrates, and toxicity of products are some of the environmental and metabolic stressors faced by yeasts used as microbial cell factories in the bioethanol industry (Ndubuisi et al. 2023). Among these stresses, the toxicity caused by high ethanol concentrations is considered one of the main factors impairing fermentation since it negatively affects cell growth and viability (Stanley et al. 2010). The complexity and extent of ethanol effect in the cells result in the simultaneous development of other associated stresses, such as osmotic stress caused by the reduction in the water activity and activation of the High-Osmolarity Glycerol pathway (HOG-MAPK) (Udom et al. 2019), and oxidative stress resulted from changes in mitochondrial membranes that affect the electron transport chain and ATP synthesis leading to ROS generation (Pérez-Gallardo et al. 2013). From an industrial point of view, yeasts should be multi-stress tolerant in order to deal with stresses while maintaining cellular functions and assuring efficient ethanol production (Deparis et al. 2017).

Cellular mechanisms allow the organisms to properly detect and respond to environmental variation, aiming to restore the internal conditions required for optimal growth and function. Stress adaptation involves several players and strategies, such as the accumulation of protective compounds (e.g. trehalose), action of stress proteins (e.g. repair systems), activation of signal transduction pathways, post-translational modifications of existing enzymes, and transcriptional activation of genes (Martínez-Pastor et al. 1996; Estruch 2000). A vast number of studies have focused on yeast stress responses at the transcriptional level using the model species *Saccharomyces cerevisiae*. Therefore, several stress-induced signal transduction pathways, stress-activated transcription factors, and stress-responsive proteins have been identified in this microorganism (Lin et al. 2022).

The transcriptional reprogramming that yeast cells undergo in stress situations might be closely related to the role played by transcription factors, regulatory proteins activated according to environmental signals by signal transduction pathways. These proteins bind to DNA elements located in the promoter region of their targets to control the activation or repression of transcription (Estruch 2000; Ma and Liu 2010). The fine regulation promoted by transcription factors in the presence of environmental stresses influences the organism's physiological adaptation ability. Thus, these proteins are considered candidates for genetic engineering approaches to remodel complex phenotypes by altering gene networks and cellular metabolism (Chen et al. 2016; Li et al. 2017). The general stress response transcription factors Msn2p/Msn4p are activated by multiple stimuli (Auesukaree 2017). These transcription factors are hyperphosphorylated in the presence of stresses, translocate to the nucleus, and periodically enter and leave this compartment. Into the nucleus, they regulate a wide range of genes in response to thermal, osmotic, oxidative, and ethanol stresses by recognition and binding of their zinc finger domain on STRE elements (5'CCCT; stress response element) (Ma and Liu 2010; Auesukaree 2017). The transcription factors Yap1p and Hsf1p respond respectively to oxidative and thermal stresses but also participate in ethanol response, probably due to the pleiotropic effects of ethanol (Ma and Liu 2010).

Most of the information regarding yeast stress responses has been acquired *for S. cerevisiae* due to its wide use in fermentation processes and ease of manipulation. However, *S. cerevisiae* is unable to naturally ferment pentoses, such as xylose (Moysés et al. 2016; Sharma et al. 2018), which constitute up to 20% of the entire lignocellulosic biomass used for second-generation ethanol production (Zabed et al. 2017). In the past few decades, much effort has been made to overcome this issue by genetic engineering *S. cerevisiae* yeast strains with xylose metabolism genes (Cunha et al. 2019; Osiro et al. 2019). However, the generated strains are usually accompanied by co-factor imbalance, low intracellular metabolic flux, and xylose fermentation rates not comparable to glucose fermentation. In addition to that, the limited knowledge about the regulation of xylose metabolism also makes difficult the reprogramming of *S. cerevisiae* xylose fermentation properties. In this context, another important strategy that arises is the amelioration and exploitation of innate xylose-utilizing yeasts (Cadete and Rosa 2018; Gao et al. 2019).

Spathaspora passalidarum is a wood-boring beetle associated-yeast that efficiently ferments xylose and other sugars under different aeration conditions (Hou 2012; Long et al. 2012; Cadete and Rosa 2018; Su et al. 2018; Ribeiro et al. 2021; Campos et al. 2022). However, the knowledge about this yeast tolerance to different stresses is currently limited, which hinders its utilization. Also, there is few information on the molecular mechanisms of gene regulation in *S. passalidarum* during stress since the studies have mainly focused on its xylose metabolism. *Spathaspora passalidarum* genome was sequenced in 2001 and is available to the public at NCBI (Wohlbach et al. 2011). Several sequences are shown as hypothetical, and a comprehensive analysis of transcription factors is still missing.

In this study, we attempted to identify possible molecular features that could influence *S. passalidarum* response to stresses by performing comparative genomics and phylogenetic approaches using *S. cerevisiae* as a model organism. Signaling pathways components and proteins responsive to different stresses were identified, as well as the inventory of putative *S. passalidarum* transcription factors. Additionally, *S. passalidarum* tolerance to stresses (ethanol, oxidative and osmotic) was characterized and the role of this yeast *MSN-like* transcription factor was evaluated in stress tolerance complementation assays with *S. cerevisiae* knockout strain for the *MSN2* and *MSN4* transcription factor genes. Thus, our study was the first to identify transcription factors in this yeast and to evaluate structural differences with *S. cerevisiae* orthologs, suggesting a potential impact of these divergences on the regulation of *S. passalidarum* stress response.

2. MATERIALS AND METHODS

2.1. Strains and maintenance

Spathaspora passalidarum NRRL Y-27907 (Laboratory of Yeast Ecology and Biotechnology of Universidade Federal de Minas Gerais), and the *Saccharomyces cerevisiae* BY4741 (background), Y07117 (Δ*msn2*), and Y04911 (Δ*msn4*) strains from the EUROSCARF collection were used in this study. In addition, the *Saccharomyces cerevisiae* Δ*msn2/4* double knockout strain was constructed in the present study using a homologous recombination approach. Cells stocks were prepared in YPD medium (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose) with 25% (v/v) glycerol and kept at - 80°C. The YPD or YPX (20 $g.L^{-1}$ xylose) solid (20 $g.L^{-1}$ agar) or liquid media were used for activation and growth assays with *S. cerevisiae* and *S. passalidarum* strains, respectively.

2.2. Sequence acquisition and OrthoMCL analysis

Protein and nucleotide sequences of *S. cerevisiae* S288C (Goffeau et al. 1996) were obtained from the Saccharomyces Genome Database (https://www.yeastgenome.org/) (Hirschman et al. 2006), and *S. passalidarum* NRRL Y-27907 sequences (assembly v2.0) (Wohlbach et al. 2011) were extracted from the NCBI website (https://www.ncbi.nlm.nih.gov/genome). Orthologs protein analysis was locally run using the OrthoMCL (v 1.4) (Li et al. 2003) software with a standard inflation index of 1.5. Filters were used to remove low-quality sequences, such as those with internal stop codons and size smaller than 10 amino acids (Fischer et al. 2011). The tool used the algorithm BLASTp (v 2.2.21) (Altschul et al. 1990) to do the all versus all sequences alignment. Sequences unique to *S. cerevisiae* were manually obtained by identifying OrthoMCL clusters that lacked *S. passalidarum* proteins. The Gene Ontology (GO) analyses of the identified proteins were performed using SGD Gene Ontology Slim Mapper with a p-value cutoff of 0.01 (https://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl).

2.3. Phylogenetic analysis and identification of putative orthologs

The transcription factors of *S. cerevisiae* were extracted from The Yeast Transcription Factor Specificity Compendium (v1.02, http://yetfasco.ccbr.utoronto.ca/index.php) and Yeastract (http://www.yeastract.com/) (Teixeira et al. 2017). The list was curated according to the presence of the transcription factor in both databases and with literature data. The sequences of the 171 transcription factors were recovered and used as queries for tBLASTn (v 2.2.21) searches against the *S. passalidarum* genome. Protein and nucleotide sequences with an E-value < 10^{-5} were manually curated and annotated. Phylogenetic analyses were carried out for tBLASTn with inconclusive results or for families that had more than five proteins.

The MEGA 7.0 software package (https://www.megasoftware.net/) (Kumar et al. 2016) was used for the construction and visualization of phylogenetic trees. The transcription factors of *S. cerevisiae* and their respective sequences identified in *S. passalidarum* were grouped according to the DNA binding domain (Hahn and Young 2011) obtained from Pfam Database with E-value cutoff of 1.0

(https://pfam.xfam.org/) (El-Gebali et al. 2018). The alignment was performed with Muscle (default settings), and the phylogenetic trees were constructed using the Maximum Likelihood (ML) method, with JTT model and partial deletion of gaps/missing data treatment (site coverage cutoff of 90%). The bootstrap tests were carried out with 1000 replicates (Baker and Rogers 2006; Rojas-Ortega et al. 2018) and nodes with support values > 50 were considered significant and used as references for the determination of putative orthologs (Kocot et al. 2013).

2.4. Identification of domains and structural characteristics

The conserved protein domains of the transcription factors were predicted using the Pfam Database (v 31.0) (E-value cutoff of 1.0) and Interpro (https://www.ebi.ac.uk/interpro/) (Hunter et al. 2009). The location of the DNA binding domain within the identified domains was also determined. The primary structure of proteins was schematized with Illustrator for Biological Sequences (http://ibs.biocuckoo.org/) and edited with Inkscape (https://inkscape.org). The length and isoelectric point were predicted using ExPASy (https://www.expasy.org/tools/). The global identity of the pairwise alignment between orthologs was estimated using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default settings (Sievers et al. 2011; Sievers and Higgins 2014).

2.5. Gene knockout by homologous recombination

The *Saccharomyces cerevisiae* Y07117 (Δ*msn2* - Genotype: BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR037c::kanMX4) strain was used as the background for constructing a double mutant line for the *MSN2/4* genes. The *MSN4* gene knockout was performed using a DNA cassette composed of primer sequences for *LEU2* amplification fused to homologous regions (~ 60 bp) flanking the *MSN4* gene locus (Supplemental Table 1 - Appendix B). The transformation of the *S. cerevisiae* Y07117 cells with this DNA fragment aimed at the complete deletion of the *MSN4* coding sequence and its replacement by *LEU2*, allowing the transformants to grow on a selective medium lacking leucine. The *LEU2* gene was PCR amplified from the plasmid YEP351, analyzed in agarose gel (1%), and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The DNA fragment was quantified using a Qubit fluorometer (Invitrogen), and one µg was used for the Y07117 (Δ*msn2*) yeast transformation by the LiAc/SS carrier DNA/PEG method with modifications (Gietz and Schiestl 2007).

Transformants were selected on synthetic defined media (SD) lacking leucine (SD-leu, 6.7 $g.L^{-1}$ yeast nitrogen base without amino acids, Difco) and supplemented with glucose. The plates were incubated at 30°C for 3 - 5 days. The confirmation of successful genome integration was performed by PCR reactions using the genomic DNA of the transformant line, BY4741, and Y07117 strains and primers for amplifying the *MSN4* (~ 1800 bp) and *LEU2* (~ 1500 bp) genes (Supplemental Figure 1 - Appendix B). Additionally, total RNA was extracted from the cells using the Trizol reagent (Invitrogen), followed by DNAse treatment (Sigma-Aldrich), cDNA synthesis (Applied Biosystems), and RT-PCR reactions to verify the synthesis of the mRNAs from the *MSN2* and *MSN4* genes (Supplemental Figure 2 - Appendix B). The list of the oligonucleotides used in the present study is in Supplemental Table 1 - Appendix B.

2.6. Stress tolerance assessment

The tolerance profile of *S. passalidarum*, *S. cerevisiae* BY4741, Y07117 (Δ*msn2*), Y04911 (Δ*msn4*), and double mutant for the *MSN2* and *MSN4* genes (Δ*msn2/4*) were evaluated under ethanol (6, 8, and 10% (v/v)), osmotic (1, 1.25 and 1.5 M NaCl) and oxidative (1, 2 and 4 mM H_2O_2) stress conditions. Three individual colonies of each strain were inoculated and grown overnight at 30°C at 150 rpm in YP media supplemented with xylose or glucose for *S. passalidarum* and *S. cerevisiae* strains, respectively. The cultures' optical densities (OD_{600nm}) were measured using a spectrophotometer (Biospectro SP-220) at 600 nm and adjusted to 0.1 in 6 mL of fresh YPX or YPD media added or not (control) of the stress compounds in screw-capped glass tubes. The flasks were incubated at 30°C and stirred at 150 rpm for 48 hours. Aliquots were collected throughout time for cell growth analysis.

2.7. Molecular cloning and yeast transformation

The coding sequence of the *MSN-like* gene of *S. passalidarum* was retrieved from the NCBI (SPAPADRAFT_61412), and a restriction analysis was performed to select enzymes for the cloning step. Primers were designed for cloning the gene's full-length CDS. The restriction enzyme sites were added to the 5' end of each primer. The genomic DNA of *S. passalidarum* was used as the template for the PCR amplification. The gene was cloned into the p426-GPD expression vector by restriction digestion with the enzymes *Bam*HI and *Xho*I (Promega). The construction was transformed by the heat shock method in *Escherichia coli* DH5α competent cells for propagation. Cloning confirmation was performed by sequencing the plasmid. The previously constructed line of *S. cerevisiae* ∆*msn2/4* double mutant and the BY4741 background strain were transformed with the p246-GPD empty plasmid (control) and cloned with the *MSN-like* gene of *S. passalidarum* as previously described. The transformants selection was performed in an SD-ura medium supplemented with glucose.

2.8. Complementation assay

Three individual colonies of each transformant line were activated overnight in an SD-ura medium supplemented with glucose under stirring at 30°C. The effects of the *S. passalidarum MSN-like* gene expression in *S. cerevisiae* ∆*msn2/4* double mutant and BY4741 strains were analyzed by measuring the growth of the cells under stress conditions as previously described, except for selective medium (SD-ura) being used instead of complete rich medium. The stress conditions assessed were 10% (v/v) ethanol, 2 mM H_2O_2 and 1.25 M NaCl. Cell growth was followed during 48 h, and samples were periodically collected in several time intervals for spectrophotometer measurements at 600 nm. The statistical analysis was performed on GraphPad Prism 5 using analysis of variance (non-parametric ANOVA) followed by the Tukey test for comparison of means at 5% significance.

3. RESULTS

3.1. Transcription factor sequences of *S. passalidarum* and *S. cerevisiae* show great diversity

The OrthoMCL analysis identified 501 proteins exclusive to *S. cerevisiae*. Among these, 300 proteins have a known function annotated in SGD (Supplemental File S1 – Attachment A). The category “transcription control” was over-represented with 39 proteins, which represents 7,8% of the total number of unique *S. cerevisiae* proteins retrieved from the OrthoMCL analysis (Fig. 1). Among these, 30 transcription factors with functions involved in different physiological processes were identified.

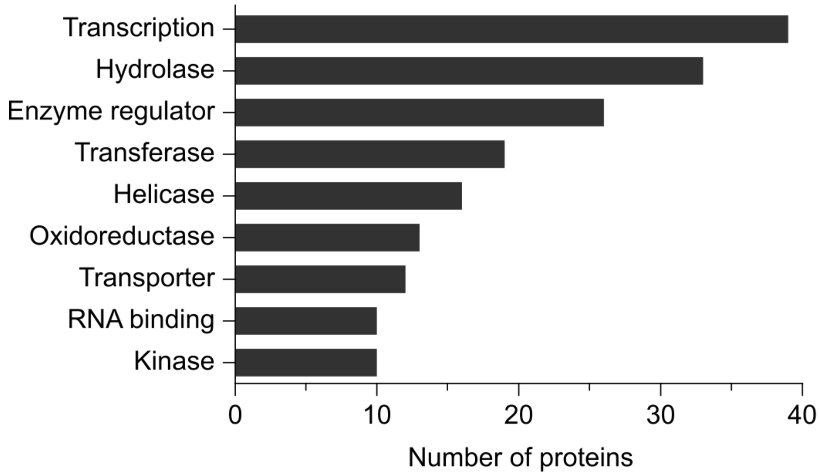


Figure 1. Gene ontology analysis of proteins exclusive to *Saccharomyces cerevisiae*. The nine most representative categories retrieved from OrthoMCL analysis were presented according to the absolute number of proteins identified for each of them.

Since transcription factors are crucial regulatory elements in different stress responses, the set of *S. cerevisiae* transcription factors were used as queries in tBLASTn searches against *S. passalidarum* genome to identify putative transcription factors in this yeast and to compare their sequences (Supplemental File S2 - Attachment A). *Saccharomyces cerevisiae* transcription factors were grouped into 33 families according to their DNA binding domain. Phylogenetic trees and the domains composition of the proteins were evaluated to define the putative orthologs (Supplemental File S3 - Attachment A). This curated approach identified 149 putative transcription factors in *S. passalidarum*. Among these, 105 were putative orthologs of *S. cerevisiae* and 44 were unique proteins of *S. passalidarum.* In addition to that, a total of 42 exclusive transcription factors of *S. cerevisiae* were identified, expanding the initial set of 30 *S. cerevisiae* unique proteins found by the OrthoMCL analysis. Twenty-six transcription factors identified in *S. passalidarum* had two or more copies in *S. cerevisiae*. Overall, the distribution of transcription factors in protein families was similar (Fig. 2A and 2B). *S. passalidarum* had transcription factors in all families except the AFT domain. A total of 43 sequences had their annotations manually corrected compared to the NCBI draft (SPAPADRAFT v 2.0).

The sequences of the orthologs transcription factors were used to determine different parameters that were binary evaluated for a graphical construction (Fig. 2C, Supplemental File S4 - Attachment A). Among the putative orthologs, about 14% of the pairwise alignments showed coverage below 70%, while about 64% had a global identity lower than 30%. In addition, 25% of the pairwise alignments had low overall identity and different domain composition.

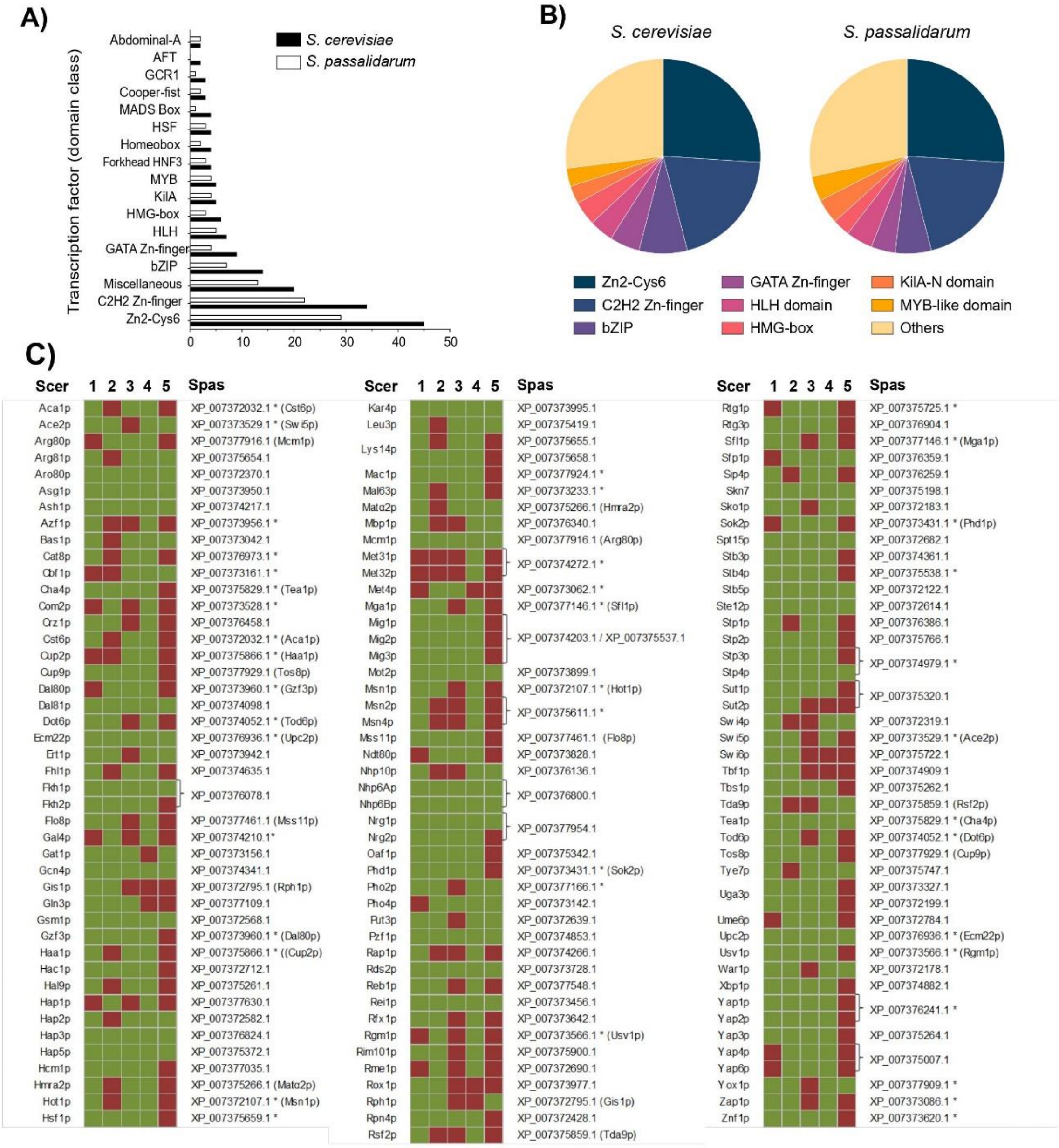


Figure 2. Inventory of transcription factors of *Saccharomyces cerevisiae* and *Spathaspora passalidarum*. Absolute (A) and relative (B) distribution of the transcription factors in different families, based on DNA binding domains. The predicted parameters (C) were distributed in columns (1 - 5) and visually compared by pairwise alignments, according to the following cutoffs: 1, length (green: coverage > 70%); 2, isoelectric point (green: pH difference ± 1.0); 3, domains (green: same

domains identified); 4, DNA binding domain location (green: similar location); 5, global identity (green: identity > 30%). Duplicated sequences were marked with curly brackets, and incomplete annotated sequences of *S. passalidarum* in NCBI were marked with an asterisk. Miscellaneous is a family that has only one member. Scer, *S. cerevisiae* and Spas, *S. passalidarum*. The numbers in the Spas column refer to the identification number of the annotated sequence in NCBI (SPAPADRAFT_number format). Access numbers can be obtained in the Supplemental File S2 - Attachment A.

3.2. Stress signaling pathways are conserved in *S. passalidarum*

Signaling pathways components of *S. cerevisiae* were identified in the literature and phylogenetic analyses were used to search for the *S. passalidarum* orthologs. The parameters of the identified putative orthologs proteins were also annotated. Figure 3A and Supplemental File S5 (Attachment A) show the signaling pathways where green proteins have similar features (length, pI, domains, and global identity), yellow proteins differ in only one parameter, and red proteins show differences in 2 or more parameters. Figure 3A represents the high-osmolarity glycerol (HOG) pathway, which is responsible to sense changes in the osmolarity of the environment and induce adaptative responses to high osmolarity. Wide conservation of signaling components was observed, although marked differences in transcription factors were seen. Regulatory proteins of the Msn2/4p pathway were highly conserved, showing identities higher than 30%, similar sizes and pI, and the same domains. The exceptions were Ssk1p (different pI) and Ssk2/Ssk22p (one copy in *S. passalidarum*). For Msn2/4p, there were many differences: *S. passalidarum* had only one copy, which showed overall identity below 30% and different pI value. The activation pathway of other transcription factors (Supplemental File S5 - Attachment A), such as Hsf1p (heat shock stress), also presented conserved proteins. However, differences in size and identity of Hsf1p were seen.

Global identity of 171 transcription factors, 300 stress response genes and 52 signaling components of seven pathways were also evaluated. About 70% of the transcription factors had identity below 30% or were exclusive to *S. cerevisiae,* revealing the low degree of conservation among them. Proteins involved in signaling pathways and ethanol response, on the other hand, were more conserved (Fig. 3B).

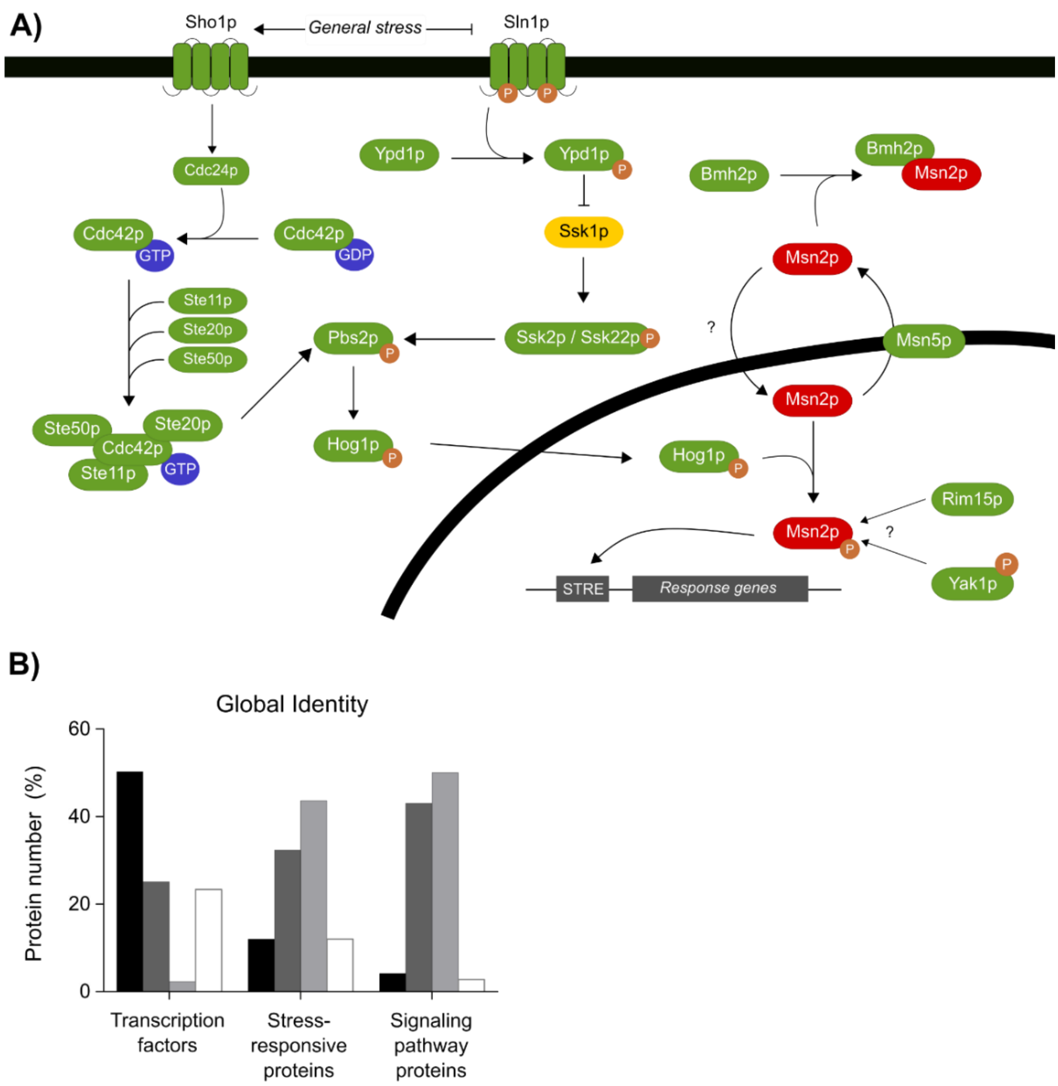


Figure 3. Conservation of signaling and response mechanisms in *Spathaspora passalidarum*. A) Reconstruction of the high-osmolarity glycerol (HOG) pathway and activation of Msn2/4p. The figure shows two independent upstream osmosensing branches (Sln1 and Sho1 sensors) that activate mitogen-activated protein kinase (MAPK) signaling cascades, leading to the phosphorylation of the Hog1 MAPK, and the activation of the MSN2/4 transcription factors promoting a series of adaptive responses. Conserved orthologs (coverage, identity, pI, and domains) are shown in green, orthologs varying in only one parameter are shown in yellow, and orthologs varying in 2 or 3 parameters are shown in red. B) Global identity of proteins involved in different cellular processes. Identity < 30% (black), identity 30 – 50% (dark gray), identity > 50% (light gray), and *S. cerevisiae* exclusive proteins (white).

3.3. Transcription factors divergence is high among different yeast species

Identity matrices using ethanol-responsive proteins and transcription factors were constructed to better understand the divergences of the stress response mechanisms among yeasts (Fig. 4; Supplemental File S6 - Attachment A). Overall, Msn2/4p, Hsf1p, and Yap1p showed divergences in sequences, with the exception seen in the genus *Saccharomyces*. The scenario was different for the responsive genes, as illustrated by Tps1p, Sod1p, and Adh1p, which had identities higher than 60% and shared similar sizes and functional domains among the yeasts.

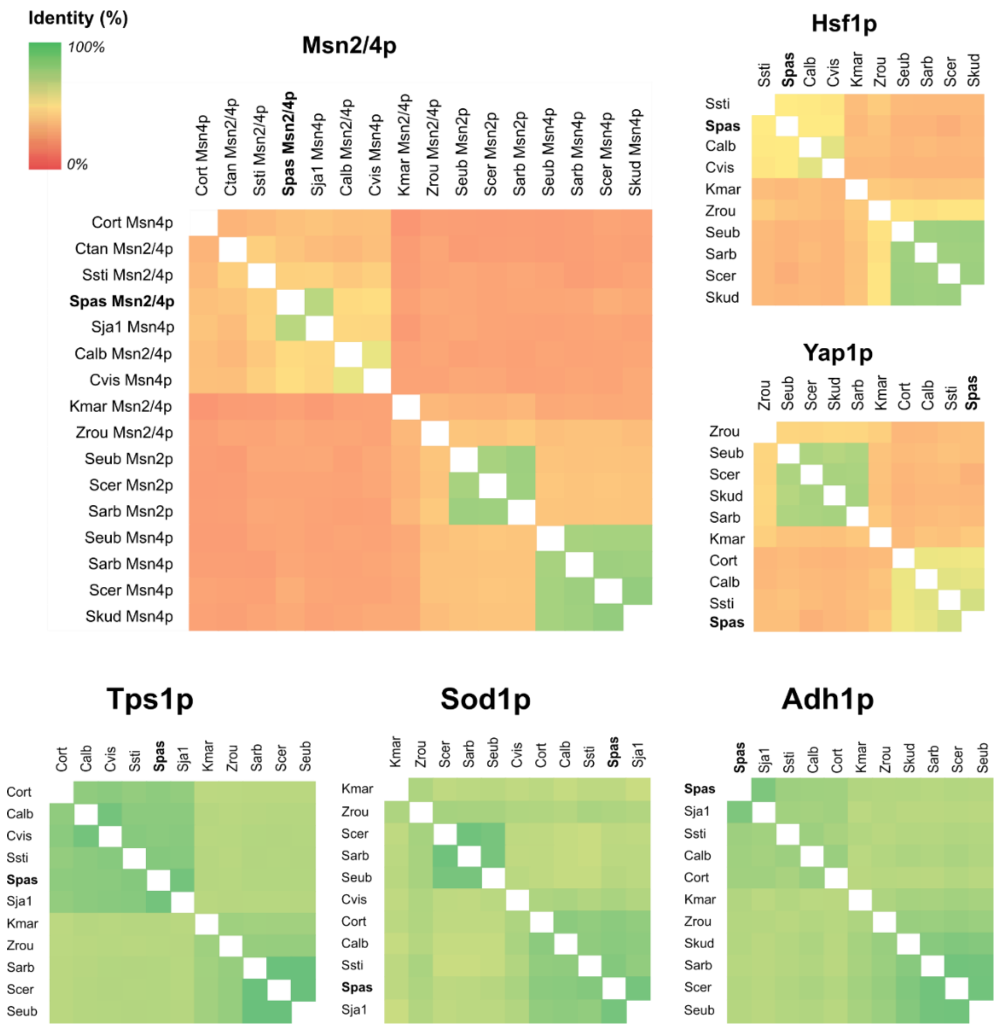


Figure 4. Identity matrices using ethanol-responsive proteins of 13 yeast species. Transcription factors (Msn2/4p, Hsf1p, Yap1p), and proteins involved in carbon metabolism (Adh1p), trehalose biosynthesis (Tps1p), and superoxide detoxification (Sod1p) were used to demonstrate the conservation of amino acid sequences among yeasts. Calb, *Candida albicans* SC5314; Cort, *Candida*

orthopsilosis Co 90-125; Ctan, *Candida tanzawaensis* NRRL Y-17324; Cvis, *Candida viswanathii*; Kmar, *Kluyveromyces marxianus* DMKU3-1042; Sarb, *Saccharomyces arboricola* H6; Scer, *Saccharomyces cerevisiae* S288C; Seub, *Saccharomyces eubayanus*; Sja1, *Spathaspora* sp. JA1; Skud, *Saccharomyces kudriavzevii* IFO1802; Spas, *Spathaspora passalidarum*; Ssti, *Scheffersomyces stipitis* CBS 6054; Zrou, *Zygosaccharomyces rouxii*.

3.4. Tolerance assessment to ethanol, oxidative and osmotic stresses of *S. passalidarum* and *S. cerevisiae* revealed different behaviors

The control experiments showed that all *S. cerevisiae* strains had very similar growth profiles regardless of the presence of gene deletions. On the other hand, *S. passalidarum* grew slower than them but reached a higher final OD value (Fig. 5). Overall, single deletions of the *MSN2* or *MSN4* genes had little or no effect on the tolerance of the yeasts to all concentrations of ethanol, hydrogen peroxide (oxidative), and sodium chloride (osmotic) evaluated in the present study compared to the growth of the background strain (BY4741) in the same conditions. On the other hand, the *MSN2/4* double gene deletion led to an increased sensitivity of the mutant strain to the stresses, more markedly to oxidative (2 and 4 mM H_2O_2) and osmotic (1.25 M NaCl) stresses, and ethanol (10% (v/v)) at a lesser extent. *Spathaspora passalidarum* was not able to grow even in the lowest concentrations of ethanol and NaCl assessed, showing higher sensitivity to these stresses than all evaluated *S. cerevisiae* strains. Surprisingly, *S. passalidarum* showed high tolerance to oxidative stress, and its growth was not impaired even in 4 mM H_2O_2, a condition in which even *S. cerevisiae* BY4741 presented considerable growth inhibition (Fig. 5).

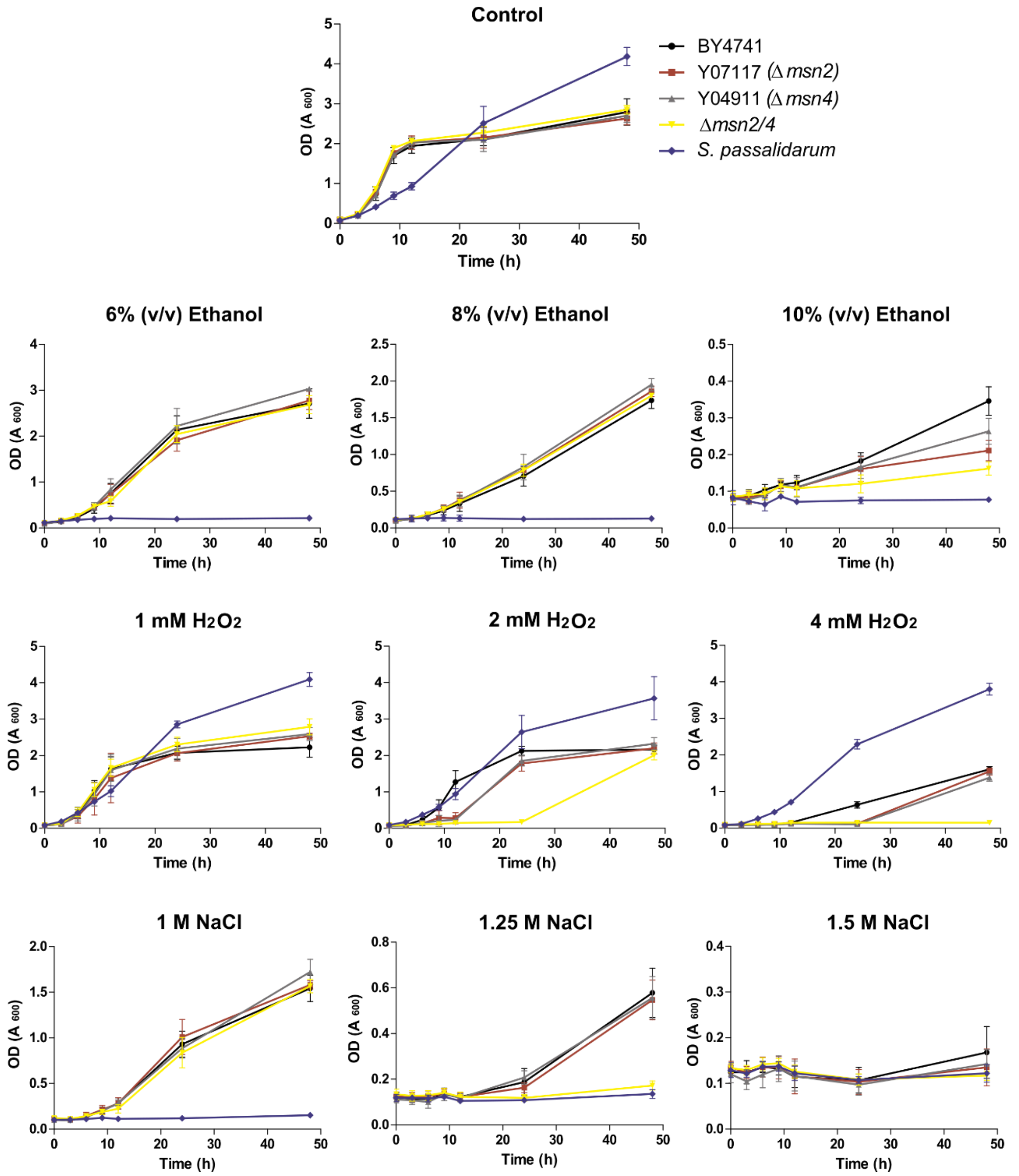


Figure 5: **Tolerance assay of *S. passalidarum* and *S. cerevisiae* strains upon ethanol (6, 8, and 10% (v/v)), oxidative (1, 2, and 4 mM), and osmotic (1, 1.25, and 1.5 M) stresses.** The control experiment was performed in YPD media. All cells were incubated at 30°C for 48 h. The plotted values correspond to the estimated mean and standard deviation of OD_{600} readings. Three individual colonies were assessed from each yeast strain. *Spathaspora passalidarum* (blue line); *Saccharomyces cerevisiae* BY4741 (black line); *Saccharomyces cerevisiae* Y07117 (red line); *Saccharomyces cerevisiae* Y04911 (gray line); and *Saccharomyces cerevisiae Δmsn2/4* (yellow line).

3.5. Complementation assay of mutant strain of *S. cerevisiae msn2 msn4* with the *S. passalidarum MSN-like* gene suggests a different role for these transcription factors

The *MSN-like* gene of *S. passalidarum* was PCR amplified from the yeast genome and cloned in the p426-GPD expression plasmid using restriction enzymes. Cloning was confirmed by sequencing (Supplemental Figure 3 – Appendix B). This construction and the empty plasmid were transformed in *S. cerevisiae* Δ*msn2/4* to evaluate the exclusive effect of the gene complementation in the double mutant strain facing stress conditions. The yeast *S. cerevisiae* BY4741 was also transformed with both plasmids to be used as a growth control (empty vector) and for evaluating the *S. passalidarum MSN-like* gene overexpression in the yeast tolerance profile (Fig. 6).

The growth of all four strains was very impaired under 10% (v/v) ethanol stress. Even though some growth was observed for the BY4741 strains expressing the empty and *MSN-like* cloned plasmids and the *S. cerevisiae* Δ*msn2/4* expressing the *MSN-like* gene under this stress condition, no statistical differences were found regarding the growth of all four strains (Table 2), which limits our conclusions about the phenotype complementation under ethanol stress. Under oxidative stress, no statistical difference was observed between the growth of the double mutant strain expressing the *MSN-like* gene and BY4741 transformed with the empty plasmid. This shows that the complementation of the double mutant strain with the *MSN-like* gene of *S. passalidarum* restored the yeast tolerance to this condition. The overexpression of this gene in the BY4741 strain did not show any statistical growth improvement though (Table 2). Conversely, the overexpression of the *MSN-like* gene in BY4741 statistically reduced the yeast tolerance to osmotic stress compared to the BY4741 control (empty vector), resulting in a growth profile similar to the double mutant lines transformed with empty and p426-GPD-MSN plasmids (Fig. 6).

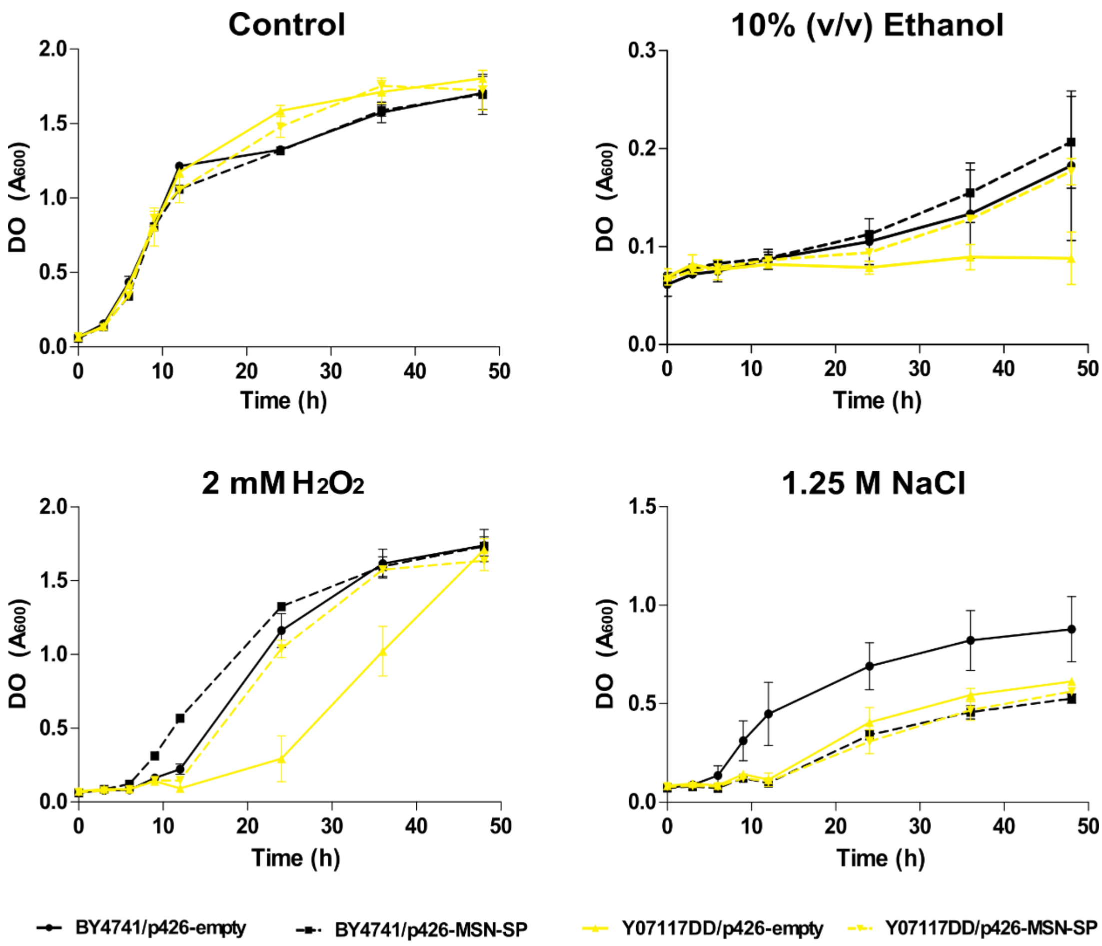


Figure 6: Tolerance assay of *S. cerevisiae* Δ*msn2/4* and BY4741 strains expressing the *MSN-like* gene of *S. passalidarum* under ethanol (10% (v/v)), oxidative (2 mM) and osmotic (1.25 M) stress conditions. The experiments were performed in SD-ura media. All cells were incubated at 30°C for 48 h. The plotted values correspond to the estimated mean and standard deviation of OD_{600} readings. Three individual colonies were assessed from each yeast strain. *Saccharomyces cerevisiae* BY4741 transformed with the empty p426-GPD plasmid (black solid line); *Saccharomyces cerevisiae* BY4741 transformed with p426-GPD-MSN-like construction (black dotted line); *Saccharomyces cerevisiae* Δ*msn2/4* transformed with the empty p426-GPD plasmid (yellow solid line); *Saccharomyces cerevisiae* Δ*msn2/4* transformed with p426-GPD-MSN-like construction (yellow dotted line).

Table 2: Optical density (OD $_{600nm}$) measures represented by the mean and standard deviation values obtained for the transformed *S. cerevisiae* strains under stress conditions. The statistical analysis was performed using ANOVA followed by Tukey's test (α = 0.05). Means identified with the same letters do not show statistical difference.

Stress	Time (h)	BY4741-p426-empty	BY4741-p426-MSN-SP	∆msn2/4-p426-empty	∆msn2/4-p426-MSN-SP
EtOH 10%	48	0.183±0.076 [ns]	0.207± 0.047 [ns]	0.088± 0.027 [ns]	0.177± 0.023 [ns]
H_2O_2 2mM	24	1.163± 0.115 [a]	1.324± 0.027 [a]	0.294± 0.156 [b]	1.039± 0.103 [a]
NaCl 1.25 M	48	0.965± 0.102 [a]	0.526± 0.023 [b]	0.613± 0.009 [b]	0.562± 0.035 [b]

4. DISCUSSION

Spathaspora passalidarum has been widely studied due to its enhanced xylose fermentation ability (Cadete and Rosa 2018; Selim et al. 2020). However, this yeast presents low ethanol tolerance (Campos et al. 2022), and little is known about its responses to other stress conditions usually found in fermentation environments, such as osmotic and oxidative stresses. Thus, the industrial use of *S. passalidarum* depends on a broader understanding of this yeast tolerance profile and the mechanisms involved in its stress adaptation.

Signaling pathways reconstruction and identification of stress-responsive proteins may contribute to a deeper comprehension of stress detection, transduction, and response in yeast species. Our results indicated that signaling components and responsive proteins are highly conserved between the yeasts, presenting similar functional domains, high coverage, and identity values. On the other hand, considerable evolutionary divergences were seen upon comparison of amino acid sequences of the transcription factors of *S. passalidarum* and *S. cerevisiae* yeasts. Morphological and physiological diversity among different species may be related to the evolution of transcriptional control (Chen and Rajewsky 2007; De Mendoza et al. 2013). In this context, two distinct processes may coexist: i) the evolution of cis-elements in the promoter region of the genes; ii) the evolution of transcription factors sequences and their ability to bind to DNA sites (Wagner and Lynch 2008; Schaefke et al. 2015; Mack and Nachman 2017; Rosanova et al. 2017). The dynamic of regulatory changes in cis and trans are complex, and mutations in both aspects can affect the abundance levels of transcripts. In general, trans changes are expected to have a more pleiotropic effect because transcription factors usually interact with multiple downstream target genes leading to a larger impact in gene expression patterns. The functional consequences of cis-regulatory evolution, such as phenotypic evolution, has been well supported by empirical studies (Wray 2007; Wittkopp and Kalay 2011). The transcription factors, however, are thought to evolve more slowly, although it still represents a primary potential source of phenotypic diversity and evolution (Wagner and Lynch 2008). Thus, the divergences in sequence found in our results may have effects on several transcriptional networks that respond to environmental stimuli and contribute to altering the expression levels of hundreds of genes.

Our study revealed a large number of transcription factors only found in *S. cerevisiae*, such as Gcr1p and Pdr1/3p, which are involved in ethanol tolerance (Teixeira et al. 2009; Święciło 2016). Among the orthologs, most *S. cerevisiae* transcription factors differed in sequence and/or had additional copies compared to *S. passalidarum*. Divergences found among the transcription factors might directly affect their post-translational modification, protein-protein interaction, and formation of protein complexes (Hahn and Young 2011). Post-translational modifications, such as phosphorylation, can alter the subcellular location of transcription factors and their DNA binding affinity, affecting gene expression (Oliveira and Sauer 2012). The substitution of serine residues (phosphorylation targets) for alanine in Msn2p impacted the fermentative ability and ethanol tolerance of *S. cerevisiae* (Vamvakas et al. 2019), while the simultaneous replacement of six residues had a lethal effect (Pfanzagl et al. 2018). On the other hand, the substitution of one serine residue for phenylalanine in the TATA-binding protein Spt15p affected the transcription of several genes probably due to the formation of Spt3p-Spt15p-SAGA protein complex increasing ethanol tolerance and fermentative efficiency (Alper et al. 2006).

The evolutionary differences in the transcription factors may be one of the factors contributing to distinct stress responses and, ultimately, to different physiological profiles. The *S. passalidarum* ortholog (*MSN-like*) for the *MSN2/4* genes of *S. cerevisiae* was used to investigate these differences in transcription factors sequence and correlate them with the *S. passalidarum* stress response profile. *Spathaspora passalidarum* has only one copy of these proteins, which shows different isoelectric point and less than 30% global identity compared to both *S. cerevisiae* transcription factors. This difference in copy number is likely related to the Whole Genome Duplication event that occurred during fungal evolution (Wolfe 2015), which might have impacted the patterns of gene regulation in both yeasts since there is evidence of the *MSN2* and *MSN4* individual regulatory contributions under different stress conditions, for instance (Estruch 2000; Ma and Liu 2010). Thus, the existing differences in copy number and the primary structures of the *MSN-like* and *MSN2/4* transcription factors might indicate that their function in stress responses have also diverged.

First, an *S. cerevisiae* knockout strain for the *MSN2/4* genes was constructed, and the stress tolerance profile of *S. passalidarum* and *S. cerevisiae* background, single and double deleted strains for these transcription factors were compared facing ethanol, oxidative, and osmotic stresses. According to our results, the *S. cerevisiae* double deleted line was more sensitive to the evaluated conditions compared to the other assessed strains of *S. cerevisiae*, which agrees with reports from the literature (Martínez-Pastor et al. 1996; Schiavone et al. 2016). The stress phenotype of *S. passalidarum*, however, was a bit surprising. While the low ethanol tolerance of this yeast was a previously known profile, its high tolerance to oxidative stress was an interesting finding that deserves further investigation. Meanwhile, it is possible to suggest that *S. passalidarum* likely presents a high antioxidant capacity due to its xylose assimilation ability and carbon flux through the pentose phosphate pathway, which is a source of reducing power (NADPH) necessary for reducing antioxidant molecules back to their redox-active forms (Meyer et al. 2009; Toledano et al. 2013). On the other hand, *S. passalidarum* was very sensitive even to the lowest NaCl concentration (1 M) tested, a condition in which all *S. cerevisiae* strains grew considerably and with very similar profiles. In the condition of 1.25 M NaCl, the *S. cerevisiae* double mutant strain behaved like *S. passalidarum* and did not show any growth, opposing the other *S. cerevisiae* strains. These results indicate that *S. passalidarum* and *S. cerevisiae* present distinct responses and tolerance phenotypes to the evaluated stresses. Moreover, the role of *MSN2/4* in stress responses is also evident, reinforcing the relevance of these transcription factors action upon stress.

Complementation tests were conducted to evaluate the function of the heterologous expression of the *MSN-like* gene of *S. passalidarum* in *S. cerevisiae* double mutant for the *MSN2* and *MSN4* genes. *Saccharomyces cerevisiae* BY4741 transformed with the empty plasmid and the p426-MSN-SP construction were used as a control and to evaluate the effect of the *MSN-like* gene overexpression, respectively. Overall, the overexpression of the *MSN-like* gene only restored the tolerance of the *S. cerevisiae msn2 msn4* double mutant to oxidative stress, indicating that this gene is clearly involved in the yeast antioxidative stress response. According to our results, oxidative stress is a stressor in which *S. passalidarum* naturally presents a high tolerance profile. Thus, the lack of tolerance improvement in the *S. cerevisiae* background strain (BY4741) overexpressing this gene indicates that probably other factors are involved in this *S. passalidarum* phenotype. Under ethanol stress, there were no statistical differences regarding the growth of all four strains, probably due to the high standard deviation values between the replicates, which limited our conclusions about the phenotype complementation by the *MSN-like* overexpression. Conversely, the *MSN-like* gene did not complement the tolerance of the *S. cerevisiae* double mutant to osmotic stress, and the overexpression of this gene in BY4741 reduced the yeast tolerance to this stress condition, resulting in a growth profile similar to the double mutant strain expressing the empty and p426-MSN-SP plasmids. Although surprising, it is worth noticing that these results agree with the very low tolerance of *S. passalidarum* to osmotic shock shown in figure 5, which suggests that the *MSN-like* transcription factor might be involved in this phenotype. The lack of functional complementation of the *MSN2/4* transcription factors by orthologs was also reported in a study with *Candida albicans* (Nicholls et al. 2004). The CaMsn4 and Mnl1 proteins of *C. albicans* weakly restored the expression of STRE-lacZ reporter genes in *S. cerevisiae msn2 msn4* double knockout strain. According to the study, the CaMsn4 and Mnl1 might have lost their functions in stress response in *C. albicans*, corroborating the authors' idea of significant divergences regarding stress signaling between the two yeast species, which is also our hypothesis in the present study. Overall, our results indicate that changes in transcription regulators affect yeasts adaptation to environmental stimuli regardless of the maintenance of the cellular response mechanisms.

5. CONCLUSION

In this study we suggest that transcription factors are a potential hot spot in the evolution of stress response in yeast. Even though *Spathaspora passalidarum* and *Saccharomyces cerevisiae* show distinct stress tolerance phenotypes, they share conserved stress-responsive mechanisms, such as signaling pathways and ethanol-responsive proteins. In contrast, divergences were found regarding their transcription factors sequences conservation, which was identified as a possible factor affecting the yeasts stress responses. Our results with the *MSN-like* gene corroborate our hypothesis that the transcription factors have diverged in *S. passalidarum* and exert distinct functions in this yeast compared to their orthologs in *S. cerevisiae*. The role of the *MSN-like* gene *of S. passalidarum* appears to have been partially reassigned in this yeast, which might be one of the factors responsible for the low tolerance of *S. passalidarum* to ethanol and osmotic stresses, and high tolerance to oxidative stress. Taken together, these results indicate that effective adaptation depends on a complex network of gene expression control, which may be strongly influenced by differences in transcription factors repertoires.

6. REFERENCES

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science (80-) 314:1565–1568. https://doi.org/10.1126/science.1131969

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/s0022-2836(05)80360-2

Auesukaree C (2017) Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng 124:133–142. https://doi.org/10.1016/j.jbiosc.2017.03.009

Baker RE, Rogers K (2006) Phylogenetic Analysis of Fungal Centromere H3

Proteins. Genetics 174:1481–1492. https://doi.org/10.1534/genetics.106.062794

Cadete RM, Rosa CA (2018) The yeasts of the genus *Spathaspora*: potential candidates for second-generation biofuel production. Yeast 35:191–199. https://doi.org/10.1002/yea.3279

Campos VJ, Ribeiro LE, Albuini FM, de Castro AG, Fontes PP, da Silveira WB, Rosa CA, Fietto LG (2022) Physiological comparisons among *Spathaspora passalidarum*, *Spathaspora arborariae*, and *Scheffersomyces stipitis* reveal the bottlenecks for their use in the production of second-generation ethanol. Brazilian J Microbiol 53:977–990. https://doi.org/10.1007/s42770-022-00693-6

Chen K, Rajewsky N (2007) The evolution of gene regulation by transcription factors and microRNAs. Nat Rev Genet 8:93–103. https://doi.org/10.1038/nrg1990

Chen Y, Sheng J, Jiang T, Stevens J, Feng X, Wei N (2016) Transcriptional profiling reveals molecular basis and novel genetic targets for improved resistance to multiple fermentation inhibitors in *Saccharomyces cerevisiae*. Biotechnol Biofuels 9:1–18. https://doi.org/10.1186/s13068-015-0418-5

Castro, AG (2019) Resposta ao estresse por etanol em *Saccharomyces cerevisiae* e em Spathaspora passalidarum. Doctoral Dissertation, Universidade Federal de Viçosa, Viçosa.

Cunha JT, Soares PO, Romaní A, Thevelein JM, Domingues L (2019) Xylose fermentation efficiency of industrial *Saccharomyces cerevisiae* yeast with separate or combined xylose reductase/xylitol dehydrogenase and xylose isomerase pathways. Biotechnol Biofuels 12:1–14. https://doi.org/10.1186/s13068-019-1360-8

De Mendoza A, Sebé-Pedrós A, Šestak MS, Matejčić M, Torruella G, Domazet-Lošo T, Ruiz-Trillo I (2013) Transcription factor evolution in eukaryotes and the assembly of the regulatory toolkit in multicellular lineages. Proc Natl Acad Sci U S A 110:E4858–E4866. https://doi.org/10.1073/pnas.1311818110

Deparis Q, Claes A, Foulquié-Moreno MR, Thevelein JM (2017) Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS

Yeast Res 17:1–17. https://doi.org/10.1093/femsyr/fox036

El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, Qureshi M, Richardson LJ, Salazar GA, Smart A, Sonnhammer ELL, Hirsh L, Paladin L, Piovesan D, Tosatto SCE, Finn RD (2018) The Pfam protein families database in 2019. Nucleic Acids Res 47:D427–D432. https://doi.org/10.1093/nar/gky995

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24:469–486. https://doi.org/10.1016/S0168-6445(00)00035-8

Fischer S, Brunk BP, Chen F, Gao X, Harb OS, Iodice JB, Shanmugam D, Roos DS, Stoechker CJJ (2011) Using OrthoMCL to assign proteins to OrthoMCL-DB groups or to cluster proteomes into new ortholog groups. Curr Protoc Bioinforma Chapter 6:1219. https://doi.org/10.1002/0471250953.bi0612s35.Using

Gao M, Ploessl D, Shao Z (2019) Enhancing the co-utilization of biomass-derived mixed sugars by yeasts. Front Microbiol 9:3264. https://doi.org/10.3389/fmicb.2018.03264

Gietz RD, Schiestl RH (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2:31–34. https://doi.org/10.1038/nprot.2007.13

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 Genes. Science 274(5287): 546, 563-567. https://doi.org/10.1126/science.274.5287.546

Hahn S, Young ET (2011) Transcriptional regulation in *Saccharomyces cerevisiae*: Transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. Genetics 189:705–736. https://doi.org/10.1534/genetics.111.127019

Hirschman JE, Balakrishnan R, Christie KR, Costanzo MC, Dwight SS, Engel SR, Fisk DG, Hong EL, Livstone MS, Nash R, Park J, Oughtred R, Skrzypek M, Starr B, Theesfeld CL, Williams J, Andrada R, Binkley G, Dong Q, Lane C, Miyasato

S, Sethuraman A, Schroeder M, Thanawala MK, Weng S, Dolinski K, Botstein D, Cherry JM (2006) Genome Snapshot: a new resource at the Saccharomyces Genome Database (SGD) presenting an overview of the *Saccharomyces cerevisiae* genome. Nucleic Acids Res 34:D442-5. https://doi.org/10.1093/nar/gkj117

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94:205–214. https://doi.org/10.1007/s00253-011-3694-4

Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Das U, Daugherty L, Duquenne L, Finn RD, Gough J, Haft D, Hulo N, Kahn D, Kelly E, Laugraud A, Letunic I, Lonsdale D, Lopez R, Madera M, Maslen J, McAnulla C, McDowall J, Mistry J, Mitchell A, Mulder N, Natale D, Orengo C, Quinn AF, Selengut JD, Sigrist CJA, Thimma M, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2009) InterPro: the integrative protein signature database. Nucleic Acids Res 37:D211–D215. https://doi.org/10.1093/nar/gkn785

Kocot KM, Citarella MR, Moroz LL, Halanych KM (2013) PhyloTreePruner: A phylogenetic tree-based approach for selection of orthologous sequences for phylogenomics. Evol Bioinforma 9:429–435. https://doi.org/10.4137/EBO.S12813

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/MOLBEV/MSW054

Li L, Christian J. Stoeckert J, Roos DS (2003) OrthoMCL: Identification of Ortholog Groups for Eukaryotic Genomes. Genome Res 13:2178–2189. https://doi.org/10.1101/gr.1224503

Li P, Fu X, Zhang L, Zhang Z, Li J, Li S (2017) The transcription factors Hsf1 and Msn2 of thermotolerant *Kluyveromyces marxianus* promote cell growth and ethanol fermentation of Saccharomyces cerevisiae at high temperatures. Biotechnol Biofuels 10:1–13. https://doi.org/10.1186/s13068-017-0984-9

Lin NX, Xu Y, Yu XW (2022) Overview of yeast environmental stress response pathways and the development of tolerant yeasts. Syst Microbiol

Biomanufacturing 2:232–245. https://doi.org/10.1007/s43393-021-00058-4

Long TM, Su YK, Headman J, Higbee A, Willis LB, Jeffries TW (2012) Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. Appl Env Microbiol 78:5492–5500. https://doi.org/10.1128/aem.00374-12

Ma M, Liu ZL (2010) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 87:829–845. https://doi.org/10.1007/s00253-010-2594-3

Mack KL, Nachman MW (2017) Gene Regulation and Speciation. Trends Genet 33:68–80. https://doi.org/10.1016/j.tig.2016.11.003

Martínez-Pastor MT, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). EMBO J 15:2227–2235. https://doi.org/10.1002/j.1460-2075.1996.tb00576.x

Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and glutaredoxins: Unifying elements in redox biology. Annu Rev Genet 43:335–367. https://doi.org/10.1146/annurev-genet-102108-134201

Moysés DN, Reis VCB, de Almeida JRM, de Moraes LMP, Torres FAG (2016) Xylose fermentation by *Saccharomyces cerevisiae*: challenges and prospects. Int J Mol Sci 17:207. https://doi.org/10.3390/ijms17030207

Ndubuisi IA, Amadi CO, Nwagu TN, Murata Y, Ogbonna JC (2023) Non-conventional yeast strains: Unexploited resources for effective commercialization of second generation bioethanol. Biotechnol Adv 63. https://doi.org/10.1016/j.biotechadv.2023.108100

Nicholls S, Straffon M, Enjalbert B, Nantel A, Macaskill S, Whiteway M, Brown AJP (2004) Msn2- and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen *Candida albicans*. Eukaryot Cell 3:1111–1123. https://doi.org/10.1128/EC.3.5.1111-1123.2004

Oliveira AP, Sauer U (2012) The importance of post-translational modifications in regulating *Saccharomyces cerevisiae* metabolism. FEMS Yeast Res 12:104–117. https://doi.org/10.1111/j.1567-1364.2011.00765.x

Osiro KO, Borgström C, Brink DP, Fjölnisdóttir BL, Gorwa-Grauslund MF (2019) Exploring the xylose paradox in *Saccharomyces cerevisiae* through in vivo sugar signalomics of targeted deletants. Microb Cell Fact 18:88. https://doi.org/10.1186/s12934-019-1141-x

Pérez-Gallardo R V., Briones LS, Díaz-Pérez AL, Gutiérrez S, Rodríguez-Zavala JS, Campos-García J (2013) Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron-sulfur cluster assembly system. FEMS Yeast Res 13:804–819. https://doi.org/10.1111/1567-1364.12090

Pfanzagl V, Görner W, Radolf M, Parich A, Schuhmacher R, Strauss J, Reiter W, Schüller C (2018) A constitutive active allele of the transcription factor Msn2 mimicking low PKA activity dictates metabolic remodeling in yeast. Mol Biol Cell 29:2848–2862. https://doi.org/10.1091/mbc.E18-06-0389

Ribeiro LE, Albuini FM, Castro AG, Campos VJ, de Souza GB, Mendonça JGP, Rosa CA, Mendes TAO, Santana MF, da Silveira WB, Fietto LG (2021) Influence of glucose on xylose metabolization by *Spathaspora passalidarum*. Fungal Genet Biol 157:103624. https://doi.org/10.1016/j.fgb.2021.103624

Rojas-Ortega E, Aguirre-López B, Reyes-Vivas H, González-Andrade M, Campero-Basaldúa JC, Pardo JP, González A (2018) *Saccharomyces cerevisiae* differential functionalization of presumed ScALT1 and ScALT2 alanine transaminases has been driven by diversification of pyridoxal phosphate interactions. Front Microbiol 9:944. https://doi.org/10.3389/fmicb.2018.00944

Rosanova A, Colliva A, Osella M, Caselle M (2017) Modelling the evolution of transcription factor binding preferences in complex eukaryotes. Sci Rep 7:7596. https://doi.org/10.1038/s41598-017-07761-0

Schaefke B, Wang TY, Wang CY, Li WH (2015) Gains and losses of transcription factor binding sites in *Saccharomyces cerevisiae* and *Saccharomyces* paradoxus. Genome Biol Evol 7:2245–2257. https://doi.org/10.1093/gbe/evv138

Schiavone M, Formosa-Dague C, Elsztein C, Teste MA, Martin-Yken H, De Morais MA, Dague E, François JM (2016) Evidence for a role for the plasma membrane in the nanomechanical properties of the cell wall as revealed by an atomic force microscopy study of the response of *Saccharomyces cerevisiae* to ethanol stress. Appl Environ Microbiol 82:4789–4801. https://doi.org/10.1128/AEM.01213-16

Selim KA, Easa SM, El-Diwany AI (2020) The xylose metabolizing yeast *Spathaspora passalidarum* is a promising genetic treasure for improving bioethanol production. Fermentation 6:1–12. https://doi.org/10.3390/FERMENTATION6010033

Sharma NK, Behera S, Arora R, Kumar S, Sani RK (2018) Xylose transport in yeast for lignocellulosic ethanol production: Current status. J Biosci Bioeng 125:259–267. https://doi.org/10.1016/j.jbiosc.2017.10.006

Sievers F, Higgins DG (2014) Clustal omega, accurate alignment of very large numbers of sequences. Methods Mol Biol 1079:105–116. https://doi.org/10.1007/978-1-62703-646-7_6

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539. https://doi.org/10.1038/msb.2011.75

Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA (2010) The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol 109:13–24. https://doi.org/10.1111/j.1365-2672.2009.04657.x

Su YK, Willis LB, Rehmann L, Smith DR, Jeffries TW (2018) *Spathaspora passalidarum* selected for resistance to AFEX hydrolysate shows decreased cell yield. FEMS Yeast Res 18:foy011. https://doi.org/10.1093/femsyr/foy011

Święciło A (2016) Cross-stress resistance in *Saccharomyces cerevisiae* yeast—new insight into an old phenomenon. Cell Stress Chaperones 21:187–200.

https://doi.org/10.1007/s12192-016-0667-7

Teixeira MC, Monteiro PT, Palma M, Costa C, Godinho CP, Pais P, Cavalheiro M, Antunes M, Lemos A, Pedreira T, Sá-Correia I (2017) YEASTRACT: An upgraded database for the analysis of transcription regulatory networks in *Saccharomyces cerevisiae*. Nucleic Acids Res 46:D348–D353. https://doi.org/10.1093/nar/gkx842

Teixeira MC, Raposo LR, Mira NP, Lourenco AB, Sa-Correia I (2009) Genome-wide identification of *Saccharomyces cerevisiae* genes required for maximal tolerance to ethanol. Appl Environ Microbiol 75:5761–5772. https://doi.org/10.1128/aem.00845-09

Toledano MB, Delaunay-Moisan A, Outten CE, Igbaria A (2013) Functions and cellular compartmentation of the thioredoxin and glutathione pathways in yeast. Antioxidants Redox Signal 18:1699–1711. https://doi.org/10.1089/ars.2012.5033

Udom N, Chansongkrow P, Charoensawan V, Auesukaree C (2019) Coordination of the cell wall integrity and highosmolarity glycerol pathways in response to ethanol stress in *Saccharomyces cerevisiae*. Appl Environ Microbiol 85:1–16. https://doi.org/10.1128/AEM.00551-19

Vamvakas SS, Kapolos J, Farmakis L, Genneos F, Damianaki ME, Chouli X, Vardakou A, Liosi S, Stavropoulou E, Leivaditi E, Fragki M, Labrakou E, Gashi EG, Demoli D (2019) Specific serine residues of Msn2/4 are responsible for regulation of alcohol fermentation rates and ethanol resistance. Biotechnol Prog 35:1–9. https://doi.org/10.1002/btpr.2759

Wagner GP, Lynch VJ (2008) The gene regulatory logic of transcription factor evolution. Trends Ecol Evol 23:377–385. https://doi.org/10.1016/j.tree.2008.03.006

Wittkopp P, Kalay G (2011) Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat Rev Genet 13:59–69

Wohlbach DJ, Kuo A, Sato TK, Potts KM, Salamov AA, LaButti KM, Sun H, Clum A, Pangilinan JL, Lindquist EA, Lucas S, Lapidus A, Jin M, Gunawan C, Balan V, Dale BE, Jeffries TW, Zinkel R, Barry KW, Grigoriev I V., Gasch AP (2011) Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. Proc Natl Acad Sci U S A 108:13212–13217. https://doi.org/10.1073/pnas.1103039108

Wolfe KH (2015) Origin of the yeast whole-genome duplication. PLoS Biol 13:1–7. https://doi.org/10.1371/journal.pbio.1002221

Wray GA (2007) The evolutionary significance of cis-regulatory mutations. Nat Rev Genet 8:206–216. https://doi.org/10.1038/nrg2063

Zabed H, Sahu JN, Suely A, Boyce AN, Faruq G (2017) Bioethanol production from renewable sources: Current perspectives and technological progress. Renew Sustain Energy Rev 71:475–501. https://doi.org/10.1016/j.rser.2016.12.076

Chapter 3

The role of the Pf2826 secreted effector protein of *Pseudozyma flocculosa* in its biocontrol activity against barley powdery mildew

This chapter contains results that are part of the article entitled "Functional analysis of a unique effector secreted by *Pseudozyma flocculosa* reveals a novel mode of action for a biocontrol agent" which has been submitted for publication in the BMC Biology Journal. Authorship: Parthasarathy Santhanam, Mst Hur Madina, Fernanda Matias Albuini, Caroline Labbé, Luciano Gomes Fietto and Richard R. Bélanger.

1. INTRODUCTION

Powdery mildew is a fungal disease that causes significant losses to agriculture and horticulture worldwide (Kiss 2003). Powdery mildew fungi are obligate biotrophic parasites that obtain food from living cells through the generation of a specialized feeding structure called haustorium (Hückelhoven and Panstruga 2011). The pathogens' actions do not kill the plant but interfere with the host immunity in order to access the plant resources and propagate (Chaudhari et al. 2014). Powdery mildew fungi belong to the *Erysiphales* order within the *Ascomycota* phylum. They comprise more than 400 powdery mildew species that can infect nearly 10,000 host species (Takamatsu 2004), including important crops such as barley, wheat, grapevines, apple, and many vegetables and ornamentals (Kiss 2003).

Blumeria graminis f. sp. hordei is a powdery mildew species that causes severe yield losses in barley (*Hordeum vulgare*) (Zhu et al. 2016), the fourth most produced grain in the world (Food and Agriculture Organization of the United Nations). To manage this disease, an alternative approach to agrochemicals comprises the use of biological control, a method that exploits the natural antagonism between microorganisms. Biological control is an efficient and environmentally acceptable approach that receives growing attention due to environmental concerns regarding the excessive use of fungicides and their adverse effects on humans and ecosystems (Kiss 2003). Overall, biocontrol agents (BCA) are natural enemies of invading pathogens that can protect crops with one or more of the following modes of action: induction of host resistance, competition for the same space and nutrients as the pathogen, parasitism, and antibiosis (Ghorbanpour et al. 2018).

Pseudozyma flocculosa is a basidiomycetous fungus that has an efficient biocontrol activity against powdery mildews (Bélanger et al. 2012). It is considered an epiphyte and was first identified in powdery mildew (*Erysiphe polygoni*) infected clover leaves (Traquair et al. 1988). *P. flocculosa* mode of action against powdery mildew has been described as antibiosis according to *in vitro* bioassays, electron microscopy studies, and chemical analyses (Bélanger et al. 2012). This classification was reinforced by the discovery of flocculosin, an extracellular glycolipid that presents antimicrobial activity (Cheng et al. 2003). Flocculosin was later investigated against several microorganisms under *in vitro* conditions and displayed a large spectrum of activity (Mimee et al. 2005; Mimee et al. 2009). However, further studies aiming to relate the production of flocculosin with the mode of action of *P. flocculosa* analyzed the glycolipid production and biocontrol activity of *P. flocculosa*, *U. maydis* and other species of *Pseudozyma* (producers and non-producers of glycolipids). The bioassays revealed that only *P. flocculosa* was able to antagonize powdery mildews, even though the other evaluated microorganisms also produced the same glycolipids profile as *P. flocculosa*. Thus the authors concluded that there was no direct correlation linking glycolipid production and biocontrol activity against powdery mildew, which indicated that flocculosin alone could not be responsible for *P. flocculosa* antagonism to powdery mildews (Clément-Mathieu et al. 2008; Hammami et al. 2011). Additionally, Santhanam and colleagues (2021) recently generated a *P. flocculosa* knockout strain for the *fat1* gene, which encodes the last enzyme of the flocculosin biosynthesis pathway, and they observed that the mutant strain lost its ability to produce flocculosin but conserved its biocontrol properties. These findings corroborate that flocculosin-mediated antibiosis is not responsible for the biocontrol activity of *P. flocculosa in vivo.*

To investigate the mode of action of *P. flocculosa* at a molecular level, Laur and colleagues (Laur et al. 2018) analyzed the simultaneous transcriptomic response of the tritrophic system *P. flocculosa*-powdery mildew (*Blumeria graminis f.sp. hordei*)-barley (*Hordeum vulgare*) over the development of the interaction. The genes within the cluster of flocculosin biosynthesis were expressed at a much lower level in the bioassay compared to *in vitro* conditions, showing no clear evidence of flocculosin central role in the biocontrol activity of *P. flocculosa*. On the other hand, some candidate-secreted effector proteins (CSEPs) such as Pf2826, Pf2382, and Pf0303 were among the highly expressed transcripts. Moreover, these CSEPs were only expressed when in contact with *B. graminis* structures and synchronized with the time at which *P. flocculosa* overwhelmed the pathogen, suggesting that these effectors are somehow involved in fungal-fungal interactions.

The role of these CSEPs in the biocontrol activity of *P. flocculosa* is yet to be experimentally investigated. To functionally characterize the Pf2826 effector protein in the tritrophic interaction, the present study aimed to knockout this gene using the CRISPR Cas9 system and analyze the biocontrol properties of the mutant against powdery mildew *in vivo* and validate the physical interaction of this effector with potential interactors previously identified in a pull-down assay by the Yeast-two-Hybrid methodology.

2. MATERIALS AND METHODS

2.1. Microorganism and growth conditions

Pseudozyma flocculosa stock cultures were grown on Petri dishes containing YMPDA medium (3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, 5 $g.L^{-1}$ peptone water, 10 $g.L^{-1}$ dextrose, and 15 $g.L^{-1}$ agar) for 5 days at 28°C. The culture was inoculated in liquid medium by transferring agar blocks (5 mm) from the margins of the culture into 500 mL baffled flasks containing 100 mL YMPD medium. Cultures were incubated at 28°C on a rotary shaker at 150 rpm for 3 days.

2.2. sgRNA synthesis and RNP complex formation

CRISPR RNA (crRNA) targeting the Pf2826 effector was designed using the E-CRISP online tool (http://www.e-crisp.org/E-CRISP/). A single-stranded oligo (5' - GGTTGTACTTGCCACACAGG - 3') corresponding to the crRNA was synthesized (Life Technologies) and used as the template for the sgRNA synthesis with the EnGen sgRNA synthesis kit from NEB following the manufacturer's instructions (https://international.neb.com/protocols/2016/05/11/engen-sgrna-synthesis-kit-s-pyogenes-protocol-e3322). The resulting sgRNAs were treated with DNAse I (RNAse-free, provided), and purified and concentrated using the RNA clean and concentrator-25 kit from Zymo Research. The Cas9 protein was purchased as a purified protein from GenScript. Ten µg of Cas9 protein was complexed with freshly prepared sgRNA (56 pmol) following the manufacturer's instructions (37°C, 30 min). The resulting ribonucleoprotein (RNP) complex was used for *in vitro* cleavage assay or for the PEG-mediated transformation targeting the Pf2826 gene of *P. flocculosa*.

2.3. *In vitro* digestion assay

The Pf2826 gene was amplified from the genomic DNA of the *P. flocculosa* wild type (WT) strain using the OneTaq DNA polymerase enzyme (NEB) and the primers Pf02826-F (ATGAAGGGATTCAAGCTCAGC) and Pf02826-R (CTAGTTGAAGGTCGGCTTGAC). The PCR product was purified using QIAquick PCR purification kit (Qiagen). For the cleavage reaction, 1.5 µl of diluted Cas9 protein (400 ng.μl^{-1}), 2 μl of 10X Cas9 reaction buffer, 100 ng of freshly prepared sgRNA and 200 ng of purified *Pf2826* PCR product were mixed in a PCR tube. The final reaction volume (20 μl) was completed with sterilized Milli-Q water. The reaction mix was incubated at 37°C for 2 h. The *in vitro* cleavage was analyzed on 1% agarose gel.

2.4. PEG mediated transformation of *P. flocculosa*

For protoplasts generation, a volume of 1.5 ml of a 3-days-old *P. flocculosa* culture was centrifuged at 3900 rpm for 1 min. The cells were resuspended in 15 ml of 5 mM EDTA supplemented with 25 mM *β*-mercaptoethanol and incubated at room temperature for 20 min with gentle shaking. The spores were collected by centrifugation (3900 rpm for 1 min) and resuspended in an enzyme solution containing 50 mg Tricho-derma lysing enzymes (Sigma), 200 mg β-D-Glucanase (Interspex), 100 μl Viscozyme L (Sigma), and 2.5 ml of 50 mM KCE buffer (600 mM KCl, 100 mM sodium citrate, 10 mM EDTA; pH 5.8). Cells were incubated at room temperature with gentle shaking for 5 h. Protoplasts were pelleted by centrifugation (3900 rpm for 2 min), washed with 15 ml of KTC buffer (0.8 M KCl, 25 mM Tris pH 8, 50 mM $CaCl_2$), and pelleted again (3900 rpm for 2 min). Finally, protoplasts were resuspended in 1 ml of KTC buffer.

For the PEG-mediated transformation, 200 μl of protoplasts, 5 μg of the plasmid pRB-Hyg (Supplemental Figure 1 – Appendix C), 20 μl of the RNP complex and 100 μl of PEG solution (PEG 4000 60% w/v, 50 mM Tris-HCl, 50 mM $CaCl_2$) were added sequentially in a 50 ml Falcon tube and incubated on ice for 20 min. The control reaction was prepared as described above but without the addition of DNA (pRB-Hyg plasmid and RNP complex). Subsequently, 2 mL of PEG 4000 solution were added to the wall of the tubes and mixed gently, following another 20 min incubation on ice. For protoplast recovery, 5 ml of YMPD supplemented with 0.8 M sucrose were added to the protoplasts, and the solution was incubated at room temperature with gentle shaking for 1-3 h. After that, 45 ml of lukewarm (42°C) regeneration medium (YMPDA with 0.8 M sucrose) containing or not (control) 50 μg.ml^{-1} of Hygromycin B (Invitrogen) were added to the protoplasts, and the final mixture was immediately plated. The plates were incubated at room temperature for 4 days.

2.5. Confirmation of CRISPR-Cas9 mediated gene edition by sequencing

Isolated colonies were transferred to new Petri dishes containing PDA (Potato Dextrose Agar, Himedia) + Hygromycin B (50 µg.ml^{-1}) medium and incubated at 28°C for approximately 7 days. The transformant colonies and the *P. flocculosa* WT strain were grown on YMPD medium for genomic DNA isolation. The regions flanking the sgRNA binding site in the Pf2826 gene were PCR amplified, and the fragments were sent for sequencing to confirm the gene edition. Stock cells were prepared and stored at -80°C.

2.6. Biocontrol activity assay

Strains of *P. flocculosa* WT and knockout for the Pf2826 effector were grown in baffled flasks containing 100 ml of YMPD at 150 rpm and 28°C for 3 days. From the cultures, spores' suspensions (2 × 106 spores.ml^{-1}) were prepared and sprayed on barley plants infected with *Blumeria graminis* until the solutions runoff. Water was sprayed on the leaves as the negative control. The inoculated leaves were left to evaporate at room temperature for 20 min before a plastic bag was used to cover the plants. Plant tissues were examined at 24 and 36 hours post-inoculation (hpi) by stereomicroscope, and samples were collected for scanning electron microscopy (SEM) analysis. The biocontrol activity of the strains was rated based on the stereomicroscope pictures and according to a scale of 1–5 as previously described by (Hammami et al. 2011), in which 1 = no collapse of conidial chains; 2 = 1 to 25%; 3 = 26 to 50%; 4 = 51 to 75%; and 5 = 76 to 100%.

2.7. Scanning electron microscopy (SEM)

The leaf segments collected from the biocontrol assay were fixed with 100% methanol for a minimum of 10 min, followed by a further dehydration step in 100% ethanol overnight. The 100% ethanol solution was changed twice. Subsequently, the critical drying point was carried out on samples previously mounted on aluminum stubs to further dehydrate them. Finally, gold coating (20 nm) was performed before the SEM observations. Samples were observed on SEM InspectTm F50 from a FEI microscope (Hillsboro).

2.8. Molecular cloning of putative interactors of the Pf2826 effector protein

The coding sequences of seven targets identified as putative interactors of the Pf2826 effector by a previous pull-down experiment, were retrieved from the National Center for Biotechnology Information - NCBI (https://www.ncbi.nlm.nih.gov/). The targets included genes from *B. graminis* and barley. Primers were designed to clone the targets' full-length CDS, excluding the signal peptide in the case of secreted proteins. The list of primers used for cloning can be assessed in the Supplemental Table 1 (Appendix C). Total RNA was extracted from samples of barley leaves infected with *B. graminis* 24 hpi with *P. flocculosa* WT spores' suspension using the RNEasy Mini Purification Kit (QIAGEN) and including a DNAse treatment step as per manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used as the template for the PCR amplifications. The genes were cloned into the pGADT7 and pGBKT7 vectors using restriction enzymes. The constructions were transformed in *Escherichia coli* TOP10 competent cells for propagation by the heat shock method. Confirmation of cloning was performed by sequencing. The cloning of the Pf2826 effector into the pGADT7 and pGBKT7 vectors was performed by a previous work.

2.9. Yeast transformation and interaction validation by the Yeast Two Hybrid (Y2H) system

For the Y2H experiment, the Yeastmaker™ Yeast Transformation System 2 (Clontech) was used following the manufacturer's instructions. The Gold Yeast Two-Hybrid strain was made competent and transformed with the plasmids pGADT7 and pGBKT7 cloned with the Pf2826 effector and the seven putative interactors in both orientations. Moreover, the cells were transformed with both empty plasmids, and with pGBKT7 empty and pGADT7 cloned with the genes to assess the targets autoactivation properties. The transformants were selected on synthetic defined medium lacking leucine and tryptophan (DDO). Plates were incubated at 28°C for 3-5 days. Three individual colonies of each yeast transformation line generated were used for interaction validation. The protein-protein interaction was assessed using synthetic defined media lacking leucine, tryptophan, histidine (TDO), and adenine (QDO) to evaluate the expression of *his3* and *ade2* reporter genes.

3. RESULTS

3.1. *In vitro* digestion assay

The RNP complex, consisting of the Cas9 purified protein and the sgRNA designed to target the Pf2826 gene of *P. flocculosa*, was first tested *in vitro* to evaluate its cleavage potential against the PCR product of the Pf2826 gene (Fig. 1). The gene is 1365 bp long, and the binding site of the sgRNA starts at the nucleotide position 174 (5'- 3'). The digestion reaction in column 2 shows a strong DNA band slightly smaller than the control (column 1) and a faint band close to the bottom of the gel, which represents the cleaved piece. The control reaction consists of the same PCR product submitted to the incubation step without the addition of the RNP complex. The cleavage results indicate that the digestion reaction worked efficiently, since digested products showed the expected sizes.

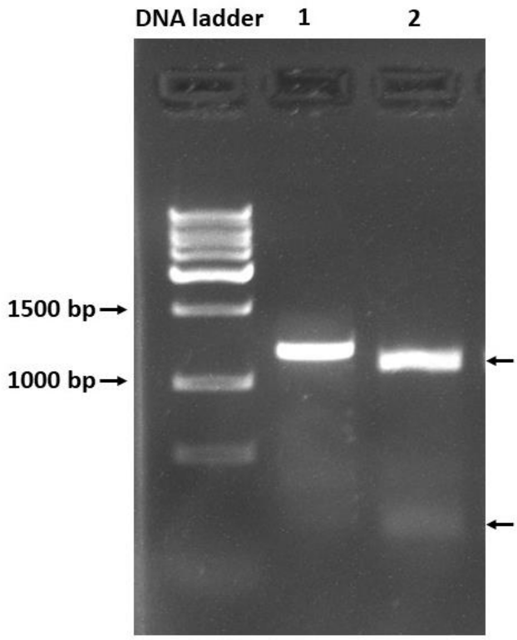


Figure 1. *In vitro* digestion of the Pf2826 PCR product by the RNP complex designed to target this gene. Column 1 represents the control reaction of the digestion in which the RNP complex was not included. Column 2 represents the digestion reaction.

3.2. CRISPR-Cas9 mediated gene edition in *P. flocculosa*

Pseudozyma flocculosa WT strain was transformed with freshly synthesized RNP complex and the pRB-Hyg plasmid by a PEG-mediated protocol. Colonies that grew on selective media containing hygromycin after transformation were propagated in liquid media for genomic DNA extraction. The Pf2826 gene was PCR amplified from the transformants, and the products were sent for sequencing to verify the presence of a CRISPR-Cas9 mediated edition event in the gene sequence. The sequencing results for one of the colonies showed a deletion of 59 nucleotides in the region surrounding the sgRNA binding site (Fig. 2). Based on this result, we can expect that this nucleotide deletion will be translated into a truncated form of the protein, compromising its function. The mutant strain was identified as Pf2826^{59x} and used in further experiments to evaluate the role of the Pf2826 effector in the biocontrol activity of *P. flocculosa*.

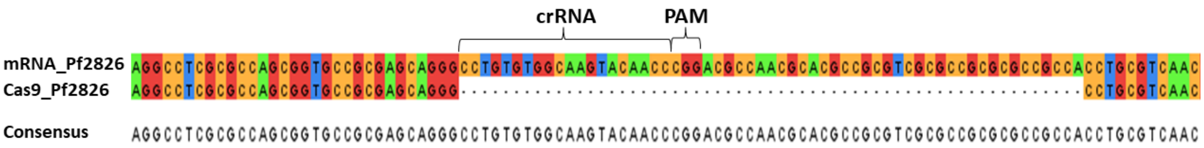


Figure 2. Comparison of the nucleotide sequences surrounding the sgRNA binding site of the Pf2826 gene of *P. flocculosa* WT (mRNA_Pf2826) and the mutant strain generated by CRISPR-Cas9 mediated edition (Cas9_Pf2826).

3.3. *In vitro* physiological characterization and biological control assay of the knockout strain

The growth profile of the Pf2826^{59x} strain was evaluated in liquid and solid media to verify if the mutation compromised the fitness of the organism. The results showed the absence of morphological differences regarding spore production (size and shape) and radial growth on agar plates between the mutant and *P. flocculosa* WT strains (Fig. 3).

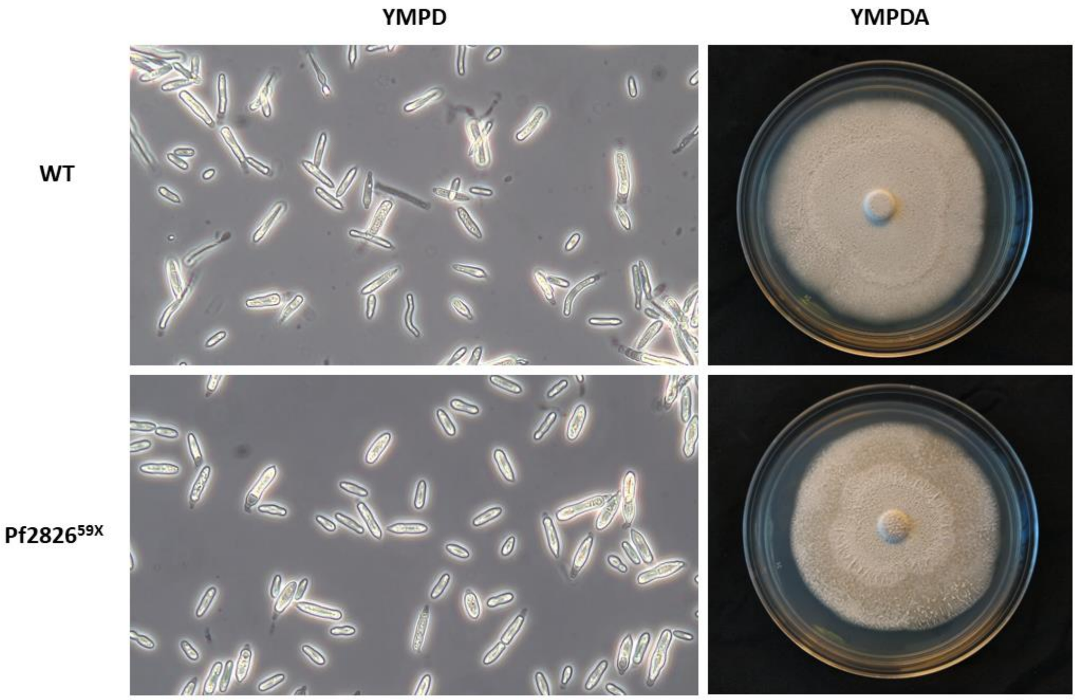


Figure 3. Physiological characterization of *P. flocculosa* Pf2826^{59x} strain regarding spore production and radial growth. Pictures were taken 3 dpi in liquid cultures and 7 dpi in solid media. WT stands for *P. flocculosa* wild type strain. Pf2826^{59x} refers to the CRISPR-Cas9 generated mutant strain.

The biocontrol assay was performed with the Pf2826^{59x} mutant strain, and *P. flocculosa* WT and water as controls (Fig. 4 a, b). After 36 h of inoculation with the *P. flocculosa* strains, the stereomicroscope observations showed that *B. graminis* conidial chains were completely agglutinated and overgrown by *P. flocculosa* WT, whereas a more moderate stage of conidia and conidiophores collapse was observed in the treatment with the Pf2826^{59x} mutant strain. These observations were rated and presented as a graph (Fig. 5 b), which shows a reduction of the biocontrol activity of the mutant strain. The SEM pictures confirmed these results in a more detailed view by presenting the mycelial filaments of the *P. flocculosa* strains either extensively colonizing *B. graminis* structures (WT) or at an earlier stage of colonization (Pf2826^{59x}). The water control showed that the *B. graminis* structures were kept intact throughout the experiment in which the plants were not sprayed with the biocontrol agent.

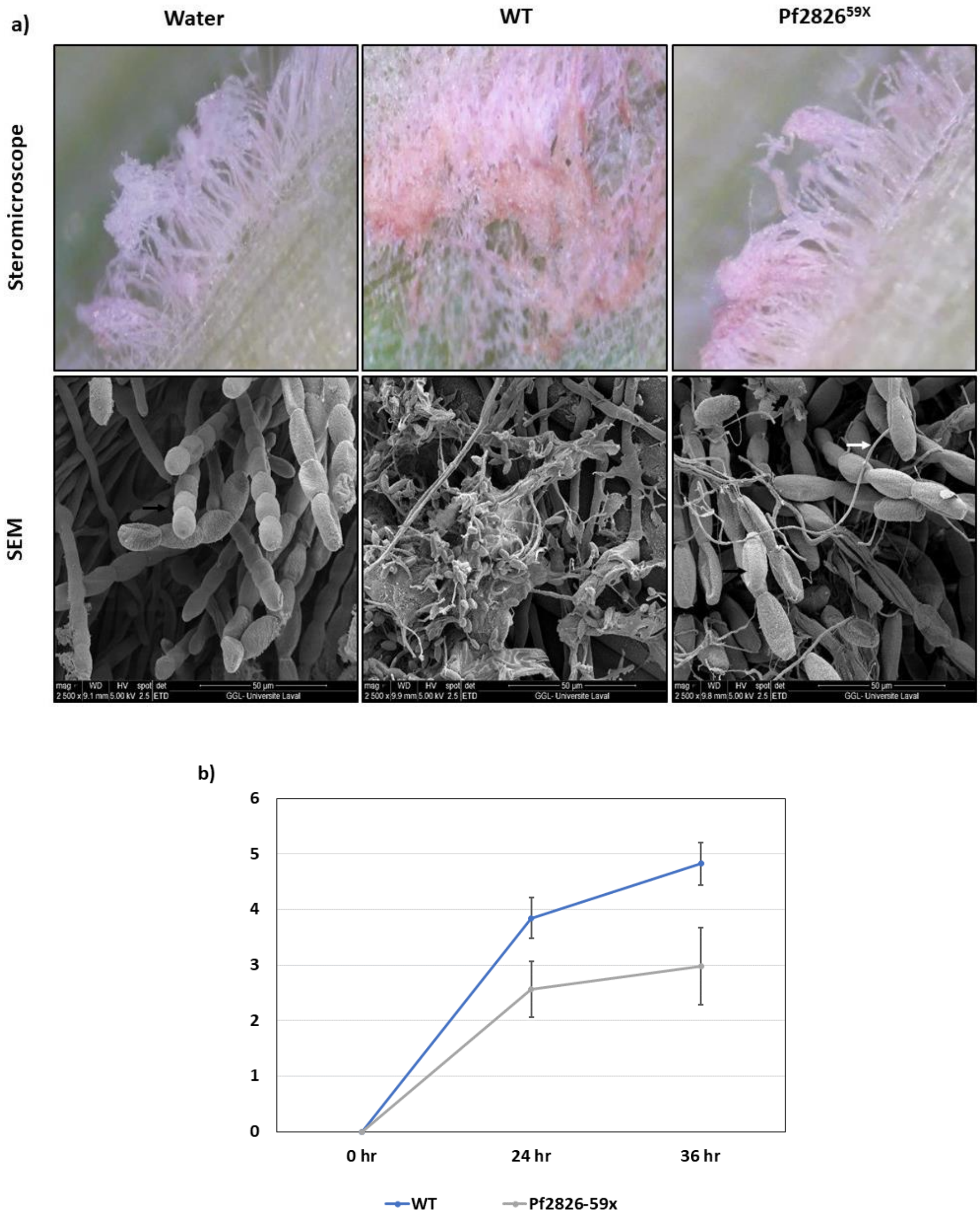


Figure 4. Comparative assessment of the biocontrol potential of *P. flocculosa* WT and Pf2826^{59x} strains against barley powdery mildew by stereomicroscope and SEM observations at 36 hpi. **a)** The stereomicroscope analysis shows the complete agglutination of *B. graminis* conidial chains in the assay with the *P. flocculosa* WT strain, while a more moderate stage of collapse was observed in the treatment with the mutant strain (Pf2826^{59x}). The SEM images confirm these results by showing the mycelial filaments of the *P. flocculosa* WT strain extensively colonizing the *B. Graminis* structures, while a lower stage of colonization is observed in the mutant

strain. *P. flocculosa* (white arrow); *B. graminis* (black arrow). **b)** Biocontrol rating graph based on the stereomicroscope pictures taken at 24 and 36 hpi, in which 0 = no activity and 5 = complete collapse of the powdery mildew colonies. Each experiment comprised five leaves and three different sites per leaf.

3.4. Validation of putative interactors of the Pf2826 effector protein

Seven putative interactors of the Pf2826 effector (Table 1) were selected for interaction validation by Y2H. The selection of the putative interactors from barley and powdery mildew was based on their molecular function and cellular localization, and on previous immunolocalization results which showed that the Pf2826 effector localizes around the haustoria and on powdery mildew spores (submitted data). The two selected targets from barley (A0A287N0S4 and A0A287RAV7) are potentially involved in stress responses and presented predicted signal peptides, which indicates that they are secreted to the extracellular region. Three out of the five targets from *B. graminis* were identified as candidate secreted effector proteins (N1J6Z3, A0A383UML4 and N1J908). *Blumeria graminis* potential interactors also included a serine/threonine-protein kinase (N1J4Z2) and an uncharacterized protein related to the COP9 signalosome complex (A0A383V2V0).

The targets and the Pf2826 effector were cloned in pGADT7 and pGBKT7 plasmids and co-transformed in the Gold Yeast Two-Hybrid strain. The interaction between the preys and the Pf2826 effector were assessed by the reconstitution of an active form of the Gal4 transcription factor and consequent transcription activation of two reporter genes (*his3* and *ade*2), allowing growth on TDO and QDO media. Some basal growth was observed for all lines in TDO medium (Fig. 5). The growth on QDO media indicated that the Pf2826 effector interacted in yeast with the barley pathogenesis-related (PR) proteins HvPR1a (A0A287RAV7) and with a putative effector protein (N1J908) from powdery mildew in both bait-prey cloning orientations. Yeast lines expressing the uncharacterized effector protein (A0A383V2V0) from powdery mildew predominantly grew in one bait-prey orientation on QDO medium, and some growth was also observed for the negative control (pGBKT7-uncharacterized effector/pGADT7-empty). The yeast lines expressing the other targets behaved like negative controls and did not grow on QDO media, which indicates that there is no interaction between them and the Pf2826 effector (Fig. 5).

Table 1. List of targets selected from a pull-down screening using the Pf2826 effector as bait used for interaction validation by Y2H.

Organism	Accession (Uniprot)	Protein name (Uniprot)	Protein length (aa)	GO (cellular component)	GO (molecular function or biological process)	Signal Peptide
Hordeum vulgare subsp. vulgare (Domesticated barley)	A0A287N0S4	Germin like protein	183	Apoplast (GO:0048046); Extracellular region (GO:0005576)		YES (cleavage between 23 and 24 aa residues)
Hordeum vulgare subsp. vulgare (Domesticated barley)	A0A287RAV7	HvPr-1a	170	Extracellular region (GO:0005576)	Response to biotic stimulus (GO:0009607)	YES (cleavage between 24 and 25 aa residues)
Blumeria graminis f. sp. hordei (strain DH14)	N1J4Z2	Serine/threonine-protein kinase Sgk2	234		kinase activity (GO:0016301)	NO
Blumeria graminis f. sp. hordei (strain DH14)	A0A383V2V0	Uncharacterized protein	511	COP9 signalosome (GO:0008180)		NO
Blumeria graminis f. sp.	N1J6Z3	CSEP0313 putative effector	384			YES (cleavage

hordei (strain DH14)		protein		between 19 and 20 aa residues)
Blumeria graminis f. sp. hordei (strain DH14)	A0A383UML4	Uncharacterized protein	171	YES (cleavage between 18 and 19 aa residues)
Blumeria graminis f. sp. hordei (strain DH14)	N1J908	Putative candi-date secreted effector protein	134	YES (cleavage between 21 and 22 aa residues)

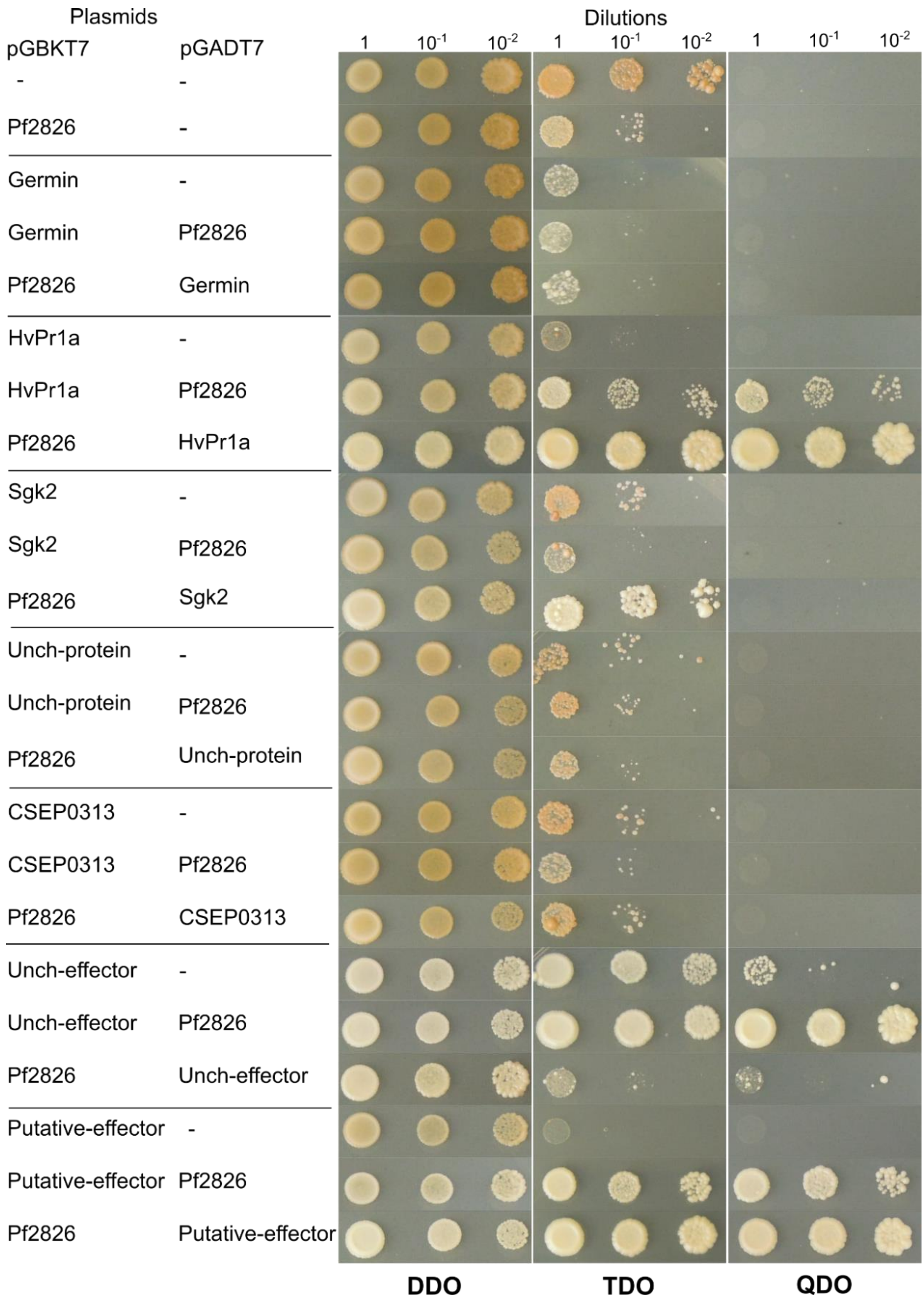


Figure 5. Validation of interaction between the Pf2826 effector and putative interactors from barley and powdery mildew by Y2H. Growth on TDO and QDO media indicates interaction. DDO (SD/−Leu/−Trp); TDO (SD/−Leu/−Trp/-His); QDO (SD/−Leu/−Trp/ -His/- Ade). Pictures were taken 5 days after spotting and incubation

at 30 °C. From top to bottom, candidate interactors are A0A287N0S4 (Germin); A0A287RAV7 (HvPR-1a); N1J4Z2 (Sgk2), A0A383V2V0 (Unch-protein), N1J6Z3 (CSEP0313), A0A383UML4 (Unch-effector), and N1J908 (Putative-effector).

4. DISCUSSION

Pseudozyma flocculosa, a yeast-like fungus recognized by its powerful antagonistic potential against powdery mildew, is the object of study of the present work. More precisely, we attempted to investigate how *P. flocculosa* exerts its biocontrol activity in the tritrophic system which includes barley and powdery mildew (*B. graminis*). Recent evidence has demonstrated that flocculosin-mediated antibiosis does not account for the *P. flocculosa* biocontrol mode of action (Santhanam et al. 2021). Conversely, a set of unique candidates secreted effector proteins (CSEPs) of *P. flocculosa* were strongly and exclusively expressed during contact with powdery mildew structures in the tripartite interaction (Laur et al. 2018). Among these CSEPs, the most expressed one was the Pf2826 effector, a 454 amino acids long protein containing a predicted N-terminal signal peptide. In this regard, the present study aimed to functionally characterize the Pf2826 effector protein by knocking out this gene and by confirming possible interaction partners belonging to barley and powdery mildew.

The knockout of the Pf2826 gene was performed using the CRISPR-Cas9 system. This methodology allows site-specific genome modifications and is especially interesting for organisms that mainly relay on non-homologous end joining (NHEJ) to repair double-strand DNA breaks (Adli 2018). While gene disruption in *Saccharomyces cerevisiae* can be easily achieved using DNA cassettes displaying short stretches of homology domains (< 50 bp) targeting the site of interest through homologous recombination (HR), a similar strategy was not effective when applied to *P. flocculosa* because it led to multiple random genomic insertions (Santhanam et al. 2021). On the other hand, the same study achieved successful gene knockout in *P. flocculosa* by using the CRISPR-Cas9 technology. In short, the technology principle relies on the ability of the Cas9 nuclease to create double-stranded DNA breaks driven by the complementarity of a designed RNA guide molecule at any locus three nucleotides upstream of an NGG protospacer adjacent motif (PAM). The cellular DNA repair pathways are then activated and result in indels (insertions or deletions) that can disrupt genes (Adli 2018). Our sequencing analysis revealed the occurrence of an edition event that led to the deletion of 59 nucleotides in the Pf2826 gene sequence, which consequently translates into a shorter protein with an altered frame and very likely compromised function.

The disruption of the Pf2826 gene allowed the interrogation of its functional significance regarding the antagonistic activity of *P. flocculosa* against barley powdery mildew. The mutant and WT strains of *P. flocculosa* were sprayed on powdery mildew-infected barley leaves, and the plants were monitored by microscopy observations. The results showed that the knockout strain was not able to overtake the powdery mildew colonies, while the WT strain developed extensively and resulted in the complete collapse of the pathogen structures. The observations demonstrated that the $Pf2826^{59X}$ strain was less aggressive against powdery mildew and showed reduced biocontrol activity compared to the WT. These results indicate that the Pf2826 effector plays an essential role in the biocontrol phenotype of *P. flocculosa*. It is well documented that effector proteins are central players in plant-pathogen interactions. These molecules are secreted by the pathogens to manipulate plant processes to allow successful infection (Asai and Shirasu 2015). However, the identification of a crucial effector belonging to a biocontrol agent brings new insight into the development of the interaction system and the biological control modes of action.

The identification of interaction partners of the Pf2826 protein was performed aiming to shed light into the function of this effector. Proteins are biological molecules that usually establish a set of physical interactions with other molecules to properly exert their biological activity. Protein-protein interactions participate in the development of many cellular processes, such as sensing and transducing environmental signals, catalyzing enzymatic reactions, and maintaining cellular organization (Braun and Gringras 2012). The screening of the putative interactors of the Pf2826 effector was previously performed by an *in vitro* pull-down assay using the Pf2826 fused to an N-terminal his-tag and the total protein extract from the tripartite assay 36 hpi with spores of *P. flocculosa* (data not shown). Mass spectrometry analysis identified putative interactors from all three organisms. From the list, two targets from barley and five from *B. graminis* were selected for interaction validation by Y2H. Pull-down and Y2H are two complementary approaches in what concerns the kind of interactors they can detect. In short, pulldown is an *in vitro* methodology that can identify many molecules that constitute larger complexes, even though not all of them interact directly with the bait. The Y2H assay is an *in vivo* system that evaluates the interaction between two proteins in yeast (Brückner et al. 2009). It is relevant to highlight that the combination of these two approaches increases the chance of identifying a real interaction.

The Y2H results confirmed the interaction of the Pf2826 effector with proteins from the plant (HvPr-1a) and the pathogen (putative effector). The pathogenesis-related protein HvPr-1a from barley (A0A287RAV7), is a putative extracellular protein involved in plant response to biotic stimulus (Uniprot). In general, PR proteins are classified into several families and described as associated with plant resistance (Muthukrishnan et al. 2001). For instance, the PR-1b protein from barley was shown to contribute to penetration resistance to powdery mildew fungus (Schultheiss et al. 2003). The abundance of several classical PR proteins was shown to be increased upon attack of pathogens (Lambertucci et al. 2019). Moreover, the characterization of some PR proteins revealed antifungal functions such as lytic enzyme activity (chitinases, glucanases), proteinase inhibitors, and peroxidases (Van Loon and Van Strien 1999). Although the function of the PR-1a protein is unknown, its interaction with the Pf2826 effector suggests that the biocontrol agent may contribute to boosting the plant defenses against *B. graminis* infection.

A putative secreted effector (N1J908) from *B. graminis* was also validated as an interaction partner of the Pf2826 effector. Fungal effectors are virulence factors delivered by the pathogen to interfere with the plant immunity or mask pathogen invasion (Chaudhari et al. 2014). The release of *B. graminis* genome allowed the identification of 491 genes classified as candidate secreted effector proteins (CSEPs) (Pedersen et al. 2012), and Y2H screenings have already identified *B. graminis* effectors that interact with barley proteins. For instance Zhang and colleagues (2012) (Zhang et al. 2012) confirmed that the CSEP0055 effector contributes to the aggressiveness of the pathogen and interacts with the PR1 and PR17 proteins from the host. Additionally, Peningthon and colleagues (2016) (Pennington et al. 2016) showed that the BEC1054 effector interacts with several barley proteins including a malate dehydrogenase, a glutathione-S-transferase (GST), an elongation factor (eEFg), and the PR5 thaumatin-like protein. Based on our results, we can infer that the PF2826 effector from *P. flocculosa* might also be involved in reducing *B. graminis* infection aggressiveness against barley by interacting with one of the pathogen's effectors.

5. CONCLUSION

The present study investigated the mode of action of the biocontrol agent *P. flocculosa* against barley powdery mildew. The use of the CRISPR-Cas9 gene edition system brought evidence of the direct role of a fungal effector (Pf2826) in the antagonistic activity of a biocontrol agent. Moreover, the validation of interaction partners by the yeast two-hybrid methodology revealed the occurrence of a fungal-fungal interaction, besides the association between a biocontrol effector and a plant defense protein, which might have both contributed to altering the plant-powdery mildew fungus biotrophic interaction resulting in the pathogen collapse.

6. REFERENCES

Adli M (2018) The CRISPR tool kit for genome editing and beyond. Nat Commun 9. https://doi.org/10.1038/s41467-018-04252-2

Asai S, Shirasu K (2015) Plant cells under siege: Plant immune system versus pathogen effectors. Curr Opin Plant Biol 28:1–8. https://doi.org/10.1016/j.pbi.2015.08.008

Bélanger RR, Labbé C, Lefebvre F, Teichmann B (2012) Mode of action of biocontrol agents: All that glitters is not gold. Can J Plant Pathol 34:469–478. https://doi.org/10.1080/07060661.2012.726649

Braun P, Gringras A-C (2012) History of protein-protein interactions: From egg-white to complex networks. Proteomics 12:1478–1498

Brückner A, Polge C, Lentze N, Auerbach D, Schlattner U (2009) Yeast two-hybrid, a powerful tool for systems biology. Int J Mol Sci 10:2763–2788. https://doi.org/10.3390/ijms10062763

Chaudhari P, Ahmed B, Joly DL, Germain H (2014) Effector biology during biotrophic invasion of plant cells. Virulence 5:703–709. https://doi.org/10.4161/viru.29652

Cheng Y, McNally DJ, Labbé C, Voyer N, Belzile F, Bélanger RR (2003) Insertional mutagenesis of a fungal biocontrol agent led to discovery of a rare cellobiose lipid with antifungal activity. Appl Environ Microbiol 69:2595–2602. https://doi.org/10.1128/AEM.69.5.2595-2602.2003

Clément-Mathieu G, Chain F, Marchand G, Bélanger RR (2008) Leaf and powdery mildew colonization by glycolipid-producing *Pseudozyma* species. Fungal Ecol 1:69–77. https://doi.org/10.1016/j.funeco.2008.09.003

Ghorbanpour M, Omidvari M, Abbaszadeh-Dahaji P, Omidvar R, Kariman K (2018) Mechanisms underlying the protective effects of beneficial fungi against plant diseases. Biol Control 117:147–157. https://doi.org/10.1016/j.biocontrol.2017.11.006

Hammami W, Castro CQ, Rémus-Borel W, Labbé C, Bélanger RR (2011) Ecological basis of the interaction between *Pseudozyma flocculosa* and powdery mildew fungi. Appl Environ Microbiol 77:926–933. https://doi.org/10.1128/AEM.01255-10

Hückelhoven R, Panstruga R (2011) Cell biology of the plant-powdery mildew interaction. Curr Opin Plant Biol 14:738–746. https://doi.org/10.1016/j.pbi.2011.08.002

Kiss L (2003) A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Manag Sci 59:475–483. https://doi.org/10.1002/ps.689

Lambertucci S, Orman KM, Das Gupta S, Fisher JP, Gazal S, Williamson RJ, Cramer R, Bindschedler LV (2019) Analysis of Barley Leaf Epidermis and Extrahaustorial Proteomes During Powdery Mildew Infection Reveals That the PR5 Thaumatin-Like Protein TLP5 Is Required for Susceptibility Towards *Blumeria graminis f. sp. hordei*. Front Plant Sci 10. https://doi.org/10.3389/fpls.2019.01138

Laur J, Ramakrishnan GB, Labbé C, Lefebvre F, Spanu PD, Bélanger RR (2018) Effectors involved in fungal–fungal interaction lead to a rare phenomenon of hyperbiotrophy in the tritrophic system biocontrol agent–powdery mildew–plant. New Phytol 217:713–725. https://doi.org/10.1111/nph.14851

Mimee B, Labbé C, Bélanger RR (2009) Catabolism of flocculosin, an antimicrobial metabolite produced by *Pseudozyma flocculosa*. Glycobiology 19:995–1001. https://doi.org/10.1093/glycob/cwp078

Mimee B, Labbé C, Pelletier R, Bélanger RR (2005) Antifungal activity of flocculosin, a novel glycolipid isolated from *Pseudozyma flocculosa*. Antimicrob Agents Chemother 49:1597–1599. https://doi.org/10.1128/AAC.49.4.1597-1599.2005

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2001) Pathogenesis-related proteins and their genes in cereals. Plant Cell Tissue Organ Cult 64:93–114. https://doi.org/10.1023/A:1010763506802

Pedersen C, van Themaat EVL, McGuffin LJ, Abbott JC, Burgis TA, Barton G, Bindschedler L V., Lu X, Maekawa T, Weßling R, Cramer R, Thordal-Christensen H, Panstruga R, Spanu PD (2012) Structure and evolution of barley powdery mildew effector candidates. BMC Genomics 13. https://doi.org/10.1186/1471-2164-13-694

Pennington HG, Gheorghe DM, Damerum A, Pliego C, Spanu PD, Cramer R, Bindschedler L V. (2016) Interactions between the powdery mildew effector BEC1054 and barley proteins identify candidate host targets. J Proteome Res 15:826–839. https://doi.org/10.1021/acs.jproteome.5b00732

Santhanam P, Labbé C, Fietto LG, Bélanger RR (2021) A reassessment of flocculosin-mediated biocontrol activity of *Pseudozyma flocculosa* through CRISPR/Cas9 gene editing. Fungal Genet Biol 153:103573

Schultheiss H, Dechert C, Király L, Fodor J, Michel K, Kogel KH, Hückelhoven R (2003) Functional assessment of the pathogenesis-related protein PR-1b in barley. Plant Sci 165:1275–1280. https://doi.org/10.1016/S0168-9452(03)00336-4

Takamatsu S (2004) Phylogeny and evolution of the powdery mildew fungi (Erysiphales, Ascomycota) inferred from nuclear ribosomal DNA sequences. Mycoscience 45:147–157. https://doi.org/10.1007/s10267-003-0159-3

Traquair JA, Shaw LA, Jarvis WR (1988) New species of *Stephanoascus* with

Sporothrix anamorphs . Can J Bot 66:926–933. https://doi.org/10.1139/b88-134

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85–97. https://doi.org/10.1006/pmpp.1999.0213

Zhang WJ, Pedersen C, Kwaaitaal M, Gregersen PL, Mørch SM, Hanisch S, Kristensen A, Fuglsang AT, Collinge DB, Thordal-Christensen H (2012) Interaction of barley powdery mildew effector candidate CSEP0055 with the defence protein PR17c. Mol Plant Pathol 13:1110–1119. https://doi.org/10.1111/j.1364-3703.2012.00820.x

Zhu J, Zhou Y, Shang Y, Hua W, Wang J, Jia Q, Liu M, Yang J (2016) Genetic evidence of local adaption and long distance migration in *Blumeria graminis f. sp. hordei* populations from China. J Gen Plant Pathol 82:69–81. https://doi.org/10.1007/s10327-016-0643-1

CONCLUSION

Understanding the molecular basis underlying a microorganism given phenotype is pivotal for accelerating biotechnological exploitation. The study of *Spathaspora passalidarum* transcriptional responses to ethanol stress revealed the repression of genes responsible for nutrient transportation and from the central carbon pathways, halting sugar consumption and fermentation. These findings suggest that ethanol stress causes a pseudo-starvation state, in which nutrients are not accessible to the cells, although available in abundant amounts in the medium. Additionally, a pseudo-hyphal morphology of *S. passalidarum* colonies was observed in ethanol-treated cells, and an increase in oleic acid content was only observed at a late stage of the fermentation growth curve. A limited osmotic stress response is another possible aspect involved in the low ethanol tolerance profile of *S. passalidarum* inferred from our data.

Changes in gene expression profiles, such as the ones shown by our transcriptome analysis, can be associated with the action of transcription factor proteins. Comparisons between the set of transcription factors from *S. passalidarum* and *Saccharomyces cerevisiae* showed wide divergences in sequence and copy numbers among them, which can be one of the factors influencing the adaptive responses of the yeasts to stress. Growth profiling showed that *S. passalidarum* and *S. cerevisiae* present different tolerance levels to ethanol, oxidative and osmotic stress. Complementation assays of the *MSN-like* transcription factor of *S. passalidarum in S. cerevisiae msn2 msn4* double knockout strain revealed a partial tolerance phenotype restoration in the mutant line. These results indicate that the *MSN-like* exerts different functions in stress responses from its orthologs in *S. cerevisiae*, which agrees with our bioinformatic data that showed high divergences in transcription factor sequences among yeast and our hypothesis that this might influence yeast stress adaption.

The study of the mode of action of *Pseudozyma flocculosa* antagonistic activity against barley powdery mildew revealed the Pf2826 effector protein as a new player involved in this phenotype. The knockout of this effector compromised the ability of the mutant strain to overtake the powdery mildew colonies, indicating that it is required for the full biocontrol activity of *P. flocculosa*. The search for interaction partners of the Pf2826 effector identified that it interacts with a pathogenesis-related protein from barley and an effector protein from powdery mildew, which might contribute to deregulating the interaction between the host and the pathogen, leading to the collapse of the powdery mildew structures. These findings point to a new mode of action with which biocontrol agents can antagonize pathogens that involves the direct action of effector proteins.

APPENDIX

APPENDIX A: Supplemental material Chapter 1

Supplemental File S1 (T1 – T6), Link:

https://docs.google.com/spreadsheets/d/12c3GK6cxvyokZEcMVEuwEB5OWi3pDspG/edit?usp=share_link&ouid=102431636824392108401&rtpof=true&sd=true

Supplemental Table 1: List of oligonucleotides used in the study.

NCBI ID	Protein annotation *S. passalidarum*	Protein annotation *S. cerevisiae*	Primer Forward	Primer Reverse	Reference
SPAPADRAFT_61341	XYL1.1	GRE3	CTCAGGTCACTTGATGCCTTTAG	TCTTTAAACCGTCACCGACTTCC	Cadete et al., 2016
SPAPADRAFT_59053	XYL2.1	SOR1	TGCTGCCAGAGTCATTGTCATTG	AACATTTGGTTCAACACCGTCAA	Ribeiro et al., 2021
SPAPADRAFT_67816	TDH3	TDH3	ACGGTTTCGGACGTATTGGT	TAAGCAGCGTAGTCGGTAGC	Campos et al., 2021
SPAPADRAFT_130940	not annotated	YOR283W	CGTGATATGGGACATTGTGAGGGA	CACCACCATGAGTACATACCAAGACA	This work
SPAPADRAFT_59873	not annotated	ADH5	TGCTTGTGTTGGCCCTAATGG	TGGTCGGATGTGACGTTATCGG	This work
SPAPADRAFT_58160	ICL1	ICL1	TGAACGTGGTGCTGCTGGTA	AGTGGCGGCTTCAGAATCAGT	This work
SPAPADRAFT_139829	MLS1	DAL7	CCCAGAAGCTCTCGCTCTTGT	GGAGGACCAGTCCAGGTTGC	This work
SPAPADRAFT_59277	not annotated	DAL7	CTGGCCGGTTTGAACTGTGG	GCAGCCATTCCTCCCATAGCA	This work
SPAPADRAFT_59267	MFS domain-containing	STL1	CTCCTGTGGCGTTTGAAAAT	CACCACTGTGCATTTGGTTC	This work

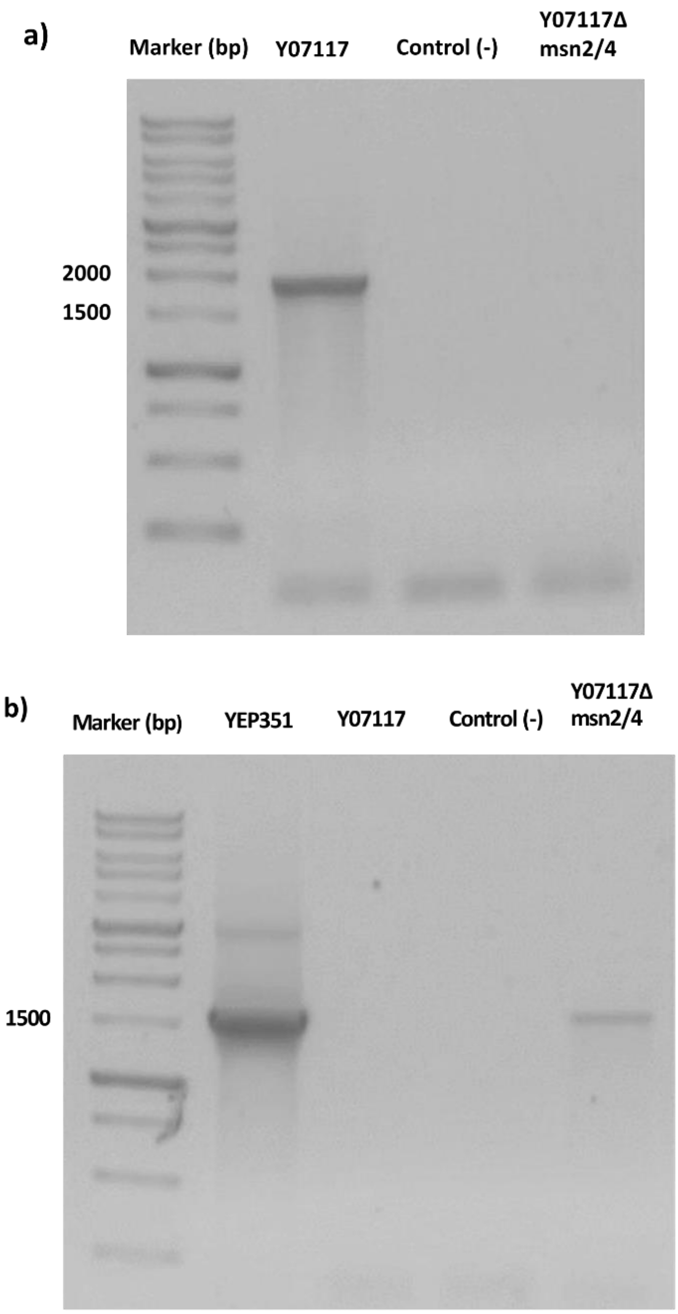
	protein				
SPAPADRAFT_59608	XUT1	RGT2	AGCTGCTGGTGTCGGTAAGA	ATCGGCGACGTAACCGTTCA	This work
SPAPADRAFT_57693	HXT2.4	HXT2	TGCTAGATTCGCTCTTGGTGG T	AGACCCTTGGCTCTGTGGG A	This work
SPAPADRAFT_55953	HGT1	HXT11	GGTGTCGGTGTTGGTTTCGG	TGACCCAACCCGTAAGAAAG GT	This work
SPAPADRAFT_145342	HGT2	RGT2	TTCGTCTGTTCCTTCGCTTC	GCGTTACCTCTTTGGGAGAT T	This work
SPAPADRAFT_61664	ACT1	ACT1	AGATACCCAATTGAACACGGT ATC	GATTTAGGATTCATTGGAGC TTCA	Cadete et al., 2016

APPENDIX B: Supplemental material Chapter 2

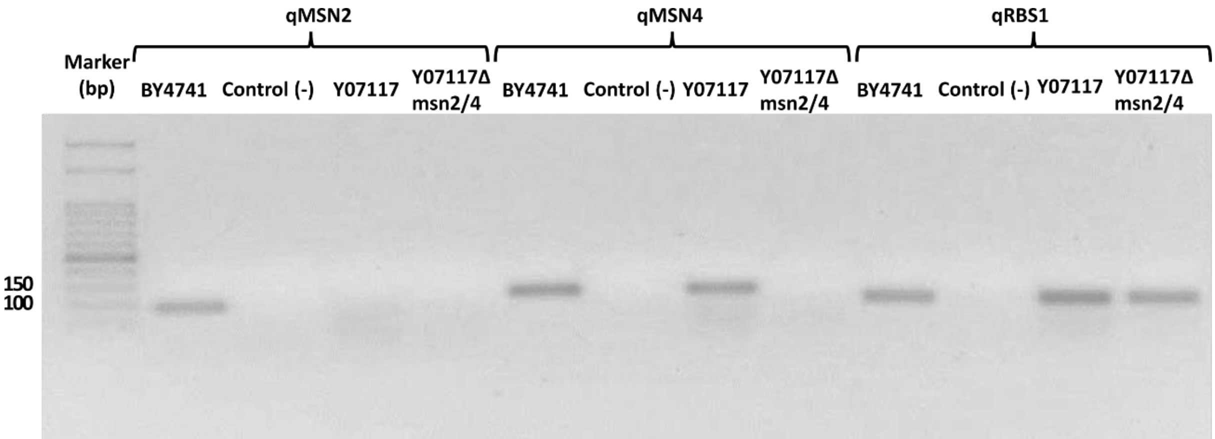
Supplemental Table 1: List of oligonucleotides used in the study.

NCBI ID	Primer ID	Primer Sequence	Description
N/A	LEU2-MSN4_F	GGGTACTGTTGTACGAAGCGGTTGGTAG CGATGACAGTGGCCAGGGCTGCTAGGCA TGGTGAATCTTTTTAAGCAAGGAT	Homologous Recombination, Confirmation
	LEU2-MSN4_R	ACGCCAAGCGTTTAATTATCAGAAAGCA AACGTCGTACCAATCCTTGAATGCTTCC CAATTAACTGTGGGAATACTCAGGT	
NM_001179628.1	pY_Msn4_ScF	CGC**GGATCC**ATGCTAGTCTTCGGACCTA	Confirmation
	pY_Msn4_ScR	CGC**GAATTC**AAAATCACCGTGCTTTTTGT	
NM_001179628.1	qMsn4_ScF	GCACAACCAACGTTTCTGCT	Confirmation
	qMsn4_ScR	AGTGGCGTCCGTTCTATGC	
NM_001182534.1	qMsn2_ScF	TCACGCCGAAGATTTAGCGA	Confirmation
	qMsn2_ScR	CGGATGGTTCCAAAGGTCCA	
NM_001180249.1	qRBS1_F	GGTGCGATTCCGAAACGTAGTG	Confirmation
	qRBS1_R	CAGTATCCCTAGATGACGGCGAA	
SPAPADRAFT_61412	pY_Msn_SpF	TTA**GGATCC**ATGAATACCCCCCAGAATTT	Cloning
	p426_Msn_SpR	TATA**CTCGAG**CTACTGCAG-TTTCTTCTTTTC	

Supplemental Figure 1: PCR reactions using genomic DNA for *MSN2/4* double knockout confirmation with primers for **a)** *MSN4* (~1800 bp) and **b)** *LEU2* (~1500 bp) amplifications. Yeast strains: Y07117 and Δ*msn2/4*. Water was used instead of DNA in the negative controls. The YEP351 plasmid was used as a positive control for the *LEU2* amplification.



Supplemental Figure 2: RT-PCR reactions using cDNA for *MSN2/4* double knockout confirmation with primers for *MSN2* (~100 bp), *MSN4* (~120 bp), and *RBS1* (~ 100 bp) amplifications. Yeast strains: BY4741, Y07117 and ∆*msn2/4*. Water was used instead of cDNA in the negative controls.



Supplemental Figure 3: Sequence alignment analysis for confirming the *MSN-like* gene cloning in the p426-GPD expression plasmid. Restriction digestion sites for the *Bam*HI and *Xho*I enzymes are highlighted in red and yellow, respectively.

```
MSN-SP          -------------------------ATGAATACCCCCCAGAATTTCAGCAATATGTCTTA 35
p426-MSN-SP     GACGGATTCTAGAACTAGTGGATCCATGAATACCCCCCAGAATTTCAGCAATATGTCTTA 60
                                         ***********************************

MSN-SP          TAATACCAATGCTACTAATACTACAGCAGCCATGCCAGAGTCATTACACATTAATGACGA 95
p426-MSN-SP     TAATACCAATGCTACTAATACTACAGCAGCCATGCCAGAGTCATTACACATTAATGACGA 120
                ************************************************************

MSN-SP          GATTTTCTTCAATAACTATCAGTTATCTAACCAATTTCCAGGCGGTGTTGCCAACAAGCT 155
p426-MSN-SP     GATTTTCTTCAATAACTATCAGTTATCTAACCAATTTCCAGGCGGTGTTGCCAACAAGCT 180
                ************************************************************

MSN-SP          CAACTATGACGAAGAATCAACTGGCAATTTGTCATCCTCAAATTCAATCAACAATTATCT 215
p426-MSN-SP     CAACTATGACGAAGAATCAACTGGCAATTTGTCATCCTCAAATTCAATCAACAATTATCT 240
                ************************************************************

MSN-SP          GAGCCATAACCCCTCGGCTTCCGCTTCTTCGCAGTCGTCGTTCTCCCTGAACTCGTCTAC 275
p426-MSN-SP     GAGCCATAACCCCTCGGCTTCCGCTTCTTCGCAGTCGTCGTTCTCCCTGAACTCGTCTAC 300
                ************************************************************

MSN-SP          GGGAGTACATTCCACTTTTCAAACTTCAGCCACAACTACACCCAAAGTCGGTTCCATGCC 335
p426-MSN-SP     GGGAGTACATTCCACTTTTCAAACTTCAGCCACAACTACACCCAAAGTCGGTTCCATGCC 360
                ************************************************************

MSN-SP          TCAGTTTGGAAATGGTCCAGGTAAACATGGAAGGCAAAACTCCTCTTCGATTCCAATCGA 395
p426-MSN-SP     TCAGTTTGGAAATGGTCCAGGTAAACATGGAAGGCAAAACTCCTCTTCGATTCCAATCGA 420
                ************************************************************

MSN-SP          CCAGTTAACTTTATTGAGTTTGAGAACTCCTCTGGTTTCAGGTCCACCTCAACAGCAACA 455
p426-MSN-SP     CCAGTTAACTTTATTGAGTTTGAGAACTCCTCTGGTTTCAGGTCCACCTCAACAGCAACA 480
                ************************************************************

MSN-SP          ACAAACCCCACAACAACAGCAAATACAACAACAATTGGCTGGGATTGCTCAACCATCATT 515
p426-MSN-SP     ACAAACCCCACAACAACAGCAAATACAACAACAATTGGCTGGGATTGCTCAACCATCATT 540
                ************************************************************

MSN-SP          TATGTTTGATCCAGTTAGTAATACCTCCTCGCCAAATGCTGATGATTTCCTCCAATCTCC 575
p426-MSN-SP     TATGTTTGATCCAGTTAGTAATACCTCCTCGCCAAATGCTGATGATTTCCTCCAATCTCC 600
                ************************************************************

MSN-SP          CACCAACTTTTCCTACTCCCAGGCCGCAGCAGCCGTAGCGGCCGCAGCAGCAGCTATTCC 635
p426-MSN-SP     CACCAACTTTTCCTACTCCCAGGCCGCAGCAGCCGTAGCGGCCGCAGCAGCAGCTATTCC 660
                ************************************************************

MSN-SP          TACAATACCAGAAGAACGAACCATTAATCCAAGACAATTATTTACCAAGTTGAATACTTC 695
p426-MSN-SP     TACAATACCAGAAGAACGAACCATTAATCCAAGACAATTATTTACCAAGTTGAATACTTC 720
                ************************************************************

MSN-SP          AATGAGCTCGCCTAGTTTATCAACATTATTTACTACACATAATTCGCAACAACAACAACA 755
p426-MSN-SP     AATGAGCTCGCCTAGTTTATCAACATTATTTACTACACATAATTCGCAACAACAACAACA 780
                ************************************************************

MSN-SP          GCAGCAGCAGCAACCGCAGCAACATCAACATCACCAACACGCGCATCATTCACAACAACA 815
p426-MSN-SP     GCAGCAGCAGCAACCGCAGCAACATCAACATCACCAACACGCGCATCATTCACAACAACA 840
                ************************************************************

MSN-SP          TCTGCACCAGCAACATATGCAAGCTCAGTTGTCTCCCCAACAACCACAAGCGGCTAATTT 875
p426-MSN-SP     TCTGCACCAGCAACATATGCAAGCTCAGTTGTCTCCCCAACAACCACAAGCGGCTAATTT 900
                ************************************************************

MSN-SP          TCCCCAGGCACTTTCTGCACAAATTTCACCAACTCAACCAACAGTGCCTTATTTCATGCC 935
p426-MSN-SP     TCCCCAGGCACTTTCTGCACAAATTTCACCAACTCAACCAACAGTGCCTTATTTCATGCC 960
                ************************************************************

MSN-SP          GCAACATACCCGCAGTGTATCTACTCCGCAATTCGACTTTAAAGTTGATGACGAGTTTTC 995
p426-MSN-SP     GCAACATACCCGCAGTGTATCTACTCCGCAATTCGACTTTAAAGTTGATGACGAGTTTTC 1020
                ************************************************************

MSN-SP          GCAAGCAATCTCTTCGTGGCTCAATGGAGGTTGGGTCGATGGGTTGAATCCGGAAATAGT 1055
p426-MSN-SP     GCAAGCAATCTCTTCGTGGCTCAATGGAGGTTGGGTCGATGGGTTGAATCCGGAAATAGT 1080
                ************************************************************

MSN-SP          CAACCCGGTTAAAAAGAATAGAATGTATAGTACTGTCAAGGGAAGAAGAAACTCGATTCA 1115
p426-MSN-SP     CAACCCGGTTAAAAAGAATAGAATGTATAGTACTGTCAAGGGAAGAAGAAACTCGATTCA 1140
                ************************************************************

MSN-SP          ACCTCAAGTACACGTTCCTATAATCCATCAGGCTCAGCAACAAGTGCAGCTGCAACAACA 1175
p426-MSN-SP     ACCTCAAGTACACGTTCCTATAATCCATCAGGCTCAGCAACAAGTGCAGCTGCAACAACA 1200
                ************************************************************

MSN-SP          ACAACAACAACAGCAACAACAACAGCAATTGCAACCACAACCACAATCGCAGCCAATGCA 1235
p426-MSN-SP     ACAACAACAACAGCAACAACAACAGCAATTGCAACCACAACCACAATCGCAGCCAATGCA 1260
                ************************************************************

MSN-SP          ACCAATTATGGATTCAATGAGTTATGCATCGACTCCAAATACATCACCTACAACTTCATC 1295
p426-MSN-SP     ACCAATTATGGATTCAATGAGTTATGCATCGACTCCAAATACATCACCTACAACTTCATC 1320
                ************************************************************

MSN-SP          TCTGAACTCCGACGATGAAGACAAGAAAAAGAAGAATAAGAATAAGAAACGGAGGAAAAG 1355
p426-MSN-SP     TCTGAACTCCGACGATGAAGACAAGAAAAAGAAGAATAAGAATAAGAAACGGAGGAAAAG 1380
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               ************************************************************
MSN-SP         TACTGCGGCTGAAATTGATCCATTGGCTACTTCACCAGCTATATCTCCCACTTCAACCAG 1415
p426-MSN-SP    TACTGCGGCTGAAATTGATCCATTGGCTACTTCACCAGCTATATCTCCCACTTCAACCAG 1440
               ************************************************************

MSN-SP         TGACGATAAGAATGCCGCCAATTCTAGCGCATTTCCATGTATGGAGTGCGACAAGCAATT 1475
p426-MSN-SP    TGACGATAAGAATGCCGCCAATTCTAGCGCATTTCCATGTATGGAGTGCGACAAGCAATT 1500
               ************************************************************

MSN-SP         CAAGAGAAGTGAACATTTGAAGAGACATATTAGAAGTGTTCATTCCAATATTCGTCCATT 1535
p426-MSN-SP    CAAGAGAAGTGAACATTTGAAGAGACATATTAGAAGTGTTCATTCCAATATTCGTCCATT 1560
               ************************************************************

MSN-SP         CCATTGTAAATATTGTGAAAAGAAGTTTAGCCGTCTGGATAATTTAGCTCAACATTTAAA 1595
p426-MSN-SP    CCATTGTAAATATTGTGAAAAGAAGTTTAGCCGTCTGGATAATTTAGCTCAACATTTAAA 1620
               ************************************************************

MSN-SP         AACCCATTATAGAATTGATGCTGAAGGAAATAGCCATGTTATTATTAACCCAAGTGGGAA 1655
p426-MSN-SP    AACCCATTATAGAATTGATGCTGAAGGAAATAGCCATGTTATTATTAACCCAAGTGGGAA 1680
               ************************************************************

MSN-SP         TAGAAGAGAAAAGAAGAAACTGCAGTAG-------------------------------- 1683
p426-MSN-SP    TAGAAGAGAAAAGAAGAAACTGCAGTAGCTCGAGTCATGTAATTAGTTATGTCACGCTTA 1740
               ****************************
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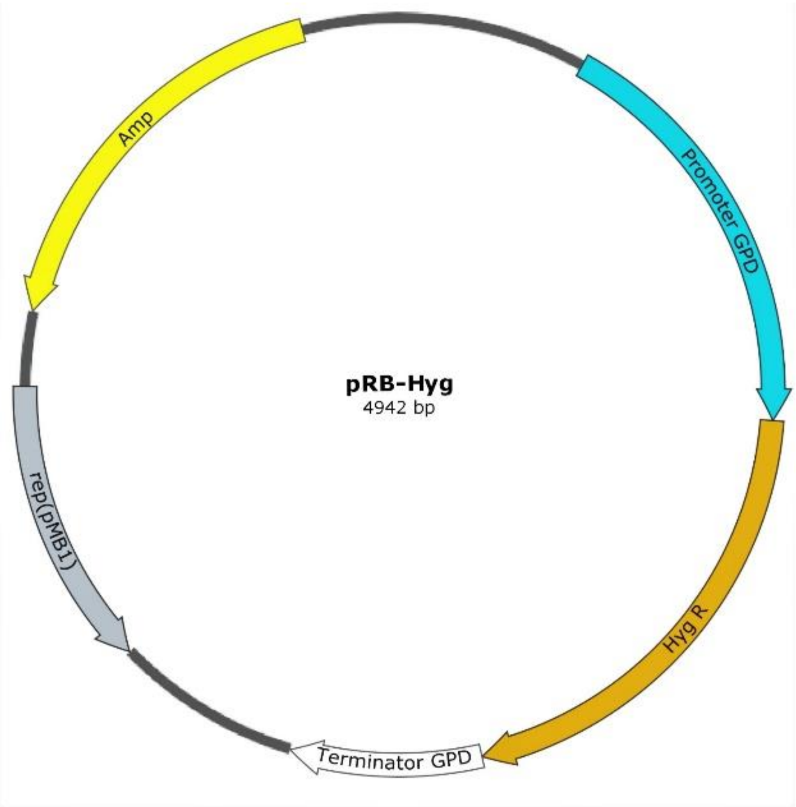
APPENDIX C: Supplemental material Chapter 3

Supplemental Figure 1: Map of the vector pRB-Hyg. The selection marks for bacteria (Amp) and *Pseudozyma flocculosa* (HygR – codon optimized) are highlighted in yellow. The GPD Promoter is highlighted in blue. The GPD terminator is highlighted in grey.



Supplemental Table 1: List of primers used for cloning the pull-down targets in pGADT7 and pGBKT7 vectors.

Acession (Uniprot)	Primer identification	Primer sequence (5'-3')	Plasmid
A0A287N0S4	Germin_NdeI_F	CACTG**CATATG**ACCGACCCTGACCCTCTAC	Both
	Germin_XmaI_R	CACTG**CCCGGG**TTAAGACCCACCGGCGAAC	
A0A287RAV7	HvPr1a_NdeI_F	ATCTG**CATATG**CAAAACTCGCCTCAGGACTAC	Both
	HvPr1a_XmaI_R	ATCTG**CCCGGG**TTAGTATGGTTTCTGTCCAACAACA	
N1J4Z2	Sgk2_NdeI_F	ATCTG**CATATG**ATGTCTGTTTCTCCAGAGG	Both
	Sgk2_XmaI_R	ATCTG**CCCGGG**TTATCCTAAAGTTTTTTCTTCATCT	
A0A383V2V0	Unch_Blumeria_BamHI_F	ATCTG**GGATCC**AAATGTTGGACAGCCATCAAT	pGADT7
	Unchar_Blumeria_XhoI_R	ATCTG**CTCGAG**TTAATAAACTCCCATAATATCCTCA	
	Unch_Blumeria_NcoI_F	ATCTG**CCATGG**AAATGTTGGACAGCCATCAAT	pGBKT7
	Unch_Blumeria_BamHI_R	CCTCC**GGATCC**TTAATAAACTCCCATAATATCCTCA	
N1J6Z3	CSEP0313_NdeI_F	ATCTG**CATATG**GAGGATTCCGTGTCAACAAGC	Both
	CSEP0313_XmaI_R	ATCTG**CCCGGG**TCAGGTGATGTCGTTTACTCGA	
A0A383UML	Unchar_effect_BamHI_F	ATCTG**GGATCC**AAATGTACTGTGTGCCGCCCTCATC	pGADT7
	Unchar_effect_X	ATCTG**CTCGAG**TTAAAAAATACGGATGGG	

4	hoI_R	AGTTTT	
	Unchar_effect_Ncol_F	ATCTG**CCATGG**AAATGTACTGTGTGCCGCCCTCATC	pGBKT7
	Unchar_effect_BamHI_R	ATCTG**GGATCC**TTAAAAAATACGGATGGGAGTTTT	
N1J908	Putative_effect_NdeI_F	ATCTG**CATATG**GTGTCAGTTCCGAATAGTGGA	Both
	Putative_effect_XmaI_R	ATCTG**CCCGGG**TTAATTGAGTGAGCAGGCTCTGA	

ATTACHMENT A: Supplemental material Chapter 2 (Castro, 2019)

Supplemental File S1 – S6, Link:

https://drive.google.com/file/d/1qVhfLEUNE9EQa2IkyboFxt-BOcmhmu24/view?usp=share_link