CHARACTERIZATION OF GENES THAT PLAY ROLE IN MANGANESE TOLERANCE IN DIFFERENT YEAST SPECIES

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by

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İZMİR

This thesis is dedicated to the loving memory of my father, Hüseyin KARAGÖZ, who never got to see this adventure ending.

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ABSTRACT

CHARACTERIZATION OF GENES THAT PLAY ROLE IN MANGANESE TOLERANCE IN DIFFERENT YEAST SPECIES

Manganese is an essential element for organisms that can also be toxic. It has been stated that more than 5 mM Mn inhibits the growth of wild-type S. *cerevisiae* BY4741 strain. In the cases when this amount is exceeded, the stress upon manganese toxicity arises and it leads to a range of responses to normalize the manganese level. However, the genes accountable for that case are unknown. Manganese toxicity is a restrictor factor in the production of agricultural products. Identification and characterization of the genes that play a role in manganese homeostasis are rather essential.

In this study, we have used Sanger Centre's Saccharomyces Genome Resequencing Project (SGRP) strains, which are collected from different regions of the world. After screened the whole collection, we have identified four manganese resistant strains; S. *cerevisiae* BY474, *S. paradoxus* Y6.5, S. *cerevisiae* 378604X and S. *paradoxus* Q74.4.

Manganese-related genes were selected via the Saccharomyces genome database (SGD). Expression levels of these genes under manganese stress in most resistant strain Q74.4 analyzed by RT-Q-PCR. As a result, GCR-1 dependent translation factor GDT1 and high-affinity phosphate transporter PHO84 were found to be upregulated in Q74.4 that endure high levels of manganese toxicity. These genes are probably accountable for manganese tolerance in Q74.4 strain. The results arising from that study, will take the lead to the development of biotechnological exercises for manganese bioremediation. Meanwhile, it might help molecular mechanisms to be able to develop resistance to stressful conditions that manganese generates and shed light to further studies.

ÖZET

FARKLI MAYA TÜRLERİNDE MANGAN TOLERANSINDA ROL OYNAYAN GENLERİN KARAKTERİZASYONU

Mangan insan, hayvan ve bitkiler için esansiyel bir elementtir, aynı zamanda toksik olabilir. Bazı canlılar bu toksisiteyi tolere edebilir. Önceki çalışmalarda, 0,5 mMdan fazla mangan konsantrasyonunun yabani maya suşu *S. cerevisiae* BY4741'I inhibe ettiği belirtilmiştir. Bu miktarın arttığı durumda mangan toksisitesinin getirdiği stress artar ve mangan düzeyini normalize etmek için bir dizi cevap meydana gelir. Fakat bununla ilişkili genler bilinmemektedir. Mangan toksisitesi tarım ürünleri üretiminde kısıtlayıcı bir faktördür. Bu sebepten mangan homeostasinde rol alan genlerin belirlenmesi ve karakterize edilmesi elzemdir.

Bu çalışmada, mangan dirençli suşları belirlemek amacıyla, dünyanın çeşitli bölgelerinden toplanmış Sanger Centre's Saccharomyces Genome Resequencing Project (SGRP) suşları kullanılmıştır. Tüm koleksiyon tarandıktan sonra dört dirençli suş tespit edilmiştir; S. *cerevisiae* BY474, *S. paradoxus* Y6.5, S. *cerevisiae* 378604X ve S. *paradoxus* Q74.4. Manganla ilişkili genler Saccharomyces genom database (SGD) kullanılarak seçilmiştir. RT-Q-PCR kullanılarak bu genlerin en dirençli suş Q74.4'da mangan stresi altında ekspresyon seviyelerine bakılmıştır. Gen ekspresyon analizinin sonucunda, GCR-1'e bağlı translasyon faktörü GDT1 ve yüksek afiniteli fosfat taşıyıcı PHO84'ün, yüksek seviyelerde mangan toksisitesine dayanan Q74.4'te upregüle edildiği bulundu. GDT1, Ca2+ ve Mn2+ taşınmasında işlev görür ve Golgi'de lokalize olurken, PHO84 bir transmembran fosfat transporterıdır. Bu genler Q74.4 suşunda mangan toleransından sorumlu olabilir.

Bu çalışmadan çıkacak sonuçlar su ve topraktan mangan biyoremediasyonı için kullanılacak biyoteknolojik yöntemler geliştirilmesine önayak olabilir. Aynı zamanda, manganın yarattığı stress koşullarına karşı geliştirilen moleküler mekanizmaların aydınlatılmasına yardımcı olabilir ve sağlık alanındaki çalışmalarda kullanılabilir.

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CHAPTER 1

INTRODUCTION

1.1. The element Manganese

Manganese is a silvery-gray chemical element with the symbol Mn and atomic number 25. Manganese is recognized by Carl Wilhelm Scheele in 1774 and isolated by Johan Gottlieb Gahn later that year. The name derives from the Latin *magnes* for "magnet" since pyrolusite (MnO_2) has magnetic properties (CIAAW, n.d.)

Mn-oxide, the most common form of Mn in nature; is a highly reactive mineral that participates in the redox reactions of various organic/inorganic species and compounds, absorbs many ions with its high absorption capacity and controls the distribution and bioavailability of toxic and essential elements (Tebo et al., 2004).

Manganese is the twelfth most abundant element on earth and the 5th most common metal among metals (World Health Organization, 2001). Manganese is not found in pure form in nature; It is found in the form of oxide, carbonate and silicate. The most common form is Pyrolusite (MnO2), a type of Mn-oxide. Although Mn can be found in different oxidation states from 0 to +7, it is generally found in the +2 (II), +3 (III), and +4 (IV) states, and all these forms have biological importance. Most of the Manganese found in nature is composed of metamorphic and sedimentary rocks, some of which originate from volcanic rocks. Manganese with many other elements and compounds can create different forms. Major Mn ores found in the earth; pyrolysis (MnO_2), hausmannite (Mn_3O_4), manganite ($Mn_2O_3H_2O$), manganese spat ($MnCO_3$), and rhodonite ($MnSiO_3$).

1.2. Manganese Biochemistry

Manganese is an essential element for all organisms (Keen et al., 2009). Manganese is known as a cofactor for many metalloenzymes such as Oxidoreductases and dehydrogenases, DNA and RNA polymerases, kinases, glutamine synthetases, integrins, transferases, decarboxylase, hydrolases, and integrins (Keen et al., 1999; Saric, 1986; Wedler et al., 1999;). These manganese-dependent enzymes are found in many different locations within the cell, such as Golgi, mitochondria, and cytoplasm. One of the best studied manganese-containing polypeptides is arginase, one of the enzymes found in lipids for ammonia elimination, and another is the antioxidant enzyme superoxide dismutase (Mn-SOD) found in mitochondria. Manganese also plays a role in the modulation of the immune system and protein, lipid and carbohydrate metabolism (Addess et al., 1997; Aschner et al., 1992; Malecki et al., 1999).

High concentrations of manganese can be highly toxic to living organisms. Overexposure to manganese can cause Parkinson's disease-like syndrome with symptoms such as rigidity, tremors and hypokinesia in humans, this degenerative brain disease is also called manganism (Calne et al., 1994; Dobson et al., 2004). Manganese toxicity damages essential pathways associated with protein biosynthesis, which is associated with neurodegenerative diseases in humans (Hernández et al., 2019). In addition, birth defects, weak bone formation and increased susceptibility to seizures are also common in manganese deficiency (Aschner, 2000; Aschner et al., 2002). Evidence also supports the idea that Mn affects stellate cell formation in astrocytes (Liao et al., 2001). In addition, the metabolism of glutamate to glutamine in the brain is driven by glutamine synthetase, an astrocyte-specific enzyme (Wedler et al., 1982; Wedler et al., 1984; Takeda, 2003).

Manganese toxicity in plants reduces the number of carotenoids and chlorophyll and carbon dioxide assimilation in leaves (Clairmont et al., 1986; Gonzalez et al., 1997). In addition, manganese toxicity causes an increase in reactive oxygen species by changing the structure of antioxidant enzymes, which damages the cell membrane and adversely affects root and shoot growth (Xue et al., 1995).

1.3. Manganese Toxicity as an environmental problem

In many countries, drinking water contamination with heavy metals such as iron, arsenic and manganese is a big issue that affects the health of millions of people (Nitzche et al., 2015). Contaminated water is not only used for drinking purposes but also for agricultural purposes. More than concentration of 0,05 mg/ L^{-1} manganese has toxic effects to cells (Environmental Protection Agency EPA, 1979). There are few methods for removing heavy metal ions from aqueous solutions such as chemical precipitation, ion exchange, electrochemical treatment, membrane technologies etc. (Wang and Chen, 2009). But these methods are not efficient enough for treating large amount of liquids containing metals at low concentrations (Schiewer and Patil, 2007).

Using biological methods for treatment is an alternative and efficient technique. And yeast is a good biosorbent material because its accumulation capacity, it's economic, non-pathogenic and easy to manipulate.

1.4. Manganese transporters

Although the biological importance of manganese and the consequences of its toxicity in soil and water have long been known, there are still some shortcomings in the mechanism of manganese homeostasis. Yeast cells adapts toxic manganese levels by enlarging their vacuoles, or by activating transport systems responsible for manganese compartmentalization, or manganese exhibition from cell. At present, some of the factors responsible for manganese transport have been identified. Most of these have been revealed by studies on yeast (Saccharomyces *cerevisiae*) and all have homologues in humans.

Two Nramp (natural resistance-associate macrophage protein) family members, Smf1p and Smf2p, carry out high affinity manganese transport. The role undertaken by Smf1p is to transport manganese across the cell surface (Supek et al., 1996; Liu and Culotta, 1999). Smf2p is found in intracellular devices and transports manganese to mitochondrial superoxide dismutase and secretory pathway enzymes (Luk and Culotta, 2001). Except for manganese starvation conditions, Smf1p and Smf2p levels are kept at a low level to prevent excessive accumulation of manganese and other metals in the cell. This down-regulation of Smf1p and Smf2p under manganese excess conditions is achieved by targeting these proteins to the vacuole by a mechanism involving Bsd2p and ubiquitination in *S. cerevisiae* (Liu and

Culotta, 1999; Portnoy et al., 2000). In manganese toxicity conditions, pathways other than Smf1p / Smf2p provide manganese uptake into the cell (Luk et al., 2003).

Phosphate transporter Pho84p protein found on the yeast cell surface also plays the role of low-affinity manganese transporter (Jensen et al., 2003). Pho84p is responsible for the uptake of the manganese or manganese-phosphorus complex. Low manganese accumulation and resistance to manganese toxicity have been observed in yeast strains that have deletions in PHO84 (Jensen et al., 2003). A manganese-resistant phenotype is caused by disruption of the PHO84 gene. This resistance is linked to yeast cells' inability to absorb significant levels of Mn^{2+}. Phosphate uptake, storage, and metabolism are all defected in S. *cerevisiae* PHO80 mutants, as are metal homeostasis problems (Jensen et al., 2003).

Detoxification of manganese in yeast is usually controlled by P-type ATPase manganese and calcium carrier Pmr1p found in Golgi membrane (Antebi and Fink, 1992; Rudolph et al., 1989). Pmr1 mutant is very sensitive to manganese and excess metal concentrations (Lapinskas et al., 1995). PMR1 is also required for calcium delivery to the secretory pathway along with manganese, where these ions are needed for protein processing and secretion (Antebi and Fink, 1992; Mandal et al., 2000). Mutations that occur in the human homolog of the PMR1 gene cause hereditary Hailey-Hailey disease, which manifests itself with blisters on the skin (Sudbrak et al., 2000; Ton et al., 2002). Indeed, once in the Golgi lumen, manganese cations can be directed out of the cell via the secretory pathway (Lapinskas et al., 1995).

Gdt1p has been identified as another transporter of both manganese and calcium presumably from the cytosol to the Golgi lumen, most likely in exchange of protons (Thines et al., 2018). In addition, Golgi manganese concentrations also controlled by a third transporter called Atx2p and it would transport Mn in the opposite direction than Pmr1p and Gdt1p (Culotta and Lin, 1996).

Protection against manganese toxicity in S. *cerevisiae* is also provided by metal separation in the vacuole. Yeast vacuoles participate in both manganese storage and detoxification, and the metal is more concentrated compared to cytosol (Li et al., 2001). Yeast cells with mutations affecting the vacuolar structure or H-ATPase are
sensitive to many metal ions. In addition to manganese (Ramsay and Gadd, 1997), zinc (MacDiarmid et al., 2003), cobalt (Conklin et al., 1994) and copper (Szczpka et al., 1997) tolerances are also affected by these kinds of mutations.

Also, mutations in the MAM3 gene confers resistance to high manganese, zinc and cobalt levels. Mam3p is a member of ACDP (ancient, conserved domain protein) family (Wang et al., 2003) and is associated with a genetic disease called ACDP1 urofacial syndrome. Mam3p functions in yeast vacuolar membrane and affects manganese toxicity (Yang et al., 2005). Total manganese deposition in the vacuole is unaffected in mam3Δ mutants. Manganese appears to be more toxic in cells expressing vacuolar Mam3p, which could alter metal ion reactivity (Yang et al., 2005).

A transporter protein called Ccc1p, suppresses manganese hypersensitivity caused by mutation in pmr1, it is known to function in calcium homeostasis (Lapinskas et al., 1996). Studies show that the Ccc1p polypeptide is located in the Golgi-like organelle and restricts intracellular access by separating manganese ions (Lapinskas et al., 1996). Ccc1p is also part of the iron storage system in the vacuole. On the other hand, there is no evidence that manganese originates from the vacuole and there is no defined carrier protein. Based on a prior research, manganese transports into the yeast vacuole by Cos16p and Ccc1p, mutations in either pathway decrease cellular tolerance, because the lack of vacuolar sequestration (Lapinkas et al., 1996). Ccc1p seems more likely to detoxify by separating manganese in the vacuole rather than storing it and releasing the metal into the cytosol like iron (Reddi et al., 2010).

Additionally, the vacuolar P-type ATPase Ypk9p would also import manganese in the vacuole and, thereby, play a role in the sequestration of divalent metal ions (Chesi et al., 2012). Ypk9Δ mutants are moderately sensitive to Cd, Mn, Ni and Se (II). Ypk9p also reported as a putative lysosomal metal transporter that involved in metal sequestration in the vacuole (Thines et al., 2019).

YBR056W-A (MNC1) is a newly identified CYTSM family protein that is essential for manganese detoxification (Andreeva et al., 2017). MNC1 chelates manganese at the cell surface, and it is a part of manganese compartmentalization system.

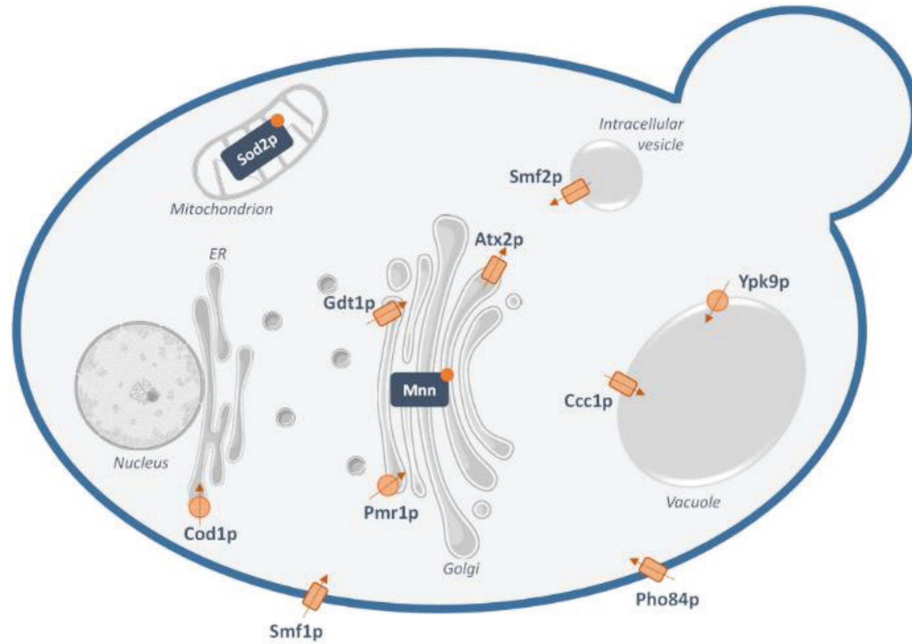

Figure.1.1. Known manganese transport pathways in yeast. Smf1p and Pho84p are plasma membrane Mn2+ transporters. Atx2p transports at Golgi. Cod1p is a P-type ATPase at endoplasmic reticulum. Smf2p functions in intracellular vesicles. Ypk9p and Ccc1p are vacuolar Mn transporters. Arrows indicate the direction of transportation (Source: Thines et al., 2019).

Table 1.1. Manganese-related genes investigated through this study and their known functions in the cell.

Gene	**Function**
MNC1	Manganese Chelating protein. Upregulated in toxic manganese levels (Andreeva et al., 2017)
MNR2	Vacuolar membrane protein is required for magnesium homeostasis and magnesium transporter (MacDiarmid and Gardner, 1998).
ATX2	Golgi membrane protein is involved in manganese homeostasis (Jin and Culotta, 1996).

(cont. on next page)

Table 1.1. (cont.)

GDT1	Gcr1 Dependent translation factor (Barbara et al., 2007). Calcium and manganese transporter localizes to golgi apparatus (Thines et al., 2018)
SMF2	Suppressor of Mitochondria import Function, Divalent metal ion transporter involved in manganese homeostasis (Portnoy et al., 2002)
CCC1	Vacuolar Fe^{2+}/Mn^{2+} transporter (Lapinskas et al., 1996)
PHO84	High-affinity inorganic phosphate (Pi) transporter; also, low-affinity manganese transporter (Bun-ya et al., 1996).
PMR1	High-affinity Ca^2+/Mn^{2+} P-type ATPase; required for Ca^{2+} and Mn^{2+} transport into Golgi (Vashist et al., 2002).
SMF1	Divalent metal ion transporter; broad specificity for di-valent and tri-valent metals (Supek et al., 1996).
SMF3	Putative divalent metal ion transporter involved in iron homeostasis; transcriptionally regulated by metal ion. (Portnoy et al., 200).
MTM1	Mitochondrial protein of the mitochondrial carrier family; high affinity pyridoxal 5'-phosphate (PLP) transporter (Luk et al., 2003)
MAM3	Protein required for normal mitochondrial morphology (Entian et al., 1999).
HIP1	High-affinity histidine permease; also involved in the transport of manganese ions (Farcasanu et al., 1998).
SOD2	Mitochondrial manganese superoxide dismutase; protects cells against oxidative stress (van Loon et al., 1986).

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Table 1.1. (cont.)

SPF1	P-type ATPase, ion transporter of the ER membrane (Cronin et al., 2002)
CAR1	Arginase catabolizes arginine to ornithine and urea (Smart et al., 1996).
PCD1	8-oxo-dGTP diphosphatase; prevents spontaneous mutagenesis via sanitization of oxidized purine nucleoside triphosphates (Cartwright et al., 2000).
MRE11	Nuclease subunit of the MRX complex with Rad50p and Xrs2p (D'Amours and Jackson, 2002)
CYS4	Cystathionine beta-synthase; catalyzes synthesis of cystathionine from serine and homocysteine (Ono et al., 1994).
SNF7	One of four subunits of the ESCRT-III complex; involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway (Babst et al., 2002)
SOD1	Cytosolic copper-zinc superoxide dismutase (Lyons et al., 2000).
CRZ1	Transcription factor, activates transcription of stress response genes (Stathopoulos and Cyert, 1997)
DID4	Class E Vps protein of the ESCRT-III complex (Amerik et al., 2004).
BRO1	Cytoplasmic class E vacuolar protein sorting (VPS) factor (Odorizzi et al., 2003).
ATP12	Assembly factor for F1 sector of mitochondrial F1F0 ATP synthase (Ackerman et al., 2002).

Genes are determined through Saccharomyces Genome Database website. As a result of a search for manganese-related genes, 32 genes are determined.

1.5. Yeast as a model organism:

Yeast has been used as a good model organism not only in industrial processes but also in scientific research because it is unicellular and has a short generation time. Most of the yeast genes have homologs in higher eukaryotes and cellular processes are similar to animals or plants. Also, Saccharomyces *cerevisiae* whole genome was released in 1996. In addition, yeast has been used as a model for several approaches such as transcriptomics, metallomics, or proteomics. For this reason, there is a large amount of accessible data obtained from studies using these approaches.

Saccharomyces *cerevisiae*, the bakers' yeast is a useful tool for metal sensitivity assays because culturing and analysis are simpler. Yeast tolerance assays have provided several data for the understanding of the function of proteins. Yeast has proven to be an excellent model system for studying manganese homeostasis (Luk et al., 2003). Much of what we currently know regarding complex pathways of metal trafficking has emerged from molecular genetic studies in yeast (Luk et al., 2003).

There are some reports of heavy metal bioaccumulation by S. *cerevisiae* (Brady and Ducan, 1994). Yeast is not only a good model organism for studying heavy metal tolerance, it also has been used as a good modern system for bioremediation of heavy metals such as manganese from water and soil. And it has several advantages; it can adapt to environmental stresses, has a better ability to absorb and accumulate manganese than other microorganisms.

CHAPTER 2

MATERIALS AND METHODS

2.1. Yeast growth and media:

Wild type strain BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ) of budding yeast, *Saccharomyces cerevisiae* were used throughout this study. Different strains around the world were obtained from Saccharomyces Genome Resequencing Project Strains, National Collection of Yeast Cultures (NCYC), as listed in Table 2.1.

Yeast cells were grown in yeast/peptone (YPD) media (2% glucose, 1% yeast extract, 2% peptone and 2% agar for solid media). Manganese chloride obtained from Sigma was added to the growth medium based on the concentration of interest for tolerance assays.

	1	2	3	4	5	6	7	8	9	10
A	DBVPG6765	BC187	NCYC 361	Y9	YJM981	N-45	Y7	A4	Q69.8	UFRJ50791
B	SK1	YPS606	K11	UWOPS03-461.4	YJM975	UFRJ50816	Q32.2	A12	W7	IFO1804
C	Y55	L-1374	YS4	UWOPS05-217.3	NCYC110	N-44	Z1.1	Y6.5	Q31.4	UWOPS91-917.1
D	YPS128	L-1528	YS9	S288c	YS2	N-17	DBVP4650	Q62.5	Y8.5	
E	DBVPG6044	Y12	3221134S	W303		T21.4	Q95.3	Q89.9	Z1	
F	DBVPG1788	BDVPG1106	378604X	UWOPS05-227.2		Q59.1	N-43	KPN3828	CBS432	
G	DBVPG1373	UWOPS83-787.3	273614N	DBVPG6040		YPS138	CBS5829	Y9.6	Y8.1	
H	DBVPG1853	UWOPS87-2421	YJM978	YIIc117_e5		S36.7	DBVPG304	Q74.4	KPN3829	

Table 2.1. The strains of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* were screened in this study.

2.2. Screening of SGRP collection set:

Before analysis, SGRP collection strains were removed from frozen stocks (-80ºC) and streak onto YPD agar plates. Cells were kept for 2 days incubation at 30ºC.

Manganese chloride was used as a manganese source in assays. YPD plates containing 0, 2,5, 5, and 10 mM of manganese were prepared. Pre-cultivation of cells were made in 5 mL of liquid YPD for 12 hours with shaking at 120 rpm at 30° C. Overnight culture of wild type and other strains of yeast were diluted into an optical density of 0.2 at 600 nm and serially diluted to 0.02, 0.002, 0.0002. A 5 µl aliquots of each dilution were spotted on the plates and incubated for 3 days at 30°C, and then plates were photographed. Yeast isolates with higher resistance to manganese concentration were selected for growth curve and transcription analysis.

2.3. Growth Curve

Growth curve assay was performed to confirm and determine the exact minimum inhibitory concentration. S. *cerevisiae* BY4741, S. *cerevisiae* 378604X, S. *paradoxus* Q74.4 and S. *paradoxus* Y6.5 strains were used for growth analysis in 0, 0.25, 0.5, 1, 2.5, 5, 10, 20 mM of Manganese. All the overnight cell cultures were diluted into optical density of 0.05 at 600 nM at the beginning and optical density was measured every two hour for 24 hours.

2.4. RNA isolation and Real-Time RT-PCR Analyses

Selected samples were grown in medium added 0,3 mM of manganese for 1 and 12 hours at 30° C. Total RNA was isolated from Manganese-treated and control samples using RNA Isolation Kit (Invitrogen). cDNAs were used as a temple for expression analysis of selected genes and reference genes. Triplicate real-time PCR analyses were performed with the IQ5 Real-Time PCR System (Bio-Rad) using Fermentas MaximaTM SYBR Green qPCR Master Mix (2X). The conditions of PCR amplification were as follows:

40 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s.

The primers were used for amplification of 144 bp fragment of control ACT1 gene were ACT1F (5'-CGGTGATTTCCTTTTGCATT-3') and ACT1R (5'-CTGCCGGTATTGACCAAACT-3')

Table 2.2. Primers used for amplification of specific genes.

Genes	Primer Sequences	Amplicon size
MNR2	Forward: 5'CTCAAGGCCATCTTCTAGTCTTT3'(23) Reverse: 5'CACTTCGCTACCCTCATCATC3'(21)	85
MNC1	Forward: 5'CCGCCACCAAGAAATGATTG3' (20) Reverse: 5'CAGCGCAGCTACAACATAGA3'(20)	112
ATX2	Forward: 5'AGGTACGACAACGAACAATGA3'(21) Reverse: 5'CCATCAAACTAGTGAGGGAAAGA3'(23)	98
GDT1	Forward: 5'GCGGAGACATCTATGGAATCTG3' (22) Reverse: 5'ATCAAGGCTGCGATCAAGAA3' (20)	116
SMF2	Forward: 5'AACGGTTCCATGAAGGGTATC3' (21) Reverse: 5'AGGTCCCAGTTTGGCATAAA3' (20)	108
CCC1	Forward: 5'CAATCAGTAGATCCTCGCGTAAT3' (23) Reverse: 5'CGCATCACCAAGTGAAGATAGA3' (22)	105
PHO84	Forward: 5'CAAACCACTGTTGCTCATTCTC3' (22) Reverse: 5'GTAGTCACCACCGATACCAATAC3' (23)	93
PMR1	Forward: 5'CCTTAGCGGTTGCTGCTATT3' (20) Reverse: 5'ACCTTCTCACGATGGCTTTAC3' (21)	105
SMF1	Forward: 5'CCGAAGAGTACGTCCGTTAAA3'(21) Reverse:5'GGCATAACAGTAGCACCTAAGA3' (22)	113
SMF3	Forward: 5'GAGGCAGAGGATGCTGATTT3' (20) Reverse:5'CGGCAGATTGACCAGAACATA3' (21)	112

(cont. on next page)

Table 2.2. (cont.)

PER1	Forward: 5'TGGTTGTGACCCTACTTGTTC3' (21) Reverse: 5'TCTGTTATACTCGCACGCATAC 3' (22)	101
MAM3	Forward: 5'CTGATGTACCCAGTCGCATATC3' (22) Reverse: 5'TCCTATGCAAGGTGACCAAAG3' (21)	115
HIP1	Forward: 5'TCCTCTCCATCATTGGCTTTAC3' (22) Reverse: 5'CCTGGGTCATGCCAGTATTT3' (20)	100
SOD2	Forward: 5'CAGTCTGGACGAGCTGATTAAG3'(22) Reverse: 5'CTTGCCTCCATTAGAGAGGTTT3' (22)	100
SPF1	Forward: 5'AACGCAGATTGGAAGGTATGA3' (21) Reverse: 5'TTTGGACCAGGTGGGAATAAG3' (21)	121
CAR1	Forward: 5'AGATCGCGTATATTGGGTTGAG3' (22) Reverse: 5'CAGCGTTGATGCCGTATTTG3' (20)	114
PCD1	Forward: 5'GAGAACTAGGGCACGAAGATTT3' (22) Reverse: 5'TCGCCGTTGATCATTCCTATC3' (21)	103
MRE11	Forward: 5'GACGAAGAGGACGCTAGTTATG3' (22) Reverse: 5'CGTCCTTGATGCTCTTCCTT3' (20)	117
APS2	Forward: 5'GAGGTATGCGGGTCTGTATTT3' (21) Reverse: 5'CATCTAGCACCTCCACAAACA3' (21)	98
SRV2	Forward: 5'GAGTCATGCAGTGTTGTTCTTG3' (22) Reverse: 5'GGAGATTTGAGGTAGGGAATGG3' (22)	102
ZEO1	Forward: 5'AAACTGCCGCCCAAGAT3' (17) Reverse: 5'GAAGCTTCAGCTTGTTCCTTTAC3' (23)	94
CYS4	Forward: 5'GCAAGCCGATTCAAGACATAAC3' (22) Reverse: 5'ATGGAACCACCTGGATTGTATAG3' (23)	138

(Cont. on next page)

Table 2.2. (cont.)

SNF7	Forward: 5’GAGGGAGCATATCAACCTTCTATC3’ (24) Reverse: 5’GTGCATTCTTCGCCATTACTTT3’ (22)	122
LHS1	Forward: 5’CGAGTTTGCGGATACCATAGAT3’(22) Reverse: 5’AGATAACGCCTTCGACACATAC3’(22)	82
CRZ1	Forward: 5’CTCGGGCATTGACTCCAATTA3’ (21) Reverse: 5’GAGACATCGCAGGTGAAGAAA3’ (21)	95
DID4	Forward: 5’GAGGTGGATGAAGACGAAGAAG3’ (22) Reverse: 5’CGTGCTTTGCAACTGTGAAT3’ (20)	90
BRO1	Forward: 5’CCCAGGCTCTTGAGTGATTT3’ (20) Reverse: 5’TGTCTGAATTTGGTCCCTCATC3’(22)	99
ATP12	Forward: 5’CCGCCACCTTAGAAACAATCT3’ (21) Reverse: 5’CAGCAATCGCAGCAGTATGA3’(20)	11
SNF8	Forward: 5’GGGAGGCTGTTAGGAGTAAATC3’ (22) Reverse: 5’CCTCTGCACCACCTTGATAAT3’ (21)	84
SOD1	Forward: 5’GGTGACATGGGTAACGTAAAGA3’ (22) Reverse: 5’AACGGAGGTAGGACCGATAA3’ (20)	93

For analysis of the results, Bio Rad iq5 using the Pfaffl model was used. Zero-hour gene expressions were taken as the reference point, and the comparative gene expression levels in the applied samples were calculated according to this reference point (fixed at 1).

2.5. Detection of intracellular Mn + 2 level

Intracellular Mn^{+2} concentrations of wild type and cells were obtained after Mn^{+2} treatment by using the method of Mizuno et al., 2005.

Cultures grown overnight were diluted 1/1000 and $MnCl_2$ was added to a final concentration of 0,5 mM and the cells were grown between 48 and 60 hours depending on their growth concentration, approximately optical density of 1 at wavelength of 600 nm. Next, the cells were washed three times with 10 mM EDTA and incubated at 70 ° C overnight. Yeast cells of 5 mg were digested with 65% HNO_3 and filtered. The Mn^{+2} amounts of the extracts were determined by Agilent 7500ce Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) (Agilent Technologies).

2.6. Tolerance tests against other metals

The wild yeast strain, *Saccharomyces cerevisiae* BY4741 (MATa; his3; leu2; met15; ura3) and *Saccharomyces paradoxus* Q74.4 were spot tested against different metals (cobalt, cadmium, nickel and zinc) in different concentrations in order to determine toxic metal concentrations.

For spot testing, overnight cultures (at 120 rpm, 30 ° C) were diluted with distilled water in the ratio of 0.2; 0.02; 0.002; 0.0002 according to their optical densities at 600 nm, and five microliters from these cultures were spotted on the YPD media treated with metals (1 mM Co^{+2}, 150 µM Cd^{+2}, 1.8 mM Ni^{+2}, 10 mM Zn^{+2} or metal free). These metal concentrations are determined as toxic concentrations for yeast in previous studies. Yeast cells were incubated and photographed in petri dishes at 30 ° C for three days.

2.7. Promoter analysis

In order to get promoter sequences of each gene in wild-type yeast, Eukaryotic Promoter Database (http://epd.epfl.ch) is used. Nation Collection of Yeast Cultures (NCYC) database is used to obtain sequences of each strain. MEME- Motif discovery tool is used to discover transcription factor binding motifs (Bailey and Elkan, 1994). Sequence alignment generations were done by using the Clustal Omega Pairwise Alignment tool.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Screening of SGRP collection set

Manganese is one of the essential transition metals that are required for plenty of biological mechanisms. However, higher concentrations of manganese interfere with other metabolic pathways by binding nonspecifically to enzymes and cause heavy metal toxicity. Tolerance to heavy metals is generally provided by the involvement of metal transporter proteins, by taking excess metal out of the cell membrane. Inhibition of the growth of wild type yeast S. *cerevisiae* was observed in 0,5 mM of Mn^{2+} (Blackwell et al., 1998). S. *cerevisiae* has the ability to adapt toxic manganese levels after an unusual long lag phase (5-6 days) (Andreeva et al., 2017). Yeasts that are tolerant to Mn^{2+} are identified by screening National Collection of Yeast Cultures (NCYC). Yeast strains were grown in the presence of 0, 2.5, 5 and 10 mM of Mn^{2+} for 5 days at 30ºC.

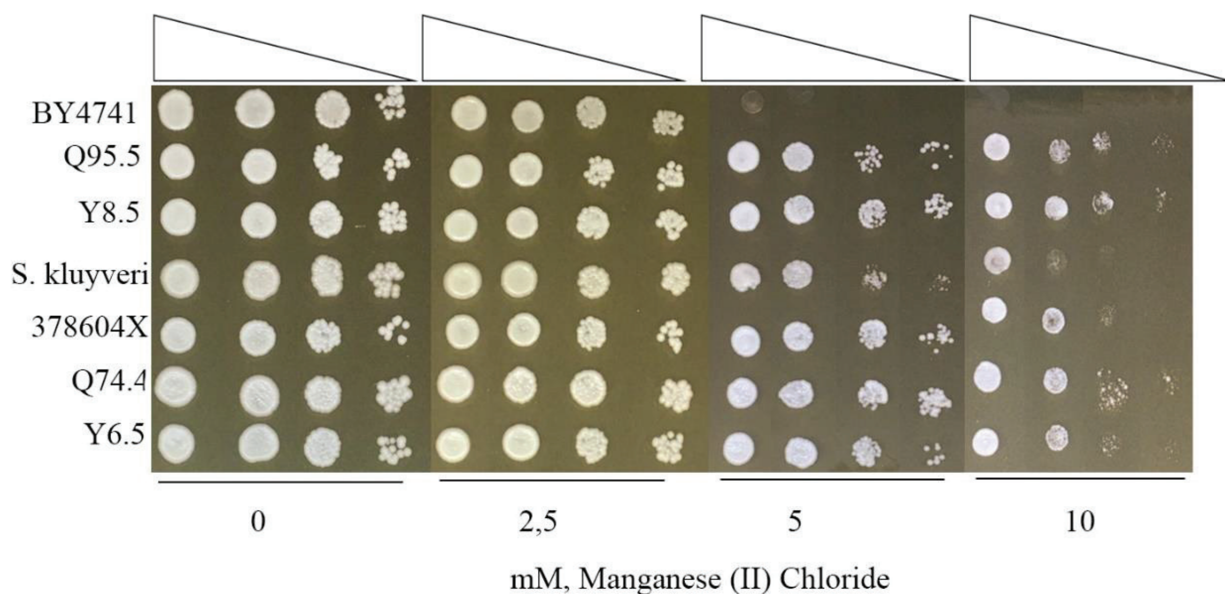

Figure 3.1. Spotting assay of different yeast strains under given manganese concentrations.

Growth of most of the strains inhibited after 2,5 mM of Mn^{2+}, including wild-type strain BY4741. Six of the strains in the NCYC collection showed significant resistance up to 10 mM of Mn^{2+} (Figure 3.1.) Yeast cells usually adapt 2,5 mM of Mn^{2+} after a prolonged lag phase. It was observed that growth of Wild type *S. cerevisiae* BY4741 is inhibited above 2,5 mM of Mn (II) concentration. Resistant strains showed resistance to 10 mM of Mn^{2+}.

3.2. Growth curve analysis

Strains confer resistance to Mn^{2+} in solid growth test re-validated with growth curve analysis. S. *cerevisiae* BY4741, S. *cerevisiae* 378604X, S. *paradoxus* Q74.4 and S. *paradoxus* Y6.5 cultured in liquid YPD medium supplemented with a range of Mn^{2+} concentrations up to 20 mM. The optical density of samples monitored over a 24-hour period.

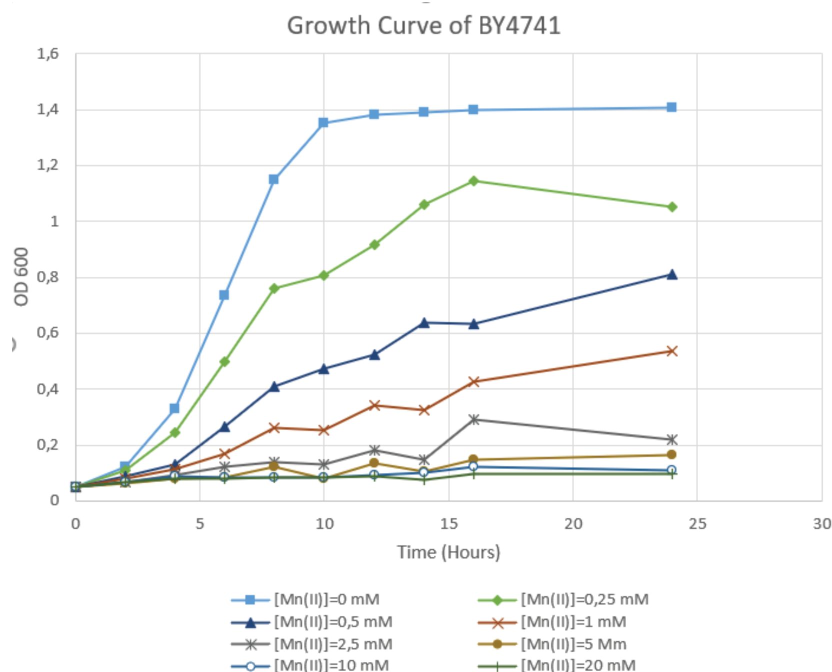

Figure 3.2. Growth curve analysis of S. *cerevisiae* BY4741 in treatment with given concentrations of Mn^{2+}.

The growth of BY4741 was influenced after treatment with 0,25 mM of Manganese. More than 1 mM of Mn^{2+} inhibited the growth of wild type, BY4741. BY7471 reaches stationary phase at 10th hour of growth in no-manganese added media. In the presence of manganese even at a minimum level such as 0,25 mM, growth has slowed down.

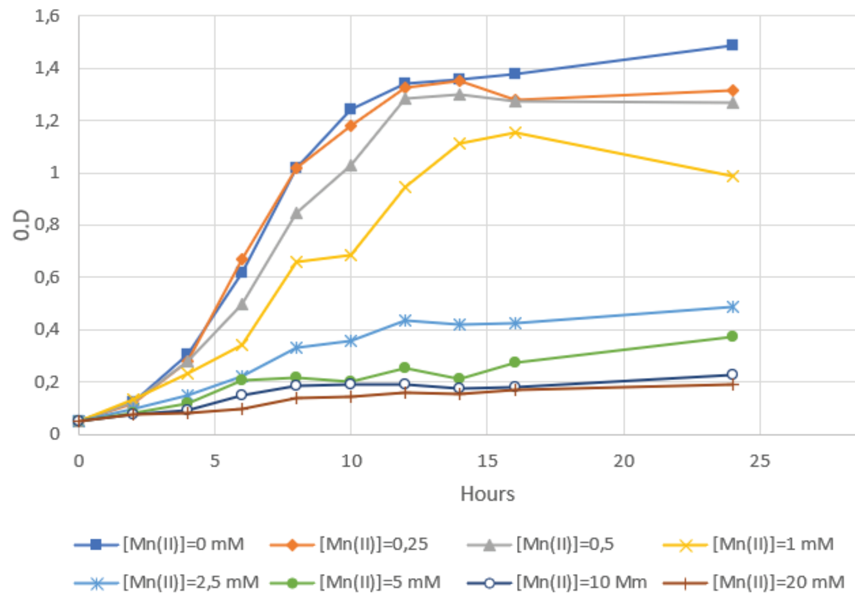

Figure 3.3. Growth curve analysis of *S. paradoxus* Y6.5 in treatment with given concentrations of Mn^{2+}.

Growth of S. paradoxus Y6.5, which is selected as a resistant strain based on solid growth tests, was affected after 1 mM of Mn^{2+} concentration, and almost inhibited in 5 mM of Mn^{2+}.

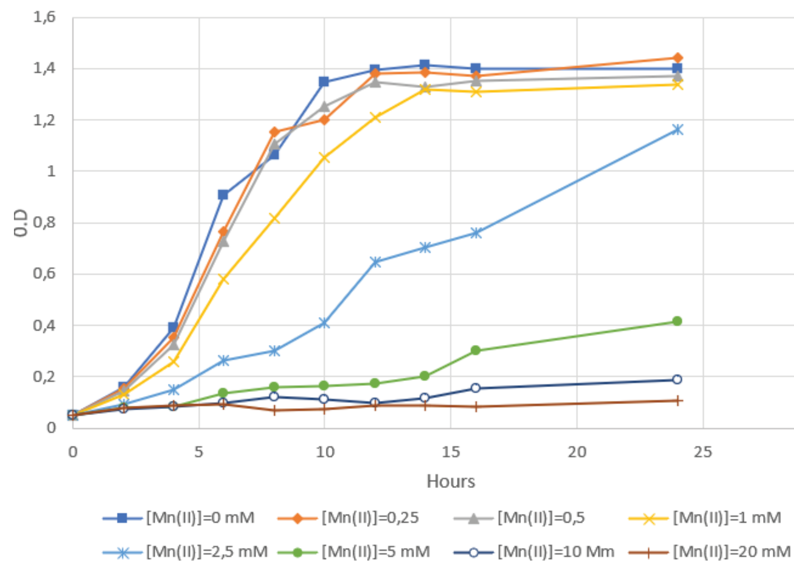

Figure 3.4. Growth curve of S. *cerevisiae* 378604X in treatment with given concentrations of Mn^{2+}.

Growth of cells slowed down after being exposed to 2,5 mM Mn^{2+}. Growth is fully inhibited when cells are exposed to 5 mM, 10 mM and 20 mM Mn^{2+}.

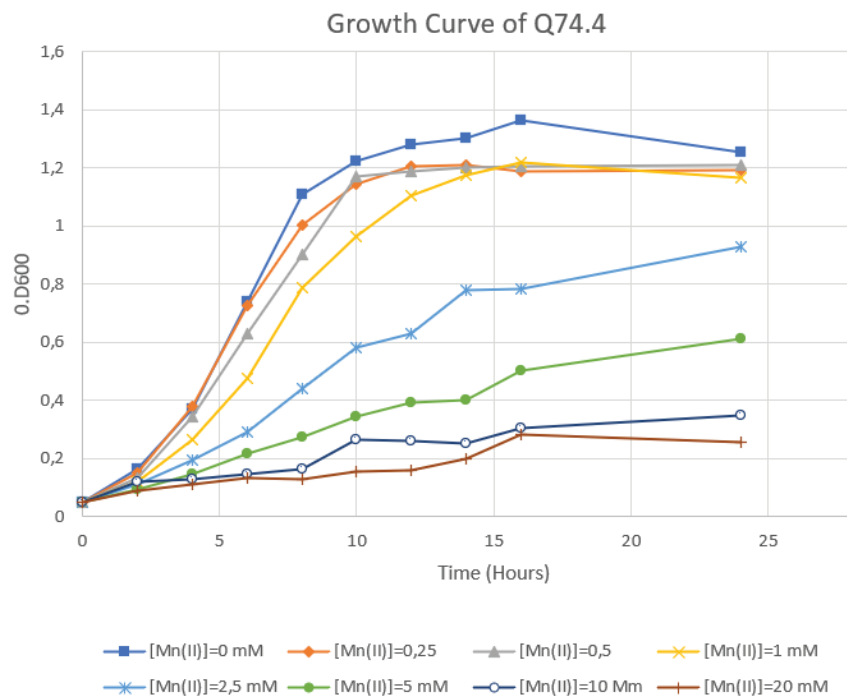

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Figure 3.5. Growth curve analysis of S. *paradoxus* Q74.4 in treatment with given concentrations of Mn^{2+}.

Growth analyses were performed to determine the subinhibitory concentration of $MnCl_2$.

Growth of Q74.4 is not significantly affected by up to 5 mM of Mn^{2+}, and is not fully inhibited until 20 mM of Mn^{2+} treatment.

Based on the spotting assays and growth curve analysis, *S. paradoxus* Q74.4 was determined as the most resistant strain above the collection. S. *paradoxus* Q74.4 was selected as a model organism for transcription analysis. All the yeast strains analyzed were sensitive to Mn^{2+} treatment and they could not grow in a medium added more than 20 mM of Mn^{2+}.

3.3. Gene expression analysis by Real Time RT-PCR

RNA isolated from zero-hour cell cultures was used as a control sample. For other samples, overnight cell cultures were re-cultivated and incubated in 1 mM of Manganese (II) containing YPD medium for one hour and twelve hours. Different exposure times were selected to analyze the effect of short-time responses versus long-term adaptation.

After total RNA isolation from harvested cell cultures and cDNA synthesis, RT-PCR was performed with three replicates for each gene. Gene expression levels were normalized using Actin-1 (ACT1) as a housekeeping gene for internal control. For data analysis, Pfaffl method was used (Pfaffl, 2001).

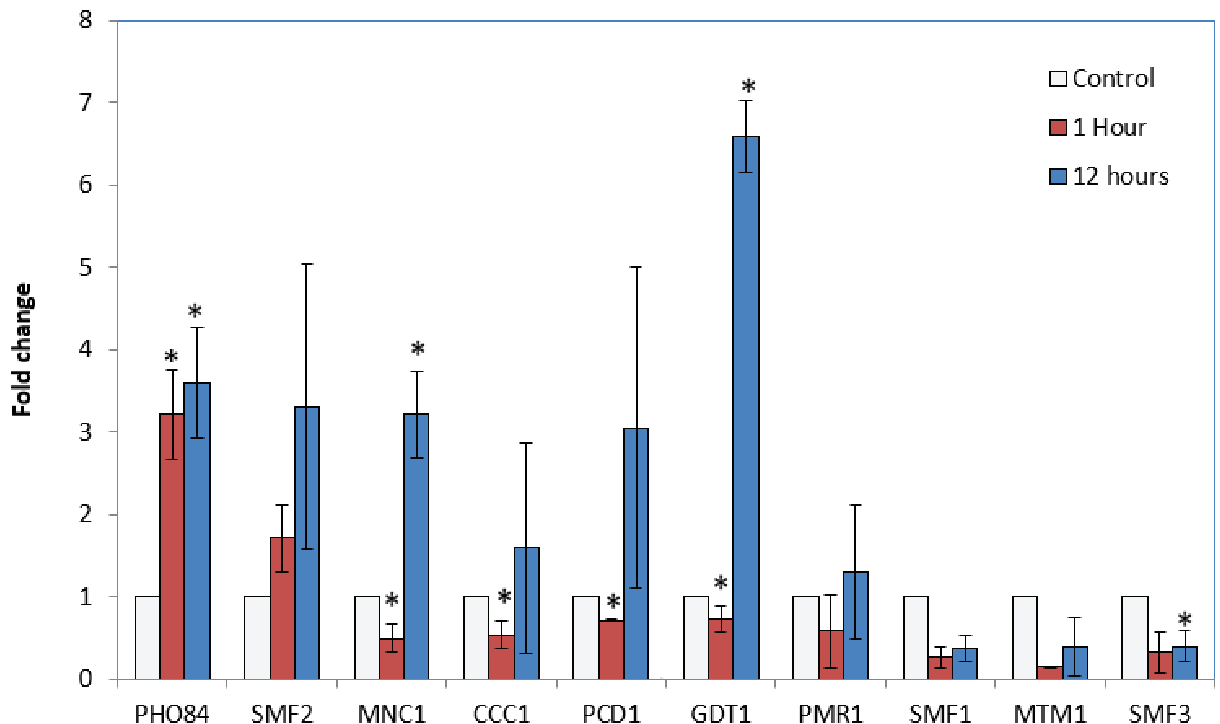


Figure 3.6. Real time RT-PCR analyses of selected genes after manganese treatment for 1 hour and 12 hours. Fold change represents relative normalized gene expression. Actin1 gene was used as internal control. Bars represent the mean of three replicates (N=3, *$p<0,005$).

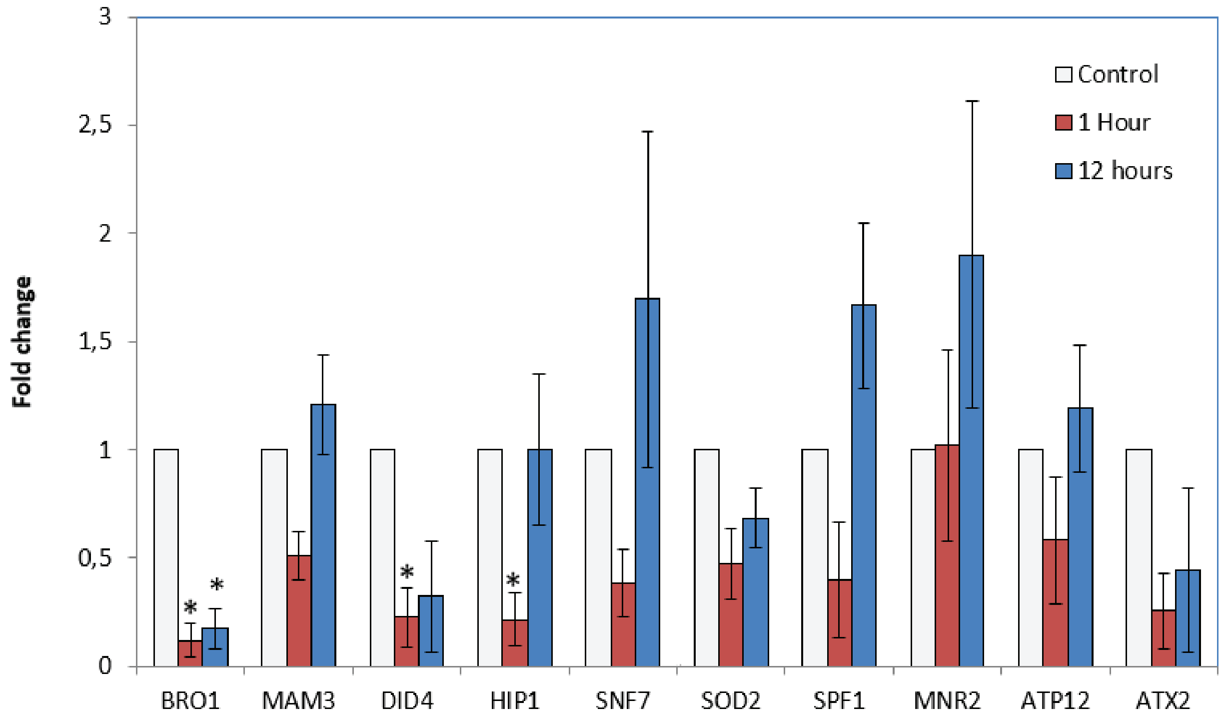


Figure 3.7. Real time RT-PCR analyses of selected genes after manganese treatment for 1 hour and 12 hours. Fold change represents relative normalized gene expression. Actin1 gene was used as internal control. Bars represent the mean of three replicates (N=3, *$p<0,005$).

Primer designs and Real Time RT-PCR analysis of twenty genes were made. Candidate genes were selected via the data obtained from SGD (Saccharomyces Genome Database). PHO84, SNF2, MNC1, CCC1, GDT1 and PCD1 genes have higher fold change rates relative to control. This suggests that they are probably upregulated as a response to Mn^{2+} stress. Results are evaluated based on their P-values and error bars. PHO84, GDT1 and MNC1 genes have lower variability and significant p-values rather than other genes with higher expression rates. Expression levels of other genes do not significantly change upon adaptation and remain almost stable after manganese exposure.

Gene expression levels differ from short-term responses and after long-term adaptation. 12th hour expression rates are significantly higher than 1st hour of treatment. Yeast adapts to environmental stress caused by manganese toxicity after several hours.

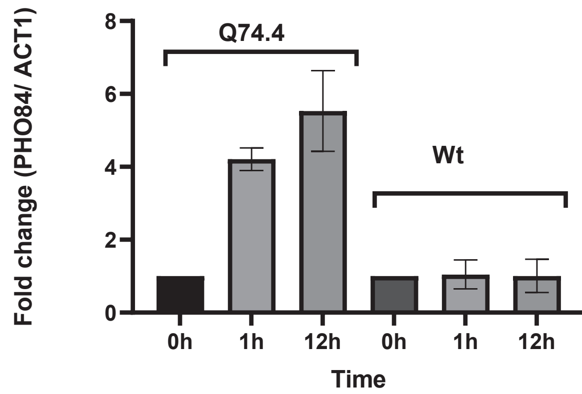


Figure 3.8. Expression levels of PHO84 in WT and Q74.4 strains under manganese stress.

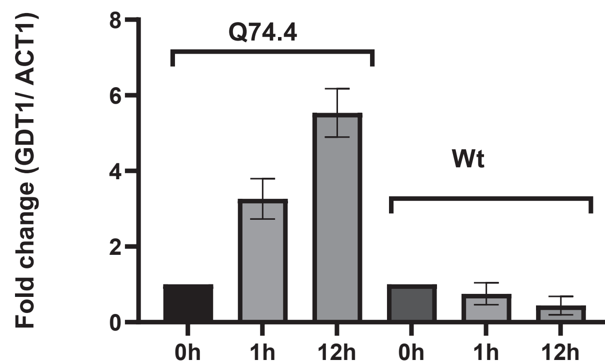


Figure 3.9. Expression levels of GDT1 in WT and Q74.4 strains under manganese stress.

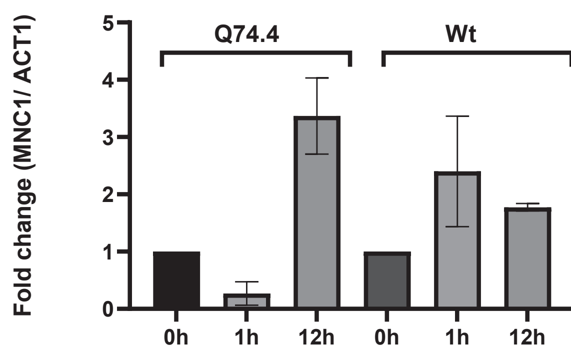


Figure 3.10. Expression levels of MNC1 in WT and Q74.4. strains under manganese stress.

Data was obtained from three different experiments.

PHO84 expression is significantly higher in Q74.4 relative to wild type under Manganese (II) stress conditions. PHO84 is a high-affinity phosphate transporter, and PHO84 deficient yeast cells have reduced metal ion accumulation, it has also suggested that resistance is due to reduced uptake of metal ions (Jensen et al., 2003). It is also been suggested that heavy metal ions may be taken up by PHO84p in a bound, less toxic form than free osmotic form, such as metal phosphates (Ofiteru et al., 2011). Osmotically free metals ions are more toxic in the cytoplasm because there are many active enzymes and biological residues. Activity of Pho84p protects cells from metal toxicity as a secondary transporter that co-transports phosphate with high-affinity and manganese with low-affinity. However, the reason behind upregulated PHO84 level could be the side-effect of phosphate pathway disruption.

Expression rate of GDT1 also increases under Mn^{2+} stress in Q74.4 cells. There is no alteration in wild type cells. Gdt1p belongs to an uncharacterized protein family that are highly conserved membrane proteins. Gdt1p is reported as a Golgi membrane protein and it is required for Ca^{2+} homeostasis. Gdt1p also mediates Mn^{2+} influx in *L. lactis*. (Thines et al.,2018). Gdt1p is a transporter of both manganese and calcium presumably from the cytosol to the Golgi lumen, most likely in exchange of protons (Thines et al., 2018). It is suggested that Gdt1p detoxifies the cytosol by transporting cations to the Golgi where excess metals get out of the cell via secretory pathways. A secondary transporter that has not been identified yet could be involved in taking excess Mn^{2+} to exit the cell. Since excretion of manganese via secretory pathways and Golgi lumen is not the only way for surplus metal detoxification.

This is also noticeable that upregulated genes found in this study have a role in compartmentalization of Mn^{2+}. For example, Smf1 is a known manganese transport protein localized in the plasma membrane and endosome and responsible for manganese homeostasis. (Liu and Culotta, 1999). However, its expression level under manganese stress remains stable (Fig 3.6.) while the genes that encode transporter proteins of Mn compartmentation are highly upregulated.

MNC1 is a putative membrane protein that chelates manganese at the surface of the cell and participates in manganese detoxication (Andreeva et al., 2017). MNC1 also involves in Cd^{2+} tolerance *of Schizosaccharomyces pombe*. Expression of MNC1 gene increased relative to control (no metal stress). When compared to WT, there is an alteration in 12th hour gene expression levels, but not as remarkable as PHO84 and GDT1 fold changes.

3.4. Measurement of intracellular Manganese (II) concentrations

Agilent 7500ce Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) was used to measure intracellular manganese concentrations. Cell cultures were incubated in YPD medium containing 0,5 mM of $MnCl_2$ until they reached stationary phase and method of Mizuno et al., 2005 used for dried cell lysates.

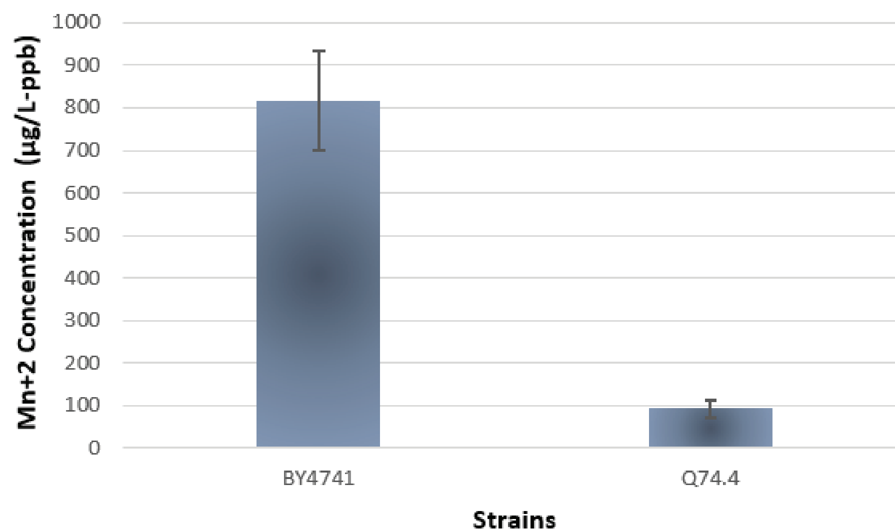


Figure 3.11. Mn^{2+} accumulation levels of wild type BY4741 and Q74.4. Bars represent standard error of the mean of three experiments.

The intracellular manganese concentrations detected to understand whether the cells overexpressed PHO84 or GDP1 genes are accumulating excess Mn^{+2} or take it out of the cell membrane. There is a significant difference in intracellular Mn^{2+} levels between wild type and Q74.4 strains. Concentration of Mn^{2+} in Wild type BY4741 cells were 817 ppb, while Q74.4 cells contain 93 ppb of Mn^{2+}.

Proteins that are conferring Manganese resistance to Q74.4 strain probably involved in pumping Mn^{2+} out of the cells or reducing the uptake of extracellular manganese to the cell.

Protection against manganese toxicity in *S. cerevisiae* can also be facilitated by sequestration of the metal in the vacuole. The yeast vacuole participates in both the storage and detoxification of manganese and the metal appears concentrated in the vacuole compared with cytosol. Nevertheless, intracellular manganese concentration of Q74.4 strain was a much lower than wild type strain. That means manganese tolerance has been not accomplished by storage in the vacuole but taking it out of the cell by transmembrane proteins. In the case of Q74.4, upregulated genes may be involved in metal export, by taking it from vacuole to the out of the cell.

In former studies that are used the budding yeast, Saccharomyces *cerevisiae*, the high-affinity inorganic phosphate transporter encoded by the PHO84 gene was found to act also as a low affinity transporter for Mn^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+}, playing a role in Mn2+ homeostasis in the S. *cerevisiae* cells (Jensen et al. 2003). It is also known that PHO84 deficient yeast cells have reduced metal ion accumulation (Jensen et al., 2003). This tolerance by PHO84 is usually due to its ability to co-transport manganese in manganese phosphate form, which is less toxic and tolerable for yeast cells. In our case, yeast overexpressing PHO84 have reduced Manganese (II) accumulation. This is probably caused by the interaction of other upregulated genes and transporter proteins. The other reason could be that yeast overexpressing PHO84 have reduced ion uptake into the cell from the environment.

3.5. Tolerance tests against other metals

BY4741 and Q74.4 cells were spotted into YPD plates containing Ni^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} at the minimum inhibitory concentration (MIC). MIC was determined by growing WT BY4741 at different concentrations of each metal.

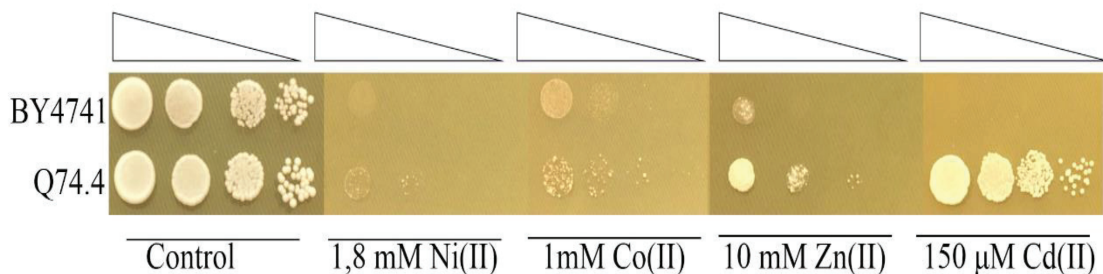


Figure 3.12. Spotting assay of wild type BY4741 and Q74.4 stains in different metal concentrations.

The manganese resistant strain Q74.4 also a had high level of Cadmium, Zinc and Cobalt resistance. PHO84 gene was found to act also as a low affinity transporter for Mn^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+}, playing a role in Mn^{2+} homeostasis in the S. *cerevisiae* cells (Jensen et al. 2003). In addition, tolerance to Nickel has not been observed.

MNC1 that belongs to the CYSTM family is responsible for Cd^{2+} tolerance of *Schizosaccharomyces pombe* (Kennedy et al., 2008). Cadmium is a worldwide environment pollutant and excess concentrations became toxic. A great example of cadmium toxicity is Itai-Itai disease, characterized by severe pain, proteinuria, bone fractures, and osteomalacia (Kennedy et al., 2008). Additionally, cadmium is also considered a human carcinogen.

Zinc is essential for numerous physiological functions due to its Lewis acidity, which makes it an ideal cofactor for many enzymes (Vallee and Falchuk, 1993). Zinc-regulated Activator Protein (Zap1p) acts as the major sensor and regulator of Zinc homeostasis (Bird et al., 2003). It is also a positive regulator of the Zn uptake transporters ZRT1 and ZRT2 (Zhao and Eide, 1997).

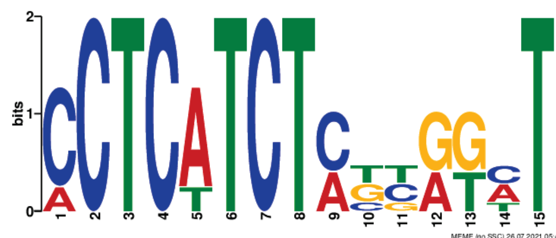
Zrc1p and Cot1p are members of the Cation Diffusor Facilitator (CDF) protein family and are responsible for metal resistance. They are both located on the vacuolar membrane and function in the transportation of metals into the vacuole (MacDiarmid et al., 2000). ATR1 is also one of the analyzed genes in our study responsible for multidrug transport involved in metal homeostasis, included boron (Gbelska et al., 2009, Kaya et al., 2009).

Cadmium is a redox-inactive metal that triggers oxidative stress by attaching to enzymes and inhibiting them. Cadmium may also influence zinc ions in metalloproteins and zinc fingers to be displaced (Faller et al 2005). Because high-affinity zinc transporters (Zrt1) transport cadmium as well, cells deficient in ZRT1 accumulate less cadmium than wild-type cells (Gomes et al., 2002). Smf1 protein expression levels were likewise found to be stable during manganese stress in our studies. Cadmium is known to be a substrate for Smf1-protein interacted pathways. Smf1 activity is regulated by metal concentrations; when manganese levels are high, Smf1 is directed to the vacuole; when manganese levels are low, Smf1 is not directed to the vacuole (Liu and Culotta, 1999). The activation of copper/zinc superoxide dismutase was affected by the BSD2 product (Liu and Culotta, 1994). Inactivation of SMF1

in cells lacking BSD2 resulted in cadmium resistance but not cobalt resistance (Liu and Culotta, 1994). Instead, by eliminating another Nramp expressing gene, SMF2, cobalt accumulation in bsd2Δ was reduced (Liu et al. 1997). Smf2 redistributes to intracellular vesicles while Smf1 shifts to the plasma membrane in response to manganese deficiency. Smf2 is also not found on the cell surface (Portnoy et al. 2000). Fet4p also acts as a Fe, Cu, and Zn uptake transporter with a low affinity (Waters and Eide, 2002). The FET4 gene encodes for a low-affinity iron transporter that is found on the plasma membrane (Dix et al., 2004). Other studies have shown that cobalt can be carried into yeast cells via the phosphate transporter Pho84, which, like manganese, transports metals in the form of metal phosphates and oxyanions (Jensen et al., 2003). Sulphur pathway of S.*cerevisiae* can also be part of metal detoxification systems, by activating the glutathione biosynthesis pathways (Vido et al., 2001).

3.6. Bioinformatic analysis of promoter regions

In order to get promoter sequences of each gene in wild-type yeast, the Eukaryotic Promoter Database (http://epd.epfl.ch) is used. National Collection of Yeast cultures (NCYC) database is used to obtain sequences of promoters of two genes, PHO84 and GDT1 in S. *paradoxus*. Protein binding motifs have been identified using MEME (Multiple Em for Motif Elicitation) tool. Sequence alignments were done by using Clustal Omega software.



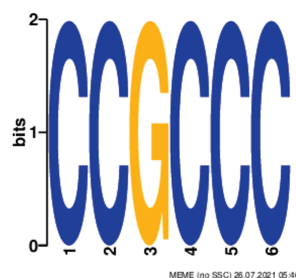


Figure 3.13. Motif logos found in promoter region of PHO84 gene in WT strain S. *cerevisiae*.

Figure 3. 14. Motif logos found in promoter region of PHO84 gene in S. *paradoxus.*

Figure 3.15. Motif logos found in promoter region of GDT1 gene in WT strain S. *cerevisiae*.

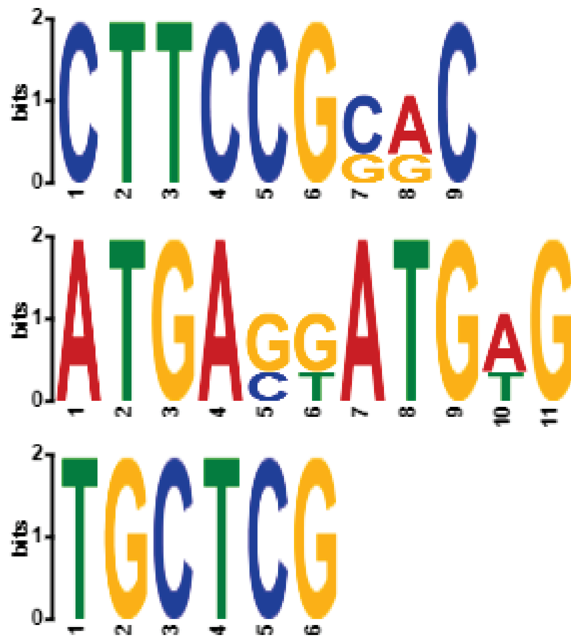


Figure 3. 16. Motif logos found in promoter region of GDT1 gene in S. *paradoxus.*

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pho84wtsc            1 -GGCCCTTTCAACTCATCTAGCGGCTATGAAGAAAATGTTGCCCGGCTGA     49
                        ||||||||.||||||||||||||||||..|||.|.|.||||||||||||
pho84sp              1 GGGCCCTTTTAACTCATCTAGCGGCTATTGAGATAGTATTGCCCGGCTGA     50

pho84wtsc           50 AAAACACCCGTTCCTCTCACTGCCGCACCGCCCGATGCCAATTTAATAGT     99
                       ||||||||||||||||||||||||||.|||||||||||||||||||||||
pho84sp             51 AAAACACCCGTTCCTCTCACTGCCGCGCCGCCCGATGCCAATTTAATAGT    100

pho84wtsc          100 TCCACGTGGACGTGTTATTTCCAGCACGTGGGGCGGAAATTAGCGACGGC    149
                       |||||||||||.||||||||||||||||| ||||||||||||.||||||.
pho84sp            101 TCCACGTGGACATGTTATTTCCAGCACGT-GGGCGGAAATTAGTGACGGG    149

pho84wtsc          150 AATTGATTATGGTTCGCCGCAGTCCATCGAAATCAGTGAGATCGGTGCAG    199
                       ||||||||||||||.||||.|||.||||||||.|||||||||||||||||
pho84sp            150 AATTGATTATGGTTTGCCGTAGTTCATCGAAACCAGTGAGATCGGTGCAG    199

pho84wtsc          200 TTATGCACCAAATGTCGTGTGAAAGGCTTTCCTTATCCCTCTTCTCCC-G    248
                       |.|||||||||||.|||||||||||||||||||||||||||||||||| |
pho84sp            200 TAATGCACCAAATCTCGTGTGAAAGGCTTTCCTTATCCCTCTTCTCCCGG    249

pho84wtsc          249 TTTTGCCTGCTTATTAGCTAGATTAAAAACGTGCGTATTACTCATTAATT    298
                       |||||||||||.||||||||||||||||||||||||||||||||||||||
pho84sp            250 TTTTGCCTGCTCATTAGCTAGATTAAAAACGTGCGTATTACTCATTAATT    299

pho84wtsc          299 AACCGACCTCATCTATGAGCTAATTATTATTCCTTTTTGGCAGCATGATG    348
                       |||||||..|||||||||||||||||||||||||||||.|||||||||||
pho84sp            300 AACCGACTCCATCTATGAGCTAATTATTATTCCTTTTTAGCAGCATGATG    349

pho84wtsc          349 CAACCACATTGCACACCGGTAATGCCAACTTAGATCCACTTACTATTGTG    398
                       ||||||.||||||||||||||||.|||||||||||.||.||||||||.||
pho84sp            350 CAACCATATTGCACACCGGTAATACCAACTTAGATTCAGTTACTATTATG    399

pho84wtsc          399 GCTCGTATACGTATATATATAAGCTCATCCTCATCTCTTGTATAAAGTAA    448
                       ..|||||||||||||||||||||||||||||||||||||.||||||||||
pho84sp            400 CTTCGTATACGTATATATATAAGCTCATCCTCATCTCTTCTATAAAGTAA    449

pho84wtsc          449 AGTTCTAAGTTCACTTCTAAATTTTATCTTTCCTCATCTCGTAGATCACC    498
                       ||||||||.||||||..|||||||||||||||||||||||||||||||||
pho84sp            450 AGTTCTAAATTCACTCTTAAATTTTATCTTTCCTCATCTCGTAGATCACC    499

pho84wtsc          499 AGGGCACACAACAAACAAAACTCCACGAATACAATCCAAATGAGTTCCGT    548
                       ||||||||||  ||||||||||||||||||||||||||||||||||||||
pho84sp            500 AGGGCACACA--AAACAAAACTCCACGAATACAATCCAAATGAGTTCCGT    547

pho84wtsc          549 CAATAAAGATACTATTCATGTTGCTGAAAGAAGTCTTCATAAAGAACACC    598
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
pho84sp            548 CAATAAAGATACTATTCATGTTGCTGAAAGAAGTCTTCATAAAGAACACC    597

pho84wtsc          599 T    599
                       |
pho84sp            598 T    598
```

Figure 3. 17. Sequence alignments of PHO84 promoter region between two strains, S. *cerevisiae* (pho84wtsc) and S. *paradoxus* (pho84sp). Transcription factor binding sites that have nucleotide variations marked as orange.

```
Gdt1wtsc         1 TAACTTCAGGTATAATTCTTTTTCACGCAAGATCAACTGGAGTCAAGTTC    50
                   ||||||.||||||||||||.||||||||||||||..|..||.||..||||
Gdt1sp           1 TAACTTTAGGTATAATTCTCTTTCACGCAAGATCTTCCAGATTCTCGTTC    50

Gdt1wtsc        51 TTCAATAAATATCAGGACATTTTGCGGAAAATTGCTATCAAATGCTCGGT   100
                   |||.|.||||||||||||.||.|||||||||||||.|||||.||||||||
Gdt1sp          51 TTCGACAAATATCAGGACGTTCTGCGGAAAATTGCAATCAAGTGCTCGGT   100

Gdt1wtsc       101 ATGTTTTTTTCTTTTATTCTACTTTTACGATTCTTTTTCTTTCTTTCCTT   150
                   |||||.|    |||||||||||.|||||.||  |||||||||||    ||
Gdt1sp         101 ATGTTCT----TTTTATTCTACCTTTACCAT--TTTTTCTTTCT----TT   140

Gdt1wtsc       151 CTTCATAAAAAAGCACTTTCACTAACTGTCTTTGTCAAACATAAATATGC   200
                   ||||||..|.||.|..||||||||||...|.|||||||..||.|.||||.
Gdt1sp         141 CTTCATCTAGAAACTTTTTCACTAACCACCCTTGTCAACAATGAGTATGT   190

Gdt1wtsc       201 GATCAATATAGACCGTTGATATCAGTGGTAGAACAATAAGGAAATTGCCC   250
                   |||.|||.|||||..|.|||||.|||||||||||||.|||||||||.||.
Gdt1sp         191 GATTAATGTAGACTCTCGATATTAGTGGTAGAACAACAAGGAAATTACCT   240

Gdt1wtsc       251 TTGATGTGTCTGTCTGAATACTTCCGGACATTTCCTGTAGACGACGATGA   300
                   ||||||||||||||.||||||||||||||||||||||||||.||||||||
Gdt1sp         241 TTGATGTGTCTGTCCGAATACTTCCGGACATTTCCTGTAGATGACGATGA   290

Gdt1wtsc       301 GTTTGTTCTGGCATTAGCTATGTCAGCGCGGAAGCTGAGCGCAGCGCGGA   350
                   ||||||||||||||||||||||||||.||||||||||.||||||||||||
Gdt1sp         291 GTTTGTTCTGGCATTAGCTATGTCAGTGCGGAAGCTGGGCGCAGCGCGGA   340

Gdt1wtsc       351 AGTGACGGAGGAGC--TTTTTCCTTTTTTTCGCTTCTTTCTTTTGTCTCA   398
                   |||.|.|||..|||  ||||||||||||||||||||||||||||||||||
Gdt1sp         341 AGTAATGGAATAGCCATTTTTCCTTTTTTTCGCTTCTTTCTTTTGTCTCA   390

Gdt1wtsc       399 TACTCAT---GTAGCATACTTTCGCGAACCC--TATTCCAACGTTTTGTT   443
                   |.|||||   |.|||||||||.|||||||||  |||||||||||||||||
Gdt1sp         391 TCCTCATGCAGCAGCATACTTACGCGAACCCTATATTCCAACGTTTTGTT   440

Gdt1wtsc       444 GTTGCTCGCTTTCATGCT-TAAAATTTTTATTATGTATT-TTACGATCAA   491
                   |||||||||||||||||| .||||||||||.|||||||| |||.||||||
Gdt1sp         441 GTTGCTCGCTTTCATGCTCAAAAATTTTTACTATGTATTCTTATGATCAA   490

Gdt1wtsc       492 GGTGTATCGTGAACCACACGTTTATGAAATCCTTATTTTAGAATCAAAAA   541
                   .||.||..||||||||.||||||||||||.||||||||||.|||| ||||
Gdt1sp         491 CGTATACTGTGAACCATACGTTTATGAAACCCTTATTTTAAAATC-AAAA   539

Gdt1wtsc       542 GTCCTACAATCTTGTTTGTGGAGTCTATATAAAGCTAAAGTTAGGTTCTA   591
                   ||||||.||.||||||||||||.||||.||||||.|||||||||||||||
Gdt1sp         540 GTCCTATAACCTTGTTTGTGGAATCTACATAAAGTTAAAGTTAGGTTCTA   589

Gdt1wtsc       592 CAAAGCAAG    600
                   ||.||||||
Gdt1sp         590 CAGAGCAAG    598
```

Figure 3. 18. Sequence alignments of GDT1 promoter region between two strains, S. *cerevisiae* (gdt1wtsc) and S. *paradoxus* (gdt1sp). Transcription factor binding sites that have nucleotide variations marked as orange.

Polymorphic motifs identified in this study could be associated with changes in gene expression levels and transcriptional variation at individual genes between two strains.

For regulatory alterations that lead to phenotypic differences across related species, the interaction between transcription factors and their binding sites is crucial (such as S. *paradoxus* and S. *cerevisiae*). Cis-regulatory elements are relatively more accountable for gene expression changes between two closely related strains, because selection in natural

populations quickly eliminated trans-acting mutations that resulted in gene expression alterations (Denver et al., 2005). According to a recent study (Connelly et al., 2013), regulatory motif variation is linked to gene expression differences amongst S. *cerevisiae* strains. With the help of motif analysis tools using mathematical models, transcription factor binding activities may be predicted and transcriptional regulators can be identified. Furthermore, combined with data on gene expression, motif analysis provides insight into the physical mechanisms of transcriptional regulation.

3.7. *Saccharomyces cerevisiae* versus *Saccharomyces paradoxus*

In this study, S. paradoxus Q74.4 strain is found to be capable of tolerating toxic metal levels. It belongs to Saccharomyces sensu stricto group that split into three species including *S. cerevisiae, Saccharomyces paradoxus,* and *Saccharomyces bayanus* based on physiological tests (Martini and Martini, 1987).

S. *cerevisiae* is widely distributed over the world, owing to human activities that lead to domestication, such as fermentation and food production. It is also found in nature, oak and maple trees and other plants in temperature zones. S. *paradoxus* is found in nature globally, sometimes together with S. *cerevisiae* and other Saccharomyces sensu stricto species. It can be isolated from plants such as oak trees, and Drosophila in regions that have optimum conditions, such as temperature that are slightly lower than the growth temperature of S.*cerevisiae*. However, there is only one report of S. *paradoxus* domestication by human intervention (Louis, 2001).

There is a significant amount of gene flow between these species, primarily due to human intervention. Unlike S. *cerevisiae*, the population structure of S. *paradoxus* seems to be well-described by geographic distance, according to recent research and whole-genome population genetic analysis (Liti et al., 2009). S. *paradoxus* populations appear to be rather well mixed within regions. However, there is a clear genetic difference between strains from Europe, America, and Asia, showing that global gene flow is a far weaker influence for S. *paradoxus* than it is for S. *cerevisiae* (Liti et al., 2009).

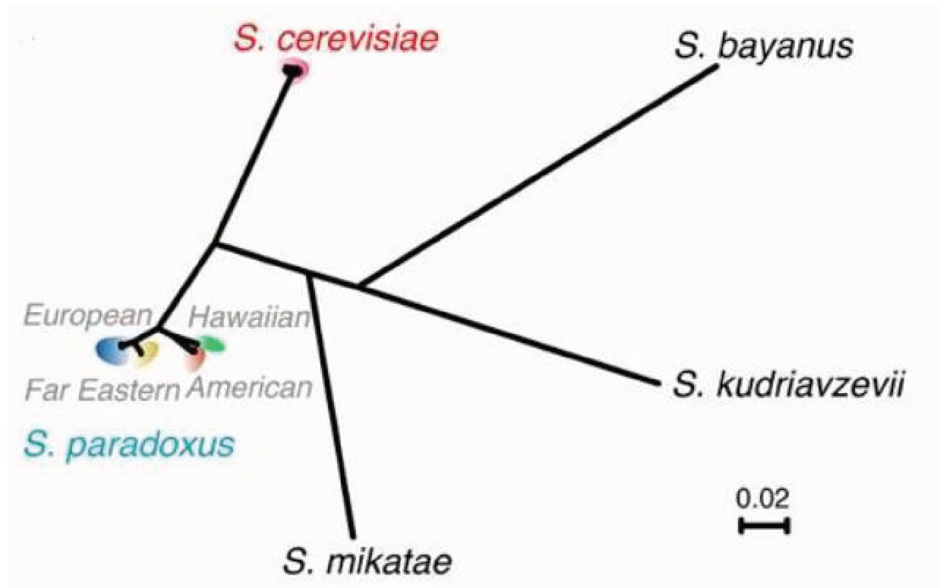

Figure 3.19. Phylogenetic tree of Saccharomyces genus (Liti et al., 2009).

Classic taxonomy, which includes phenotypic traits like cell shape, makes it difficult to identify these two species. But they can be distinguished by spore viability and based on genomic sequence divergence (Martini, 1989). The growth temperature was also found to differ between strains in other studies, but they were based on allopatric isolates (Martini and Martini, 1998). Hence, discovering other phenotypic characteristics such as metal tolerance gives some insights about the isolation of these strains.

CONCLUSION

Manganese is an essential metal for yeast growth and one of the most abundant ions in organisms. Surplus manganese concentration in cells became toxic because metals are capable of making strong coordination, disrupt enzymes or affecting functional groups (Blackwell et al., 1998). Several mechanisms conferring metal tolerance or resistance to yeast have been revealed but not fully understood. Furthermore, the Genome-wide detection of genes that are involved in metal detoxification, sequestration or transport can be done by using DNA microarrays. Mutations of protein motifs of these genes can be investigated to analyze different transporter activities.

We screened whole SGRP set 1 which contains 71 different yeast strains from around the world. Four of them that showed maximum resistance to manganese were selected for transcriptional analysis. Two genes, GDT1 and PHO84 observed to be upregulated in the presence of Manganese stress. Intracellular manganese concentrations were measured by ICP-MS and resistant strain Q74.4 has a lower intracellular level of manganese. A high percent of genes examined in this study have homologs in higher organisms such as animals and plants. Hence, outcomes of our research could be applicable for investigating tolerance and resistance mechanisms to heavy metals in higher organisms. For example, manganese toxicity causes neurodegeneration in humans called Parkinson-like syndrome. Our studies may provide some new clues about the mechanisms conferring Manganese resistance/ tolerance to eukaryotic cells. Mutant strains could be useful to assess the involvement of alternative genes in manganese tolerance or validate our results. Heavy metal accumulating cells that have a high tolerance to toxic metals would be suitable for bioremediation procedures. The data obtained from this study still could be useful for designing bio-accumulator mutant strains. We also demonstrated that S. *paradoxus* Q74.4 strain also has Cobalt, Zinc and Cadmium resistance at high levels. The accumulation status of surplus metals can be investigated in further studies, S. *paradoxus* Q74.4 can be used as a potential agent for cadmium, cobalt, and zinc remediation.

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