ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

qRT-PCR ANALYSIS OF GENE EXPRESSION LEVEL DIFFERENCES BETWEEN FREEZE-TOLERANT LABORATORY AND INDUSTRIAL STRAINS OF *Saccharomyces cerevisiae*

M.Sc. THESIS

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Department of Molecular Biology - Genetics and Biotechnology

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JANUARY 2015

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

DONMA-ERİME STRESİNE DİRENÇLİ *Saccharomyces cerevisiae* LABORATUVAR VE ENDÜSTRİYEL SUŞLARININ GEN ANLATIM DÜZEYLERİNDEKİ FARKLILIKLARININ qRT-PCR İLE ANALİZİ

YÜKSEK LİSANS TEZİ

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OCAK 2015

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To my family and friends,

FOREWORD

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ABBREVIATIONS

β-ME	: Beta-Mercaptoethanol
bp	: Base pairs
cDNA	: Complementary Deoxyribo Nucleic Acid
C_T	: Cycle Threshold
dH_2O	: Distilled Water
g	: Gram (s)
hr(s)	: Hour (s)
L(s)	: Litre (s)
M	: Molar (s)
mM	: Millimolar (s)
mg	: Milligram (s)
min(s)	: Minute (s)
ml	: Milliliter (s)
OD_{600}	: Optical Density at 600 nanometers
PCR	: Polymerase Chain reaction
qPCR	: Quantitative Polymerase Chain Reaction
rpm	: Revolutions per minute
RT-PCR	: Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	: Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	: Ribo Nucleic Acid
sec(s)	: Second(s)
YMM	: Yeast Minimal Medium
µl	: Microliter(s)
wt	: Wild-type
[ ]	: Concentration

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qRT-PCR ANALYSIS OF GENE EXPRESSION LEVEL DIFFERENCES BETWEEN FREEZE-TOLERANT LABORATORY AND INDUSTRIAL STRAINS OF *Saccharomyces cerevisiae*

SUMMARY

The aim of this thesis study was to analyze gene expression level differences between freeze-tolerant laboratory and industrial strains of *Saccharomyces cerevisiae*, to gain insight into the molecular mechanisms of freeze tolerance in this yeast. For this purpose, *S. cerevisiae* laboratory strain '905', industrial strain 'R625' and their freeze-thaw resistant mutant individuals 'F1' and 'P8' were used. Freeze-thaw stress-related genes were chosen according to whole-genome transcriptomic analysis results of 905 and F1, which have been obtained previously.

S.cerevisiae is a small, unicellular fungus that has been used as a model eukaryotic organism in biological research for a long time. Also, it has been commonly used in industrial processes since ancient times. The ability to ferment sugars makes the budding yeast is commercially important in bread-making and wine industries. The organism is also being used in bioethanol and fuel ethanol production, recently.

S. cerevisiae is also named as baker's yeast because of its ability to leaven dough. It is also known that the yeast cells are exposed to freeze-thaw stress during long term storage conditions. Thus, cryopreservation process is a stressful condition for yeast cells.

The freeze-thaw resistant mutants F1 and P8 were obtained in a previous study by an inverse metabolic engineering approach, based on random mutation and selection under freeze-thaw stress conditions. Whole-genome transcriptomic analyses of 905 and F1 were also done previously.

To verify the transcriptomic analysis results obtained by DNA microarray technique, gene expression levels of selected genes were determined by Quantitative RT- PCR. The genes were selected from microarray data according to their high up- or downregulation levels. Highy upregulated genes *HSP12, FMP45, HSP26, HXK1* and highly downregulated genes *PHO84, NSR1, ZRT1* were chosen for gene expression analysis by qRT-PCR.

The qRT-PCR experiments were performed under control and stress conditions for '905', 'F1' 'R625' and 'P8' strains. The expression profile of 'F1' was normalized to that of the wild type laboratory strain '905', and the expression profile of 'P8' was normalized to that of the wild type industrial strain 'R625'.

In conclusion, it was found that freeze-thaw stress causes significant changes in gene expression patterns of both laboratory and industrial strains. In addition, some differences were also found between gene expression level results of microarray and qRT-PCR analyses. This indicates the importance of verifying DNA microarray results by qRT-PCR, as qRT-PCR is a more sensitive method.

DONMA-ERİME STRESİNE DİRENÇLİ *Saccharomyces cerevisiae* LABORATUVAR VE ENDÜSTRİYEL SUŞLARININ GEN ANLATIM DÜZEYLERİNDEKİ FARKLILIKLARININ qRT-PCR İLE ANALİZİ

ÖZET

Bu çalışmanın amacı daha önce donma-erime stresine direnç kazandırılmış olan *Saccharomyces cerevisiae* laboratuvar ve endüstriyel suşlarının, kontrol ve donma erime stres koşullarında gen anlatım düzeylerindeki değişikliklerin Gerçek Zamanlı PCR (qRT-PCR) yöntemi ile belirlenmesidir. Gen anlatım düzeyi değişiklikleri, dirençli suşların gen anlatım değerleri referans suşlarınkine normalize edilerek belirlenmiştir.

S.cerevisiae küçük, tek hücreli ve tomurcuklanan bir maya türüdür. Bu maya türü uzun yıllardır bilimsel çalışmalarda model organizma olarak kullanılmaktadır. Endüstrideki yaygın kullanımından ve tek hücreli basit ökaryotik yapısından dolayı en çok çalışılan model organizmalardan biri olmuştur. Kültürlenmesinin kolay olması ve ökaryotik bir organizma olmasından dolayı bitki ve hayvanların karmaşık hücre yapısını araştırmaya yönelik moleküler biyolojik çalışmalarda kullanımı yaygındır.

Fermentasyon yapabilme yeteneğinden dolayı *S.cerevisiae* bira, şarap ve ekmek yapımında çok eski çağlardan beri kullanılmaktadır. Bu yaygın kullanım *S. cerevisiae'*yi ticari olarak önemli hale getirmiştir. Bu organizma son zamanlarda biyoetanol ve biyoyakıt üretiminde de kullanılmaktadır.

S. cerevisiae hamur mayalayabilme yeteneğinden dolayı ekmek yapımında da kullanıldığı için, endüstriyel alanda ekmek mayası olarak adlandırılmakta ve kullanılmaktadır. Uzun süreli muhafazası için dondurulduğunda ise, hücrelerin donma-erime stresine maruz kaldığı bilinmektedir. Bu stres koşulu, oldukça karmaşık hücresel tepkilere yol açmaktadır.

Bu çalışmada haploid *S. cerevisiae'*nin CEN.PK113-7D (MAT a) adlı laboratuvar suşu ve R625 adlı poliploid endüstriyel suşları ve bunlardan önceki çalışmalarda elde edilmiş olan donma-erime stresine dirençli mutant suşları F1 ve P8 kullanılmıştır. Önceki çalışmalarda bu mutant suşların elde edilmesinde öncelikle mutajenik bir ajan olan etil metan sülfonat (EMS) kullanılmış ve bu sayede donma-erime stresine dirençli bir başlangıç popülasyonu elde edilmiştir. EMS ile mutajenize edilmiş bu başlangıç popülasyonu ise tekrar eden donma-erime streslerine maruz bırakılmış ve fenotipik olarak donma erime stresine en dirençli suşlar olan F1 (laboratuvar suşu) ve P8 (endüstriyel suş) seçilmiştir.

Donma-erime stresine dirençli mutant bireylerin genetik altyapısının anlaşılabilmesi amacıyla, F1adlı mutant laboratuvar suşunun tüm genom transkriptomik analizleri daha önceden yapılmış ve bu amaçla DNA mikroarray tekniği kullanılmıştır. Elde edilen mikroarray sonuçları GeneSpring GX 9.0 yazılımı kullanılarak analiz edilmiştir. F1 suşuna ait olan mikroarray verileri, bu yazılım ile yaban tipe normalize edilmiştir.

Bu tez çalışması için ise mikroarray verilerinden yararlanılmış ve F1'de anlatımı en fazla artmış olan *HSP12, FMP45, HSP26, HXK1* genleri ve anlatımı en fazla azalmış olan *PHO84, NSR1*, *ZRT1* genleri çalışma için seçilmiştir. Donma erime stresi ile ilişkili olabileceği düşünülen bu genlerin bağıl transkripsiyon değişimleri F1 ve P8 de kontrol ve donma-erime stresi koşullarında çalışılmıştır.

Bağıl transkripsiyon analizleri için qRT-PCR tekniği kullanılmıştır. *S. cerevisiae* hücreleri -80º C'den alınıp 30ºC'de kültürlendikten sonra kültürün OD_{600} değerinin 1'e ulaşması beklenmiş ve ardından RNA izolasyonu yapılmıştır. RNA izolasyonunun ardından cDNA sentezlenmiş ve gerçek zamanlı PCR yöntemi uygulanmıştır. Uygulanan gerçek zamanlı PCR yönteminde ise 'SYBR Green' boyası kullanılmıştır.

Özet olarak, çalışmada haploid laboratuvar suşu '905've onun-donma erime stresine dirençli mutant suşu 'F1', endüstriyel suş R625 ve onun donma erime stresine dirençli mutant suşu 'P8' kullanılmıştır. Donma erime stresine direnç fenotipinin altında yatan genetik faktörlerin araştırılması amacıyla *HSP12, FMP45, HSP26, HXK1 PHO84*, *NSR1* ve *ZRT1* genlerinin transkriptomik analizleri yapılmıştır. Analizler hem kontrol hem de stres koşulları için ayrı ayrı gerçekleştirilmiştir.

Yapılan analizler, laboratuvar suşunda ve endüstriyel suşta aynı genler için gen anlatım seviyelerinin farklı olabileceğini göstermiştir. Ayrıca, farklı zamanlarda fakat aynı koşullarda belirlenen gen anlatım seviyelerinin mikroarray ve qRT-PCR'da farklı olabileceği görülmüştür.

Elde edilen sonuçlar, incelenen genlerde laboratuvar suşu ile endüstriyel suş arasında anlatım düzeyi farklılıkları olabildiğini göstermiştir. Bu durumun endüstriyel *S.cerevisiae* suşlarının poliploid olmasından kaynaklanabileceği düşünülmektedir. Aynı zamanda sonuçlar, F1 suşuna ait mikroarray ve qRT-PCR anlatım düzeyi değerlerinde de farklılıklar olabileceğini göstermiştir. Bu durum da iki farklı analiz yöntemi arasında qRT-PCR lehine bir hassasiyet farkı olmasıyla açıklanabilir.

1 INTRODUCTION

1.1 Purpose of Thesis

The aim of the present study was to determine expression levels of the freeze-thaw stress related genes in a laboratory and industrial strain of *Saccharomyces cerevisiae* and their freeze-thaw resistant mutants by Quantitative Reverse Transcriptase PCR, and to compare the results with those obtained previously for the freeze-thaw stress-resistant laboratory strain by DNA microarray analysis.

1.2 *Saccharomyces cerevisiae* as a Model Organism

Saccharomyces cerevisiae, the baker's yeast, is a small, unicellular fungus (Figure1.1). Unlike more complex eukaryotes, it can be grown on defined media, under completely controlled environmental conditions (Feldmann, 2005). *S.cerevisiae* has been the model organism for many molecular biology studies, because of the similarity between yeast and complex eukaryotes, including mammals, in the basic cellular processes like replication, recombination, cell division and metabolism (Schneiter, 2004).

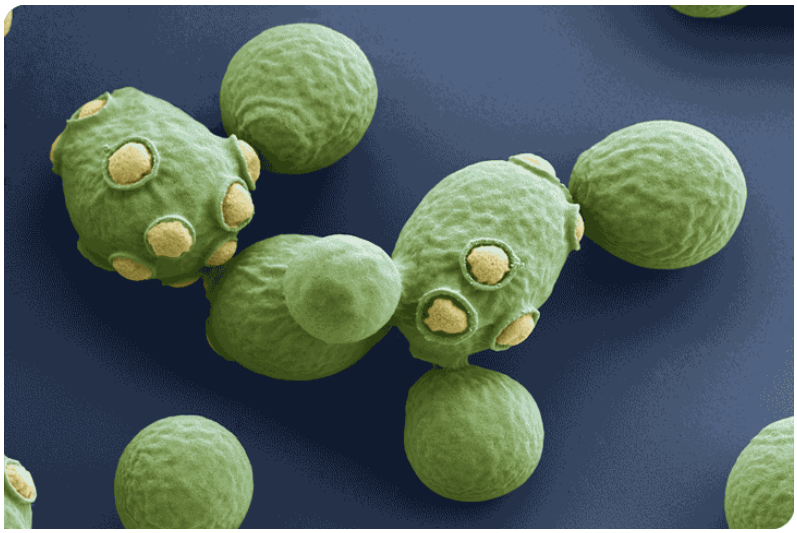

Figure 1.1 : Scanning Electron Microscope (SEM) Image of *Saccharomyces cerevisiae* (Url-1)

S. cerevisiae reproduces both sexually or asexually by budding, direct division or growing as simple irregular filaments (Schneiter, 2004**).** Mother cell multiplies by budding to a daughter cell which has a completely new cell surface (Herskowitz, 1988). The yeast divides asymmetrically to form a mother cell and a bud (Gershon, 2000).

During the exponential phase of growth, doubling times of laboratory haploid strains are approximately 90 minutes in complex YPD medium and approximately 140 minutes in synthetic media, at the optimum temperature of 30°C (Schneiter, 2004).

Unlike many other microorganisms, *S. cerevisiae* has both a stable haploid and diploid state (Figure1.2.). It has three mating types; MAT a (haploid cells), MAT α (haploid cells) and MAT a/α (diploid cells). *S. cerevisiae* is unicellular organism, but it can be present in three different cell types which has important roles in the *S. cerevisiae* life cycle (Herskowitz *et al.*, 1988). Vegetative proliferation occurs via mitotic cell division. MAT a, MAT α and MAT a/α cells can have mitotic cell proliferation. In sexual proliferation, haploid MAT a and MAT α cells fuse to generate diploid MAT a/α cells. MAT a/α cells do not have the ability to mate, however, they can undergo meiotic division to form haploid cells (Astell *et al.*, 1981).

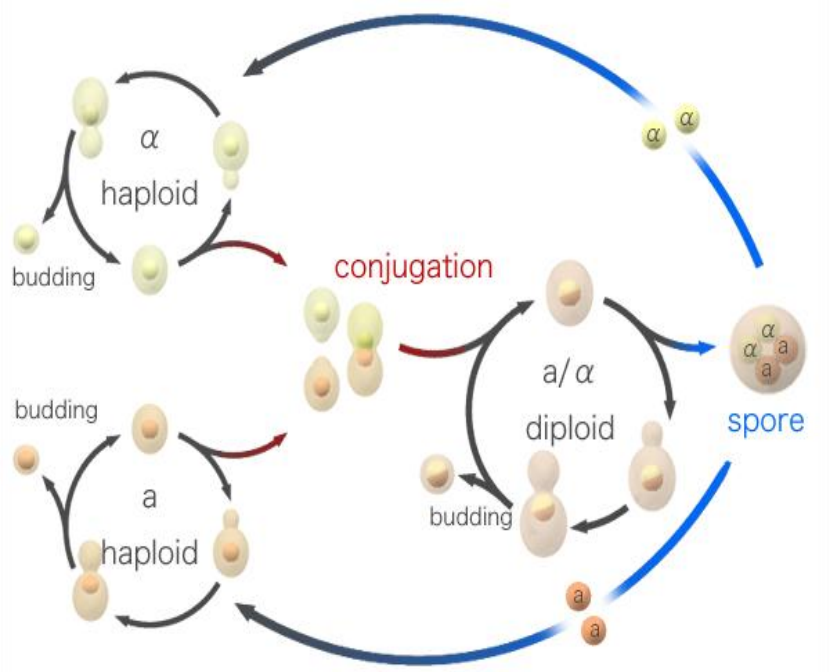

Figure 1.2: Life cycle of *S. cerevisiae* (Url-2)

S. cerevisiae can be maintained as either heterothallic or homothallic life cycle (Figure 1.3). Homothallic strains can switch their mating type which causes diploid progeny that is capable of undergoing meiosis. In a heterothallic life cycle, the strains can be maintained as diploids and haploids (Herskowitz, 1988; Schneiter, 2004**).** However, heterothallic life cycle is preferred in laboratory haploid strains, whereas natural and domesticated industrial yeast strains show homothallic life cycle (Zeyl, 2009; Perez-Torrado *et al*., 2010).

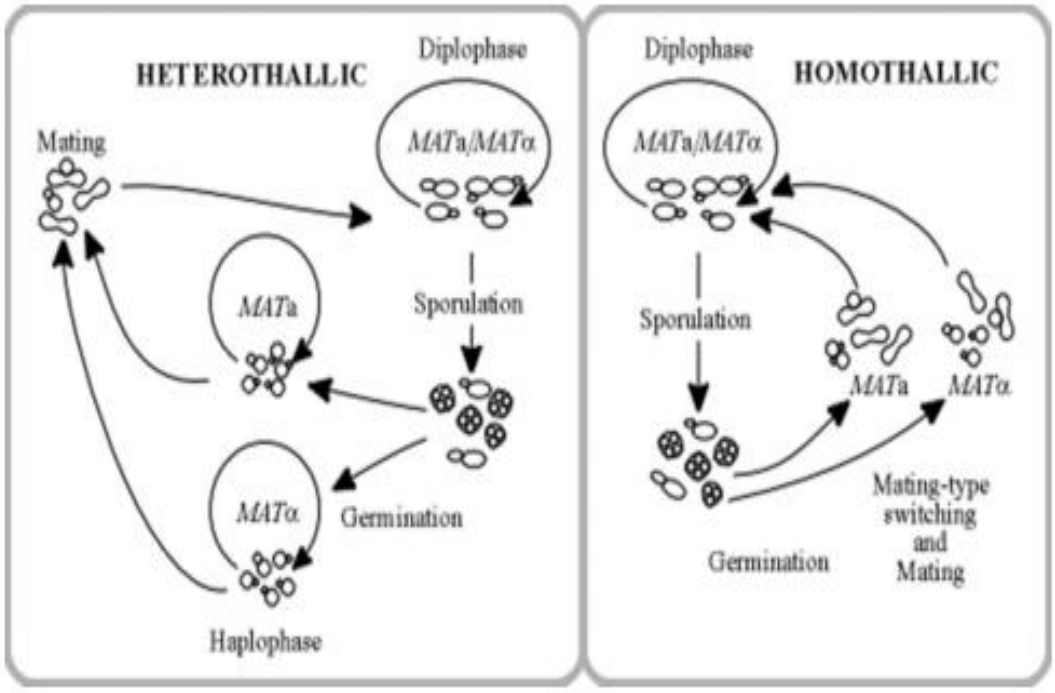

Figure 1.3: Life cycles of heterothallic and homothallic strains of *S. cerevisiae* (Schneiter, 2004)

Morphologically, *S. cerevisiae* cells are round and have a diameter of approximately 5-10 μm and the genome includes 16 chromosomes in haploid form, consisting of DNA approximately 12.5 mega base pairs long (Goffeau, 1996; Link and Olson, 1991). In addition, *S. cerevisiae* is the first eukaryotic organism whose genome was completely sequenced (Nevoigt, 2008).

Despite using the yeasts for baking, brewing, and wine making since the ancient times, the tiny "animalcules" existence was found by van Leeuwenhoek in 1680. The fermentation ability of yeast was then recognized by Louis Pasteur in 1866 (Gerald, 1990). Research activities have focused on *S. cerevisiae* as a result of its common

usage in traditional biotechnological processes such as baking, brewing, wine making, and also the biochemical and genetic characteristics of yeast.

Chronology	Milestones
6000-2000 BC	Brewing (Sumeria, Babylonia)
1680	Yeast under the microscope (van Leeuwenhoek)
1835	Alcoholic fermentation associated with yeast
1837	Name (S.cerevisiae) created for yeast observed in malt
1839	Sugar as a food source for yeast growth
1857	Fermentation correlated with metabolism (Pasteur)
1876	'Etudes sur la levure de bière' (Pasteur)
1877	Term "enzyme" (in yeast) introduced (Kühne)
1880	Single yeast cells and pure strains for brewing (Hansen)
1883	Alcohol and CO2 by cell-free extracts (Buchner)
1915	Production of glycerol
1920	Yeast physiology reviewed
1949	First genetic map (Lindegren); mating type system
1930-1960	Yeasts' taxonomy by Kluyver
1966	First tRNA structures from yeast
1978	First transformation of yeast (Hinnen, Hicks & Fink)
1990-1994	First commercial pharmaceutical products from recombinant yeast (Hepatitis B vaccine)
1990-1996	Completion of the yeast genome project

Figure 1.4: The chronological milestones of *S. cerevisiae* (Hernández-López, 2007)

The chronological milestones of *S. cerevisiae* in the life of humankind are shown in Figure 1.4.

1.3 Industrial Use of Yeast

The yeast *S. cerevisiae* has been used by mankind for thousands of years for winemaking, baking and brewing (Heer, 2009). *S. cerevisiae* and related strains and species are commonly used and commercially important microorganisms. *S. cerevisiae* can also ferment the sugars in the baking industry, and is used for leavening of dough, and producing alcoholic beverages.

Yeasts are also a rich source of vitamin B, niacin, and folic acid, and are also used as a vitamin supplement (Schneiter, 2004). Furthermore, *S. cerevisiae* and other yeasts are used for industrial and medical applications beneficial to human life (Feldmann, 2005).

Saccharomyces carlsbergensis is used in brewing industry, in the production of beers, including lager. In brewing process, *S. carlsbergensis* is used for bottom fermentation and *S. cerevisiae* is used for top fermentation. Recently in modern

brewing, original top fermentation strains can be modified and used for bottom fermenters (Schneiter, 2004).

Yeasts have also been used in the complex process of winemaking since ancient times. The role of winemaking yeast is to catalyze conversion of grape juice into wine by transforming the grape sugars to carbon dioxide ethanol, and other metabolites. This process includes variety of microorganisms such as fungi, yeasts, lactic acid bacteria, acetic acid bacteria, and also grape-associated microorganisms like mycoviruses and bacteriophages (Fleet, 1993). Yeasts are critical in biochemical interactions betweeen different types of *Vitis vinifera* and other grape species. (Pretorius, 2000)

The origin of bread predates recorded history, with suggestions that bread was baked in Egypt as early as 10000 BC (Wood, 1989). It has been produced by human from dawn of civilization and is one of the most common foods in many societies (Jenson, 1998). The functions of the yeast in bread baking are basically as follows: (I) to ferment sugar and raise the dough volume by carbon dioxide generation. The carbondioxide accumulates and causes expansion of dough (Schneiter, 2004). (II) to provide dough texture and structure. (III) to give a special flavor to the bread (Shima and Takagi, 2009).

The fermentation ability is not special to *S. cerevisiae*. Yeast strains such as *Issatchenkia orientalis, Pichia membranaefaciens* and *Torulaspora delbrueckii* also have dough leavening ability (Almeida, 1996). Besides, one of the *T. delbrueckii* strains, PYCC5321, has both freeze resistance and osmotolerance. Hence, it is convenient to use it in the frozen dough industry (Almeida, 1996). When *T. delbrueckii* cells are compared to *S. cerevisiae*, the *T. delbrueckii* cells have smaller size. For that reason, the small cells cause clogging in the filtration process before packaging. Sasaki and Oshima (1987) tried to solve this problem by diploidization of *T. delbrueckii.* On the other hand, this diploidization might cause loss of some features for technological applications, especially those related to stress (Hernández-López *et al*., 2007).

1.4 Stress Resistance and Freeze-Thaw Stress for *S. cerevisiae*

All living cells are exposed to various stress conditions and suffer from internal or external hazards. Consequently, to repair damages and eliminate the damaged

components, living systems have developed stress response strategies. The adaptive stress responses maintain cellular homeostasis (Arya , 2007).

In biotechnological applications, yeast cells are exposed to various changes in the environmental conditions that cause stress, such as oxidative, osmotic and thermal stress, in the cell. Prolonged exposure to stress may help cells develop stress tolerance. These stress responses are developed based on the activity of repair functions, transcriptional and posttranslational control, cell sensing, protein-targeting to organelles, signal transduction, accumulation of protectants (Attfield *et al*., 1997).

Stress resistance is a desirable feature for microorganisms which are used in biotechnological processes (Heer, 2009). Baking-associated stresses, such as oxidative stress, air-drying stress, high sugar concentration and freeze-thaw stress, show up during the bread making process when production of baker's yeast or fermentation of dough.

Cryopreservation is one of the long-term storage methods which is mostly used in food industry, medical, agricultural technology and scientific studies, for a variety of applications such as organ preservation, cryosurgery and strain and product preservation. Freeze-thaw stress resistance is desirable for these kinds of technologies as freeze-thaw stress may cause lethal damage to cells. Under freeze-thaw stress, cells are exposed to multiple stress types, such as ice crystal formation, osmotic stress, cold stress, dehydration in freezing and oxidative stress. Cells can be damaged physically during freezing by ice crystal formation and dehydration, and biochemically during thawing, as reactive oxygen species are formed (Odani *et al*., 2003; Park *et al*., 1997).

Although yeast cells have higher survival rates when frozen at -80 °C rapidly, they are generally stored at -20 ° C for preservation in frozen dough industry. Storage at -20°C for commercial products leads to lethal damage because the membrane integrity is disturbed by ice crystal formation and high osmotic pressure (Odani, 2003).

The molecular mechanism of freeze-thaw stress tolerance is not well-known, but previous studies showed that cellular factors and components are important for freeze-thaw tolerance in the cell. It was suggested that the freeze-thaw tolerance mechanism is related to growth phase, lipid composition of the membrane, respiratory metabolism, heat shock protein levels and accumulation of trehalose and glycerol (Tanghe *et al*., 2002; Park *et al*., 1997).

Environmental stress conditions during bread-making process are shown in Figure 1.5.

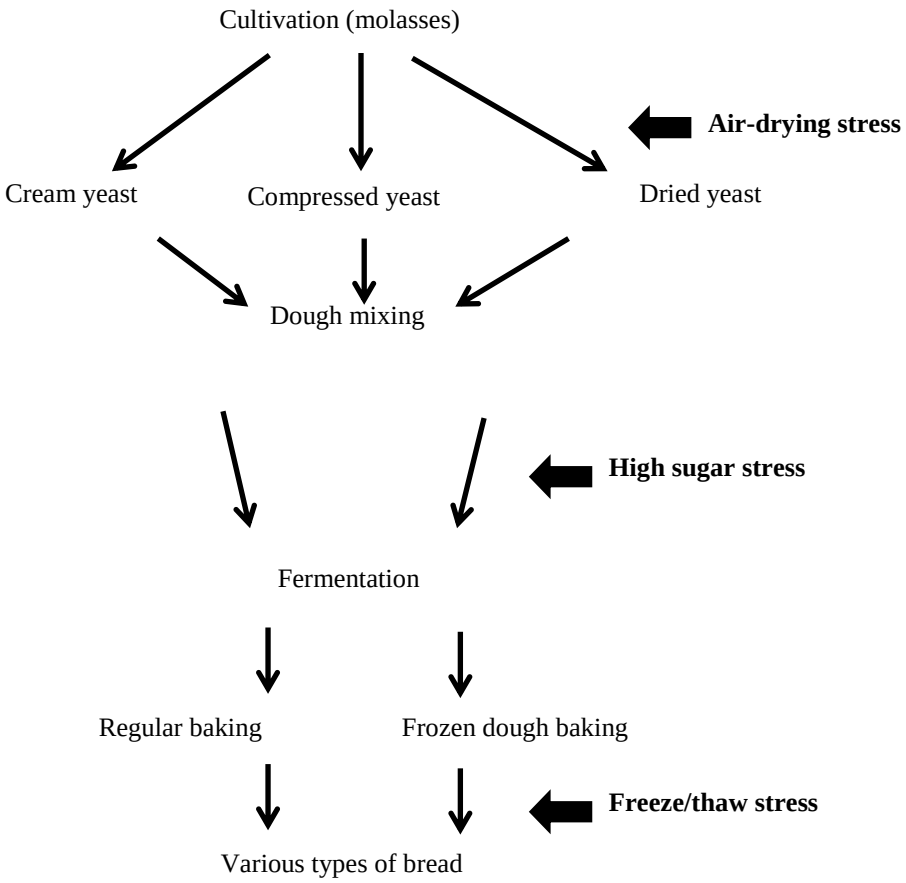

Figure 1.5: Environmental stress conditions during the bread-making process (Shima and Takagi, 2009)

1.5 *Saccharomyces cerevisiae* and *HSP12*

The most notable event among various changes in cellular activity upon stress is production of highly conserved proteins, the Heat Shock and Stress Proteins (Hsps). The Hsps are classified on the basis of their molecular weight. The heat shock genes are the genes family whose expressions are related to temperature changes and various stress conditions (Arya *et al*., 2007).

Hsp12 (YFL014W) is a plasma membrane protein that preserves membrane organization. It is positively regulated by the high osmolarity glycerol (HOG) pathway, and it is negatively regulated by Ras-protein kinase A (Ras/PKA) pathway. It exists associated to the plasma membrane and as soluble cytosolic protein. It

maintains the organization during a variety of stress conditions (Park *et al*., 1997; Welker *et al*., 2010).

HSP12 is expressed in small quantities in an exponentially growing culture. Expression of *HSP12* is induced during stationary phase and stress conditions. Hsp12 accumulates in *S. cerevisiae* when the cells are subjected to a stress like oxidative stress, glucose depletion, osmotic stress or heat shock. Phenotypic analysis demonstrated that, in order to survive during stress conditions, Hsp12 is an important protein (Welker *et al*., 2010).

In a previous study, interestingly, the null mutant of *HSP12* that was exposed to freezing stress, showed increased resistance to freezing when compared to the wild type strain. It was also shown that the intracellular trehalose concentration in the cell, which has impaired heat-induced trehalose accumulation, was higher in hsp12Δ cells during stationary phase (Pacheco *et al*., 2009).

As a result of comparative gene expression analysis, *HSP12* is a member of Environmental Stress Response (ESR). It has a specific gene expression profile due to sudden environmental changes (Gasch *et al*, 2002).

1.6 *Saccharomyces cerevisiae* and *FMP45*

YDL222C, which is the systematic name of *FMP45*, is an untagged integral membrane protein involved in sphingolipid metabolism that is localized to mitochondria and the cell cortex. It is also required for efficient sporulation (Young *et al*., 2002).

FMP45 has three paralogs, *SUR7*, *YLR414C* and *YNL194C* and their transcripts are integral membrane protein family members in *S. cerevisiae.* Most of the functional characteristics analyses were studied in *Candida albicans* (Wang *et al*., 2011).

The plasma membrane of the *S. cerevisiae* has two distinct domains. One of them includes proteins that readily diffuse, such as the plasma membrane ATPase Pma1. The other domain is immobile and looks like a punctate patch, and is named as membrane compartment occupied by Can1 (MMC). This immobile plasma membrane subdomain is the static site of the endocytosis which is named as eisosome. Sur7 and its paralogs *FMP45* are the transmembrane proteins localized to eisosomes/MMC in *Candida albicans* (Walther *et al*., 2006).

It is shown that triple mutants of *SUR7, FMP45* and YNL194C, cause a decrease in sporulation capacity and changes in the membrane composition (sphingolipid)

(Alvarez *et al*., 2008). In previous studies it was shown that the sphingolipid synthesis is downregulated during stress conditions to increase the lifespan of the yeast (Huang *et al*., 2012). It was also shown that YDL222C is induced during osmotic shock or pseudohyphal growth (Rep *et al*., 2000).

1.7 *Saccharomyces cerevisiae* and *HSP26*

Hsp26 is one of the cytosolic small heat-shock proteins which reveals 214 amino acids. It is best characterized member of the sHsps (Ferreira *et al*., 2006). Hsp26 has temperature-regulated chaperone activity. The molecular chaperone binds to unfolded substrate proteins and prevents them from forming large irreversible protein aggregate (Richter *et al*, 2005). It was found that null mutations in HSP26 cause abnormality in cell morphology (Haslbeck *et al*., 2004).

Hsp26p has activity only during stress conditions and the transcript levels of the *HSP26* could not be detectable in control conditions (Haslbeck *et al*., 2004). The transcription is induced by oxidative stress, low pH, nitrogen starvation, carbon starvation, cell cycle arrest, salt shock and heat shock. Upregulation of transcription is provided via transcription factors Hsf1p and Msn2p/Msn4p. Hsf1p and Msn2p/Msn4p are heat shock elements and stress elements which are bound to the *HSP26* promoter, respectively (Amorós, 2001). But it is also shown that deletion of *HSP26* does not cause temperature sensitivity. This deletion mutation does not affect the growth rate and survival at high temperatures under numerous conditions (Haslbeck *et al*., 1999).

Hsp26 is a component of the heat shock response. In *S. cerevisiae*, Hsp26 assembles into 24 oligomers. The oligomers are activated under heat shock and generate a large and stable complex. Hsp26 conformations are changed by heat shock and the conformational changes prevent the complex from binding substrate proteins (Richter, 2005).

In previous studies, it was found that the *HSP26* transgenic of *Arabidopsis thaliana* showed stronger growth than the wild type after freezing stress. Overexpression of Hsp26 caused increased amount of proline and soluble sugar, increased expression of stress defense genes and enhanced freezing tolerance (Xue, 2009).

1.8 *Saccharomyces cerevisiae* and *HXK1*

Hexokinase isoenzyme 1 (*HXK1*) is a cytosolic protein that is responsible for catalyzing glucose phosphorylation during glucose metabolism. During growth on non-glucose carbon sources, expression level is highest (Rodriguez, 2001). Phosphorylation of glucose is catalyzed by three enzymes, hexokinases Hxk1p and Hxk2p and glucokinase Glk1p, in intracellular glucose metabolism (Walsh *et al*., 1983). Although Hxk2p is the main protein in glucose phosphorylation during growth on glucose, all of the three proteins are responsible in the uptake of glucose (Bisson *et al*, 1983; Walsh *et al*., 1983).

During growth on fermentable carbon sources like glucose, fructose or mannose, *HXK2* gene is expressed. But if the carbon sources are changed to non-fermentable ones, *HXK2* gene is repressed, *HXK1* and *GLK1* genes are started to be expressed rapidly (Lobo *et al*., 1977; Rodriguez *et al*., 2001).

Hxk1p and Glk1p are localized in cytosol, whereas Hxk2p is found in both cytosol and nucleus. Hxk2p is responsible for glucose-induced repression of *HXK1* and *GLK1* genes and glucose-induced expression of itself in nucleus, while it is responsible for glycolysis in cytoplasm (Gancedo, 1998; Rodriguez *et al*., 2001).

Homologs of *HXK1*, *HXK2* and *GLK1* genes were identified in various organisms (Cho *et al.*, 2006; Petit, 1996). In human pancreatic cells, the functional homologs of these genes participate in regulation of insulin secretion and behave like a glucose sensor (Mayordomo, 2001).

1.9 *Saccharomyces cerevisiae* and *PHO84*

Phosphorus is a very important component in all living organisms which is involved in a variety of cellular structures as a biomolecule and to store the cellular energy for survival. It is very important for living organisms to maintain phosphate homeostasis. Therefore, a phosphate signaling and response pathway, known as PHO pathway, is evolved in baker's yeast. PHO pathway controls the phosphate levels in cytoplasm and regulates genes which are required for maintaining phosphate homeostasis (Tomar & Sinha, 2014) (Figure1.6).

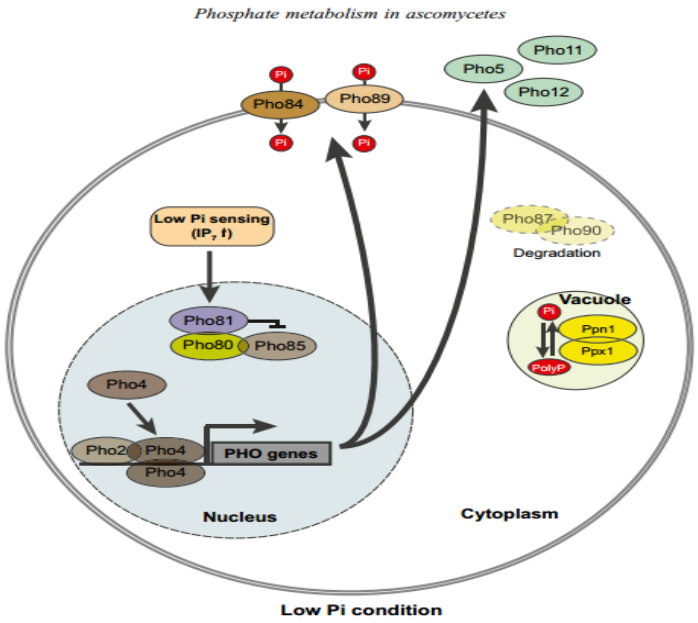

Figure 1.6 Phosphate metabolism in ascomycetes (Tomar & Sinha, 2014)

The product of the *PHO84* gene includes 596 amino acids and it also has sequence homology with sugar transporters. Transcription of the gene is regulated by Spt7p and Pho4p (Bun-Ya, 1991).

PHO84 gene encodes a protein which transports the inorganic phosphate across the plasma membrane in *S. cerevisiae.* Hence it is known as high-affinity phosphate transporter (Ofiteru *et al*., 2012). Previous studies showed that Pho86p and Pho87p are responsible in P_i uptake with Pho84p (Bun-Ya, 1996). The gene is upregulated during phosphate starvation (Wykoff, 2001).

The high affinity phosphate transporter takes charge in low affinity of heavy metal uptake. Pho84p is a low affinity heavy metal transporter (for Mn^{+2}, Cu^{+2}, Co^{+2} and Zn^{+2}), and is included in Mn^{+2} homeostasis in budding yeast (Ofiteru *et al*., 2012). Overexpression of Pho86p causes accumulation of the heavy metals. Yeast cells with *PHO84* deletion are more resistant to Mn^{+2}, Cu^{+2}, Co^{+2} and Zn^{+2}, and cause decrease in accumulation of metal-ion (Jensen, 2003).

1.10 *Saccharomyces cerevisiae* and *NSR1*

Nuclear signal recognition protein which is named as Nsr1 is a 44 kDa nuclear localization sequence-binding protein in *S. cerevisiae*. It has two RNA recognition motifs (RRM) (Gamberi, 1994). The cells which have disruption in the *NSR1* gene

showed that *NSR1* is involved in RNA processing at the rRNA maturation level (Lee, 1991).

Nsr1p recognizes the nuclear localization sequences which direct the proteins to the nucleus. It is structurally similar to mammalian nucleolin. One of the previous studies showed that although the structure was similar with mammalian nucleolin, expression of mammalian nucleolin in yeast cells that have *NSR1* mutation cannot alter the slow growth phenotype. It was also demonstrated in same study that, nucleolin is a nuclear localization sequence-binding protein like Nsr1p, and N-terminals of both proteins are recognized through nuclear localization sequences (Lee, 1992). The *NSR1* gene of *S. cerevisiae* and the structurally similar protein mammalian nucleolin are involved in pre-rRNA processing (Kondo, 1992).

NSR1 gene is not essential for cell viability but deletion of the gene causes defect in the pre-RNA processing, decreased production in 18 S rRNA and 40 S ribosomal subunits and slow growth (Kondo & Inouyes, 1992).

Several genes are induced during cold shock response in *S. cerevisiae. NSR1* is one of the cold shock inducible genes. It was demonstrated that basal transcript level of *NSR1* was detected at 30 Cº in the exponentially growing yeast cells. It was increased approximately 3-fold after cold shock to 10 Cº. After the cold shock, Nsr1 protein level also increased about 3-fold (Kondo *et al*., 1992).

1.11 *Saccharomyces cerevisiae* and *ZRT1*

Ion homeostasis for zinc in budding yeast is regulated through transcriptional regulation of zinc uptake system according to zinc levels of the cell. Two different zinc uptake systems were determined in *S. cerevisiae*. One of them is high affinity zinc transport system and its activity is increased in case of deficiency in zinc uptake systems. The second one is low affinity transport system and its activity is not increased when zinc is available in the cell (Zhao, 1996).

Both high and low affinity transport systems for zinc are regulated by zinc. Zinc-regulated transporter 1 (*ZRT1*), gene encodes the protein that is responsible for high affinity zinc uptake (Gitan, 1998). In zinc-limited cells, expression level of the zinc is increased more than 30-fold. *ZRT2* encodes the low affinity transport system.

ZRT1 shows sequence similarity with *IRT1* in *Arabidopsis thaliana.* The high affinity zinc transporter Zrt1p and the low affinity zinc transporter protein Zrt2p are

similar and they are members of Zrt- and Irt- Protein family, ZIP, which are heavy metal ion transporters (Eng *et al*, 1998) .

In response to intracellular zinc levels, Zap1 protein regulates transcriptional regulation of the *ZRT1* and *ZRT2* mRNA levels (Zhao, 1997). *ZRT1* levels are also controlled by regulated degradation. Zrt1p is located in plasma membrane in zinc-limited cells. When the cells are exposed to high zinc levels, Zrt1p is transferred to vacuole for degradation (Gitan *et al*., 1998).

1.12 Metabolic Engineering

Metabolic engineering was defined as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey *et al*., 1991).

Metabolic engineering is used for targeted and purposed alteration in the metabolic pathways in order to understand, utilize and improve cellular properties, and to use them in biotechnological process.

There are two metabolic engineering strategies, which are named as rational and inverse metabolic engineering (Figure 1.7). Rational metabolic engineering requires detailed genomic and metabolic knowledge of the organism of interest. To overcome this limitation, inverse metabolic engineering is used as a "bottom-up" approach, as an alternative strategy (Bailey *et al*, 1996).

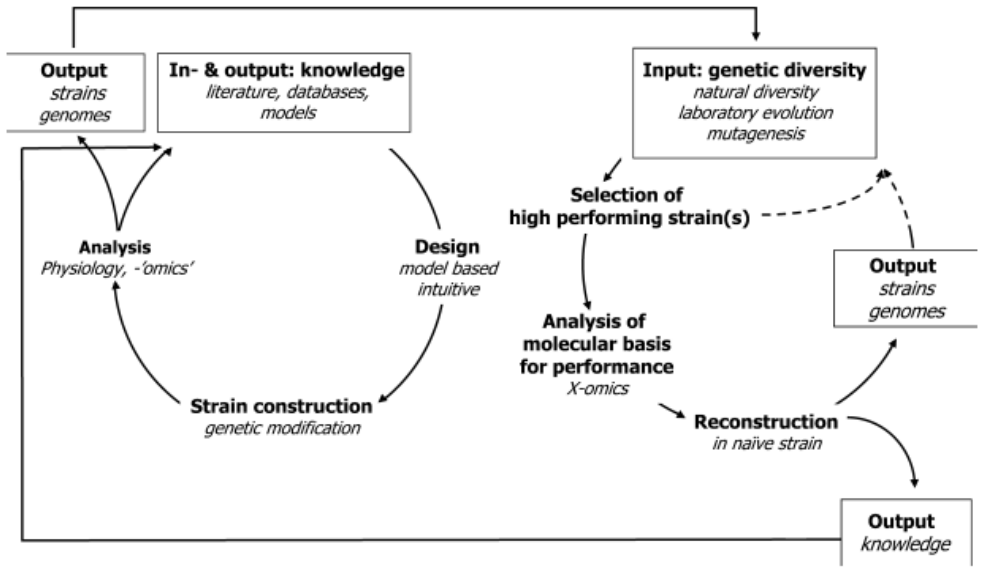

Figure 1.7: Rational and inverse metabolic engineering (Oud *et al*., 2012).

1.12.1 Inverse metabolic engineering

The aim of inverse metabolic engineering is to obtain mutant strains with desired phenotype and then to characterize their properties by using high throughput systems. The main advantage of this approach is that it does not require extensive knowledge of the organism of interest. This method is based on "natural design principles" (Nevoigt, 2008).

1.12.2 Evolutionary engineering

Evolutionary engineering is an inverse metabolic engineering strategy which is based on selection of the desired phenotype by continuous evolution procedures. To obtain a highly stress-resistant strain by evolutionary engineering, the starting point is physical or chemical mutagenesis that is followed by cultivation that provides selective conditions such as high and low temperatures, high salt, or metal concentrations. This random, non-targeted, combinatorial, and repetitive evolution process ensures to obtain highly resistant mutants.

1.13 Quantitative Reverse Transcriptase PCR (qRT-PCR)

Quantitative reverse-transcriptase PCR (qRT-PCR) is a technique that is used to quantitatively determine mRNA expression levels of samples. The basis of the method is monitoring and quantifying the fluorescent emission of the PCR products in each PCR cycle. It enables to determine the amount of PCR products during an ongoing PCR reaction.

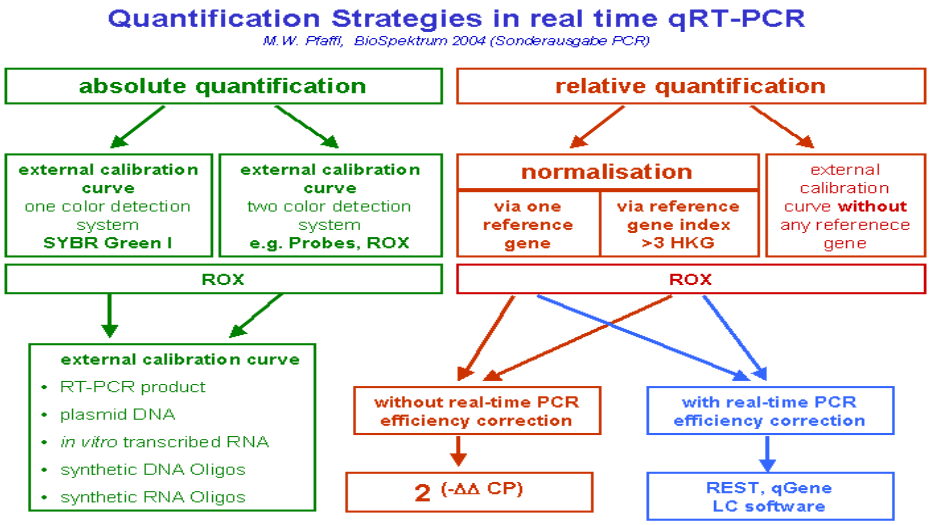

Figure 1.8: Quantification strategies in qRT-PCR (Pfaffl, 2004)

There are two types of quantification methods in qRT-PCR (Figure1.8). One of them is relative quantification that is based on relative expression of target gene versus a reference gene. "Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at the time zero in a time-course study" (Livak and Schmittgen, 2001). To determine the physiological change in gene expression, relative quantification is sufficient for that purpose. Generally, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), β–actin and 18S RNA are mostly preferred eukaryotic reference genes or housekeeping genes. Relative fold change method is usually used for analyzing the differences in gene expression. The other quantification methods is absolute quantification. The absolute quantification is based on an internal or external calibration curve. The method allows calculation of the amplicon sizes by comparing the PCR signal to a standard curve (Schmittgen and Livak, 2008).

Messenger RNA quantification can be done in a variety of ways like serial analysis of gene expression (SAGE), Northern blotting and RNA protection. All of these methods are still used, but reverse transcription PCR and microarray analysis became the most popular methods (Sinicropi, 2006). The qRT-PCR is a semi-quantitative and labor-intensive method and it allows to analyze only one or a very few genes for each assay.

Both methods have pros and cons, when we compared qRT-PCR to microarray. In the case of small differences in gene expression, qRT-PCR is more sensitive. In addition, qRT-PCR is rapid, accurate and has low sample requirements. The costs of both methods depends on the number of the targeted genes; the qRT-PCR is much less expensive if the experiments is aimed only for a few genes, but microarray is cost-effective if many genes or whole genome analysis is aimed to assessed. Moreover, microarray has the advantage of high standardization. The gene expression levels that are reported by microarrays should be analyzed by an independent method and qRT-PCR can be used for that purpose.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strain

Saccharomyces cerevisiae haploid strain CEN.PK113-7D (MAT a) which is called *'*905' was used in this study. The strain was kindly provided by Dr. Laurent Benbadis from Toulouse University, INSA-Toulouse Research Center.Assoc.

Prof. Dr. Mustafa Turker provided *Saccharomyces cerevisiae* R625 polyploid strain from Pakmaya Industries.

Freeze-thaw stress resistant mutant strains of 905 and R625, which are called 'F1'and 'P8', respectively, were obtained previously by evolutionary engineering (Ulku Yilmaz, Ph. D. Thesis, ITU, 2013). Briefly, freeze-thaw stress was applied ten times to the chemically mutagenized population of 905 and R625.The mutant generations were obtained. According to the results of phenotypic, physiological and genetic analyses, mutant individuals F1 and P8 were selected for detailed analysis.

2.1.2 Yeast cultivation media

Media were prepared as described in Table 2.1 and Table 2.2. Cultivations of the cells were done in Yeast Minimal Medium (YMM) at 30 °C and 150 rpm in a shaker. Yeast Nitrogen Base Medium (YNB) was used for washing the cells before RNA isolations. All media were autoclaved after preparation, at 121°C and 1.2 atm. pressure.

2.1.2.1 Yeast minimal medium (YMM)

The composition of Yeast Minimal Medium (YMM) was given in Table 2.1. YMM was used for regular growth and stress application cultivations.

Per liter of distilled water;

Table 2.1: Composition of YMM.

Components	Quantity
D-Glucose	20 g
Yeast Nitrogen Base (YNB) without amino acids	6.7 g

2.1.2.2 Yeast nitrogen base medium (YNB)

The composition of yeast nitrogen base medium (YNB) was given in Table 2.2.

Per liter of distilled water;

Table 2.2: Composition of YNB

Components	Quantity
Yeast Nitrogen Base (YNB) without amino acids	6.7 g

2.1.3 Laboratory equipment and kits

Table 2.3: The list of laboratory equipment

Laboratory Equipment	**Supplier**
Autoclave	Tomy SX 700E (China)
Scale	Precisa BJ 610C (Switzerland)
Benchtop Centrifuge	Eppendorf 5424 (Germany)
Laminar Flow	Biolab Faster BH-EN 2003 (Italy)
LightCycler 480 II	Roche (Switzerland)
Nano Drop 2000	Thermo Scientific (USA)
Orbital Shaker Incubators	Certomat S-2 Sartorius (Germany)

Refrigerators	+4 °C Arcelik (Turkey) -20 °C Arcelik TM-1280 (Turkey) -80 °C Sanyo (Japan)
Thermal Cycler	BioRad T100™ Thermal Cycler
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan)
Vortex Mixer	Nüve NM 110 (Turkey)

Table 2.4: The list of kits

QiagenRNeasy Mini Kit (Cat: 74104)	Qiagen, USA
LightCycler ® 480 SYBR Green I Kit	Roche (Switzerland)
Transcriptor First Strand cDNA Synthesis Kit (Cat. No. 04 707 516 001)	Roche (Switzerland)

Table 2.5: The list of softwares

Primer3Plus	http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi
S. cerevisiae Genome Database	http://www.yeastgenome.org

2.2 Methods

2.2.1 Cultivation of *Saccharomyces cerevisiae*

S. cerevisiae was cultivated at 30 ˚C and 150 rpm for each cultivation.

2.2.2 Primer design

The specific primers were designed for genes of interest by "Primer3 Plus Software" and the primers were checked by "*Saccharomyces* Genome Database". *HSP12*, *FMP45*, *HSP26*, *HXK1*, *PHO84*, *NSR1* and *ZRT1* genes were studied for transcriptomic analysis. The qRT-PCR-specific primers are listed in Table 2.6.

The specific primers were designed to amplify maximum 200 bp product. As the housekeeping gene, β-actin gene (*ACT1*) was used, and the primer concentrations for each study were 10 mM.

Table 2.6: The list of primer sequences

Gene Name	***Primer Sequence***	
	Forward (5'-3')	Reverse (5'-3')
HSP12	CGCAGGTAGAAAAGGATTCG	AAAGGCAAGGATAACGCTGA
FMP45	CTGGTTGGCCTTTTTCTTCA	TACCCACAAGGCCCACTCTA
HXK1	TGGGTGAATTGTTGCGTCTA	TGTCAAGACCACTCTGCCAG
PHO84	CATCATGGGTGCTGTCTTTG	CGTTCTAGGGTTGGCATGTT
ZRT1	ACCAGATACGTCAGCGGTTC	TGCTGGTATCATGGCTTTGA
HSP26	CCATCTGGTTTCGGTTTCCC	AGGTCAAGGTCAAGGAGAGCA
NSR1	AAGCTAAGGCCGTCTCTTCC	CGCTGTCAGAAGACGATGAA

2.2.3 Total RNA isolation from yeast cells

Laboratory and industrial strains (905, R625) and their freeze-thaw resistant mutants (F1, P8) cultures were inoculated in culture tubes containing 10 mL fresh YMM, and incubated overnight at 30 °C and 150 rpm shaker. After overnight incubation, OD_{600} of the cultures were adjusted to 0.5, and the cultures were incubated at 30 °C in a shaker at 150 rpm speed. When OD_{600} values reached at the OD_{600} value of 1.0 (~$5x10^7$ cells/ml), one mL from each culture was transferred to 2 mL microcentrifuge tubes. As control group and stress group, two sets of each cultures were prepared. The cells were centrifuged at 12000 rpm for 5 min and the supernatant was discarded. Cells were washed twice with YNB (YMM without dextrose). The control groups' RNA isolations were started and at the same time stress groups were exposed to freeze stress at -20 °C freezer for 120 min. The stress

groups cells were then thawed for 10 min at 30 °C. This freeze-thaw cycle was repeated twice. After two cycles of freeze-thaw stress application, the stress groups total RNA isolation was performed, too. "Qiagen RNeasy Mini Kit" (Cat: 74104, Qiagen, USA) was used to isolate total RNA from yeast cells.

Before starting RNA isolation, some solutions were freshly prepared as follows: per 1 ml Buffer RLT, 10 µl B-Mercaptoethanol (β-ME) was added. Only for first use, 4 volumes of ethanol (96-100 %) was added to buffer RPE and working solutions were obtained. Enzymatic lysis buffer Y1 was prepared, which contained 1 M sorbitol, and 0.1 M EDTA, pH 7.4. Just before using, 0.1 % β-ME and lyticase were added to Y1 solution.

Firstly, the cells were centrifuged at 12000 rpm for 5 minutes and the supernatants were discarded and pellets were washed with YNB Buffer (Table 2.2) twice. Cells were then centrifuged at 1000 x *g* for 5 min and the supernatants were decanted and any remaining media was carefully removed. For enzymatic lysis, the pellet was resuspended in 1.5 ml of freshly prepared Buffer Y1, and incubated for 30 min at 30 °C in the shaker to disrupt cell walls. Spheroplasts were centrifuged at 300 x *g* for 5 min and the supernatant was discarded. 350 µl Buffer RLT was added and the samples were vortexed vigorously. 350 µl 70% (v/v) ethanol was added to homogenized lysate and mixed well by pipetting. All samples were transferred to an RNeasy spin column in 2 ml collection tubes and centrifuged for 15 sec. at 8000 x *g* and flow-throughs were discarded. In order to wash the spin column membranes, 700 µl of Buffer RW1 were added to each RNeasy spin columns and centrifuged for 15 sec. at 8000 x *g*. The flow-through was discarded. Then 500 µl Buffer RPE were added to columns and centrifuged for 15 sec at 8000 x g, and the flow-through was discarded. 500 µl Buffer RPE was added to columns again, for the second time, to wash the spin columns, but the centrifugation was done for 2 min at 8000 x *g* at the end of second washing. After that, the RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at 15000 x *g* for 1 min. At the last step of isolation, the RNeasy spin column was placed in a new 1.5 ml collection tube and 40 µl RNase-free water was added directly to spin column membrane and centrifuged for 1 min at 8000 x *g*.

Each sample's RNA concentrations (µg/µl) were measured using NanoDrop 2000 (Thermo SCIENTIFIC, USA). Additionally, 260/280 and 230/280 absorbance ratios were also determined by Nanodrop. The total RNAs were stored at -80 °C.

2.2.4 cDNA synthesis

cDNA synthesis from total RNA sample was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Cat. No. 05 081 955 001). The volumes were determined according to lowest RNA amount between the samples. The calculations were done and the RNAs were adjusted to 12 µl final volume. Then 1 µl Anchored-oligo $(dT)_{18}$ Primer was added to each sample. The template-primer mixtures were denatured by heating the tube for 10 min at 65 °C in a thermal cycler (BioRad T100™ Thermal Cycler). The tubes were held on ice after this stage. Master mixes were prepared for every sample as indicated in Table 2.7. Seven µl of Reverse Transcriptase (RT) master mix was added to each sample to a final volume of 20 µl. qRT-PCR was performed at 55 °C for 30 min and the enzyme was inactivated by heating to 85 °C for 5 min in a thermal cycler. The reactions were then stopped by placing the tubes on ice.

The cDNA master mix composition is shown in Table 2.7.

Table 2.7: cDNA master mix composition

Components	**Volume for 1 reaction (µl)**
Transcriptor Reverse Transcriptase Reaction Buffer	4.00
Protector Rnase Inhibitor	0.50
Deoxynucleotide Mix	2.00
Transcriptor Reverse Transcriptase	0.50

2.2.5 qRT-PCR Experiments

For the expression determination of *ACT1, HSP12, FMP45, HSP26, HXK1, PHO84, NSR1* and *ZRT1* genes in 905, F1, R625 and P8, Quantitative Reverse Trasncriptase PCR (qRT-PCR) was used with Roche-LightCycler® 480 DNA SYBR Green I Master Kit (Cat. No. 04 707 516 001) and Roche LightCycler® 480 equipment. qRT-PCR was performed according to Table 2.8.

To obtain a standard curve, 1:5, 1:50, 1:500 serial dilutions from wild types (without stress) were prepared. The other samples were also diluted (1:5). Ten µl of LightCycler®480 SYBR Green I Master Mix and 1 µl from each forward and reverse

primer were prepared in different tubes for each genes as qPCR mix. Twelve µl of qRT-PCR master mix were inoculated for each well on 96 well plates.

Table 2.8: Light Cycler operating conditions

	Temperature (°C)		Time	Cycle
Pre-incubation	95		10 min	1
Amplification	Denaturation	95	10 sec	45
	Annealing	56	18 sec	
	Elongation	72	20 sec	
Melting	95		5 sec	1
	65		1 min	
	97			
Cooling	40		10 sec	1

For one set of reaction, seven wells in 96-well plates were used; three of the wells were prepared for 1:5, 1:50, 1:500 dilutions for the wild type, one well for 1:5 dilution of the wild type with stress application, one well for 1:5 dilution of the stress-resistant sample without stress and one well for 1:5 dilution of stress-resistant sample with stress application, and the last well was used as a negative control. After inoculation of 12 µl of qPCR master mix, 8 µl cDNA sample was added to corresponding wells except for the negative control. For the negative control, 8 µl RNAse-free dH_2O was added instead of the cDNA sample. (Table 2.9)

Table 2.9: qRT-PCR reaction mix composition

PCR Components	Volume
PCR Primers (F+R)	2 µl
SYBR Green I Master Mix	10 µl
Template	8 µl
Total Volume	20 µl

3 RESULTS

3.1 Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Expression level analysis of of freeze-thaw stress-related genes was performed by qRT-PCR according to the protocol of DNA SYBR Green I Master (Roche) kit by using Roche-LightCycler® 480. The expression levels of these genes were calculated according to the $2^{(-(\Delta Ct))}$ method (Pfaffl *et al*., 2004; Livak *et al*., 2001).

Expression levels of freeze-thaw stress related genes *HSP12, FMP45, HSP26, HXK1, PHO84, NSR1* and *ZRT1* were studied under control and freeze-thaw stress conditions. Relative quantification was used for analyzing the data such that the relative expression levels of these genes were normalized to wild type gene expression levels at normal growth conditions. The experiments were performed three times in order to calculate the standard deviations.

3.1.1 Expression levels of freeze thaw stress related genes *HSP12, FMP45, HSP26, HXK1, PHO84, NSR1* and *ZRT1* in 905 and F1

When the OD_{600} values reached 1, total RNA was extracted from wild type and the mutant F1 control group. Before total RNA extraction, stress groups were exposed to freeze-thaw stress. After obtaining total RNA, cDNA was synthesized to be used as a template for qRT-PCR. Total RNA and cDNA amounts were measured with Nanodrop 2000.

qRT-PCR analysis was repeated three times and their $2^{-\Delta\Delta CT}$ values were calculated in order to identify the relative expression levels of the genes as fold-changes (Livak, 2001). Beta actin (*ACT1*) was used as the housekeeping gene. Relative expressions profiles of related genes were normalized to wild-type gene expression levels at normal growth conditions.

Efficiency and error values of the target genes are shown in Table 3.1. The values were within the acceptable limit range (Url3). The efficiency values were between 1.8 and 2.0.

Table 3.1: Error and efficiency values for qRT-PCR in 905 and F1.

Genes Names	***HSP12***	***FMP45***	***HXK1***	***HSP26***	***PHO84***	***ZRT1***	***NSR1***
Error	0.06447	0.03325	0.01981	0.03917	0.05942	0.03947	0.05263
Efficiency	1.88733	1.95933	1.94500	1.93056	1.92067	1.93207	1.95433

3.1.1.1 Determination of *HSP12* expression levels in 905 and F1

HSP12 expression levels of wild type and the F1 mutant significantly decreased during freeze-thaw stress conditions. It could be said that freeze-thaw stress decreased the *HSP12* gene expression levels. However, relative expression level of F1 strain was up to 4-fold of the wild type under control conditions.

Relative expression profiles of *HSP12* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.1.

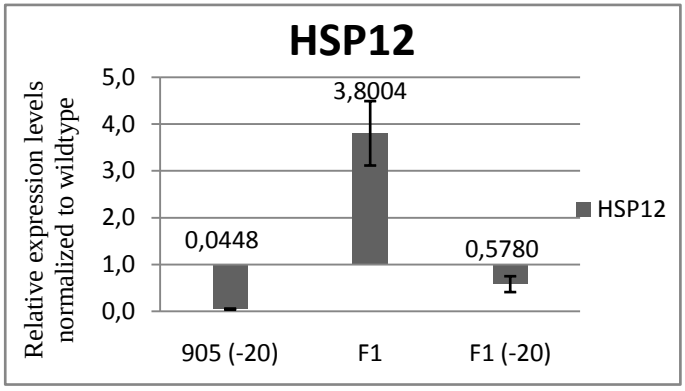

Figure 3.1: Expression levels of *HSP12* gene in control and freeze-thaw stress conditions

3.1.1.2 Determination of *FMP45* expression levels in 905 and F1

The expression level of *FMP45* gene decreased in both wild type and F1 strains under freeze-thaw stress conditions. However, under stress conditions, the expression level was up to 7-fold when normalized to wild type control. The results showed that the expression profiles of *FMP45* genes were completely different under control and freeze-thaw stress conditions.

Relative expression level profiles of *FMP45* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.2.

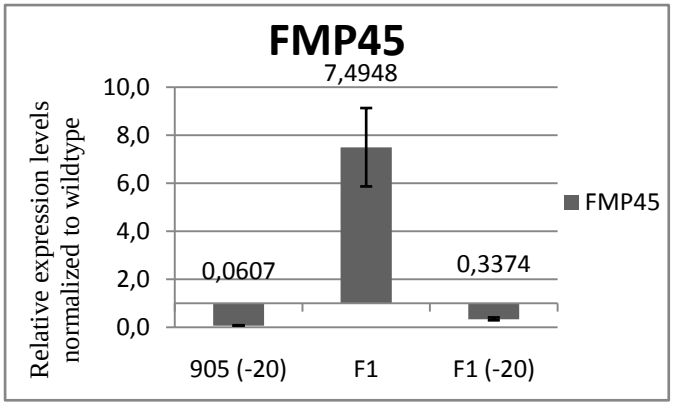

Figure 3.2: Expression levels of *FMP45* gene in control and freeze-thaw stress conditions.

3.1.1.3 Determination of *HSP26* expression levels in 905 and F1

The expression levels of *HSP26* gene decreased under freeze-thaw stress conditions in both wild type and the freeze-thaw stress-resistant mutant individual F1. However, the normalized expression level of F1 was up to 5-fold. The results indicate that the expression profiles of *HSP26* were totally different under stress and control conditions.

Relative expression profiles of *HSP26* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.3.

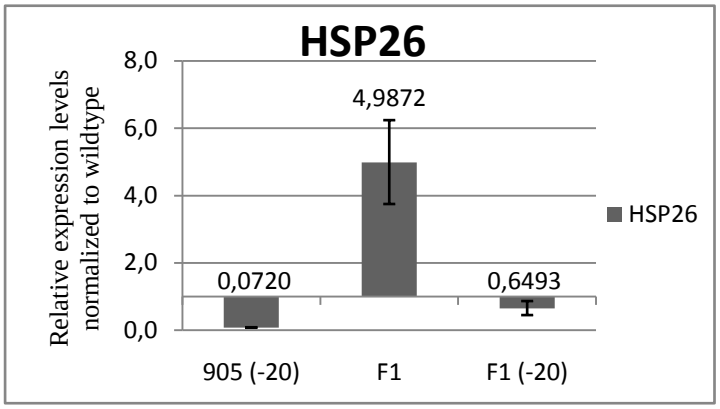

Figure 3.3: Expression levels of *HSP26* gene in control and freeze-thaw stress conditions

3.1.1.4 Determination of *HXK1* expression levels in 905 and F1

According to the relative expression levels, the *HXK1* gene was downregulated during stress conditions for both wild type and the F1 strain, and also for F1 in control conditions. The downregulation was higher during stress conditions, when compared to F1 strain control group. Relative expression profiles of *HXK1* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.4.

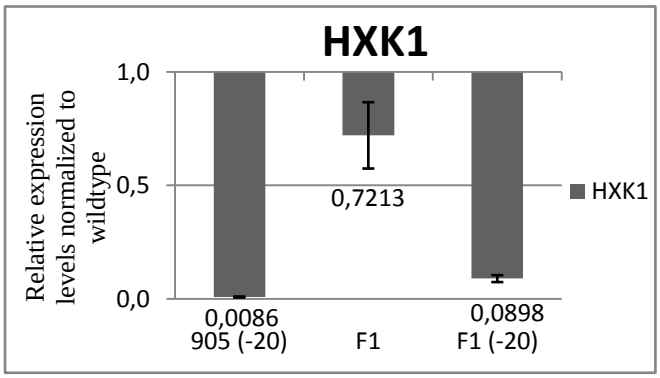

Figure 3.4: Expression levels of *HXK1* gene in control and freeze-thaw stress conditions.

3.1.1.5 Determination of *PHO84* expression levels in 905 and F1

The expression level of *PHO84* decreased under both freeze-thaw stress conditions and in control groups, when compared to wild type control conditions data. According to the results, the decrease was significantly high in both wild type and F1 strains under both conditions.

Relative expression profiles of *PHO84* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.5.

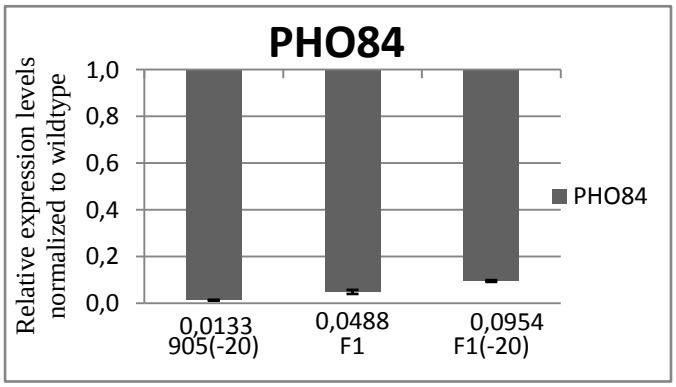

Figure 3.5: Expression levels of *PHO84* gene in control and freeze-thaw stress conditions.

3.1.1.6 Determination of *NSR1* expression levels in 905 and F1

The expression level of *NSR1* gene increased at freeze-thaw stress conditions in both wild type and F1. The expression level of *NSR1* in wild type under stress increased up to 5.5-fold. The expression level difference between wild type and F1 was significantly high under stress conditions.

Relative expression profiles of *NSR1* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.6.

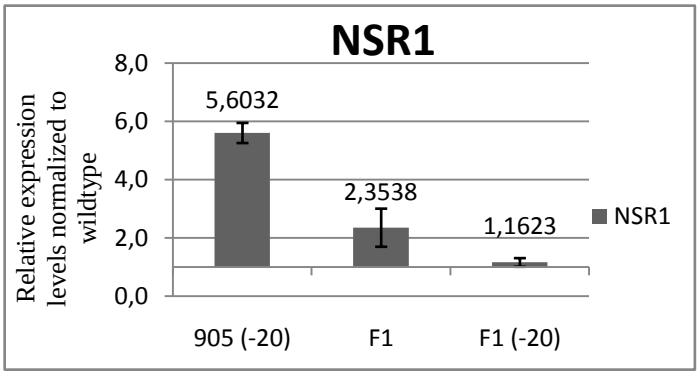

Figure 3.6: Expression levels of *NSR1* gene in control and freeze-thaw stress conditions.

3.1.1.7 Determination of *ZRT1* expression levels in 905 and F1

The expression levels of *ZRT1* were completely different in wild type and F1 strain. However, there was an increase of up to 3-fold in wild type during stress conditions, and the expression decreased in F1 strain during both stress and control conditions. Relative expression profiles of *ZRT1* as fold of wild-type for both '905' and 'F1' strains under control and freeze-thaw stress conditions are shown in Figure 3.7.

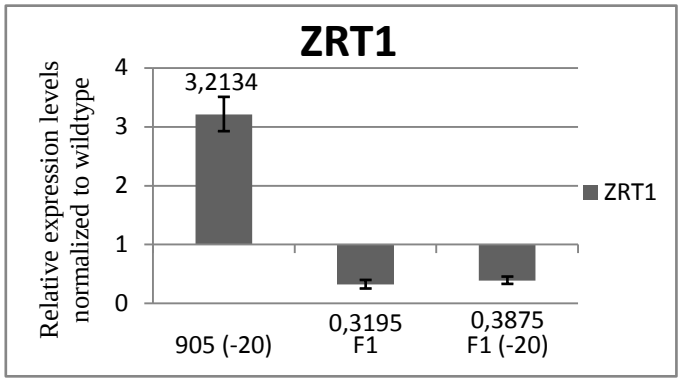

Figure 3.7: Expression levels of *ZRT1* gene in control and freeze-thaw stress conditions.

3.1.2 Expression levels of freeze thaw stress related genes *HSP12, FMP45, HSP26, HXK1, PHO84, NSR1* and *ZRT1* in R625 and P8

3.1.2.1 Determination of *HSP12* expression levels in R625 and P8

The expression level of the *HSP12* increased in P8 under control and stress conditions, although there was no significant difference in R625 under control and stress conditions.

Relative expression profiles of *HSP12* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.8.

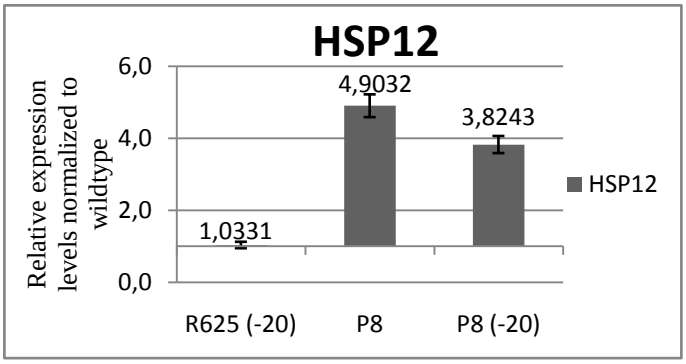

Figure 3.8: Expression levels of *HSP12* gene in control and freeze-thaw stress conditions.

3.1.2.2 Determination of *FMP45* expression levels in R625 and P8

The expression level of *FMP45* increased during both control and stress conditions in R625 and P8 strains. During control conditions, the expression level was up to 4.5-fold in P8 when normalized to wild type control.

Relative expression profiles of *HSP12* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.9.

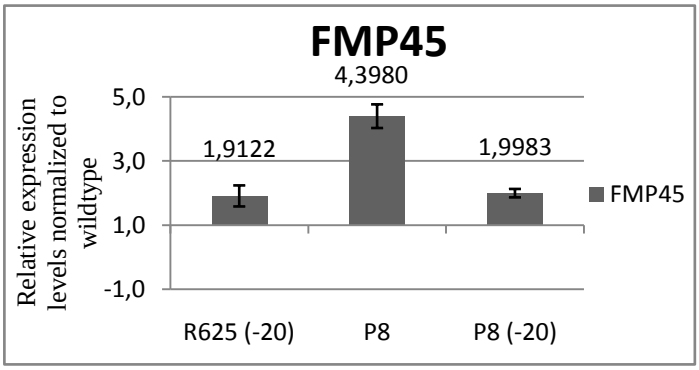

Figure 3.9: Expression levels of *FMP45* gene in control and freeze-thaw stress conditions.

3.1.2.3 Determination of *HSP26* expression levels in R625 and P8

According to the relative expression levels, the *HSP26* gene was downregulated during stress conditions for both wild type and P8 strain. Also, the expression level decreased in P8 under control conditions. The downregulation was higher in P8 when compared to R625 under control and stress conditions.

Relative expression profiles of *HSP26* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.10.

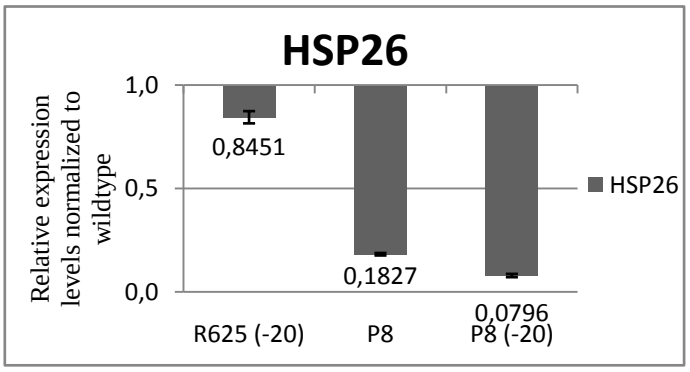

Figure 3.10: Expression levels of *HSP26* gene in control and freeze-thaw stress conditions.

3.1.2.4 Determination of *HXK1* expression levels in R625 and P8

The expression level of *HXK1* increased in both R625 and freeze-thaw stress-resistant mutant, P8. The expression level of *HXK1* gene increased up to 4-fold. In addition, increases were obtained for both control and stress conditions. The expression levels of *HXK1* gene in P8 under control and stress conditions were higher than R625. Relative expression profiles of *HXK1* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.11.

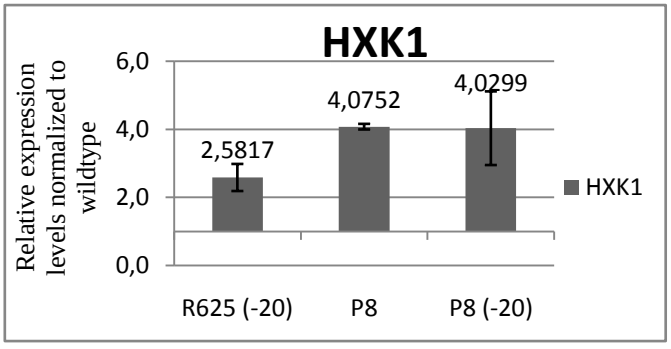

Figure 3.11: Expression levels of *HXK1* gene in control and freeze-thaw stress conditions.

3.1.2.5 Determination of *PHO84* expression levels in R625 and P8

The expression level of *PHO84* gene decreased under freeze-thaw stress conditions for both R625 and *P8*. The expression level of *PHO84* in P8 control conditions increased up to 5-fold when normalized to wild-type. The normalized expression level of P8 under freeze-thaw stress was slightly lower than P8 under control conditions. In addition, at stress conditions, the expression level of *PHO84* in P8 was higher compared to wild type. It was observed that the expression profile of *PHO84* gene was different in mutant individual P8.

Relative expression profiles of *PHO84* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.12.

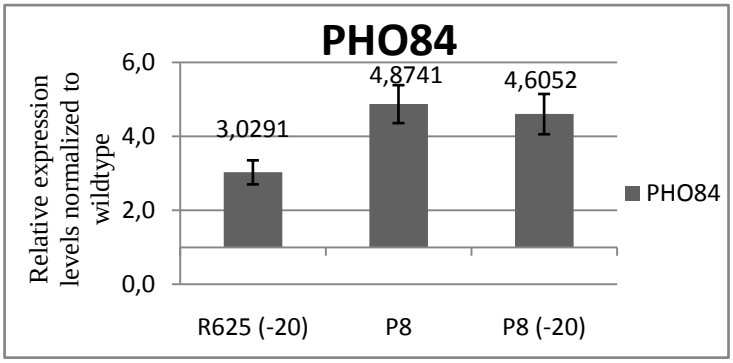

Figure 3.12: Expression levels of *PHO84* gene in control and freeze-thaw stress conditions.

3.1.2.6 Determination of *NSR1* expression levels in R625 and P8

The expression level of *NSR1* gene increased in freeze-thaw stress-resistant mutant control group up to 3-fold. There was no significant difference in the expression level of wild type under stress conditions. In addition, the expression level of *NSR1* gene decreased in P8 under stress conditions.

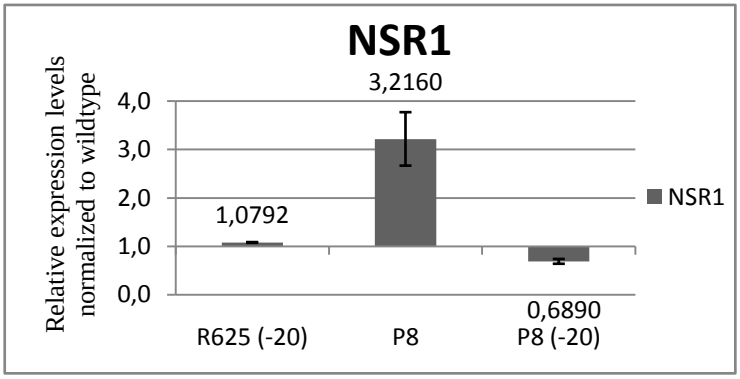

Figure 3.13: Expression levels of *NSR1* gene in control and freeze-thaw stress conditions.

Relative expression profiles of *NSR1* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.13.

3.1.2.7 Determination of *ZRT1* expression levels in R625 and P8

The expression level of the *ZRT1* gene decreased in freeze-thaw resistant mutant strain in control conditions. However, the expression level of the *ZRT1* gene increased in both wild type and mutant strains during stress conditions. In addition, the expression level of P8 was slightly lower than R625 under stress conditions.

Relative expression profiles of *ZRT1* as fold of wild-type for both 'R625' and 'P8' strains under control and freeze-thaw stress conditions are shown in Figure 3.14.

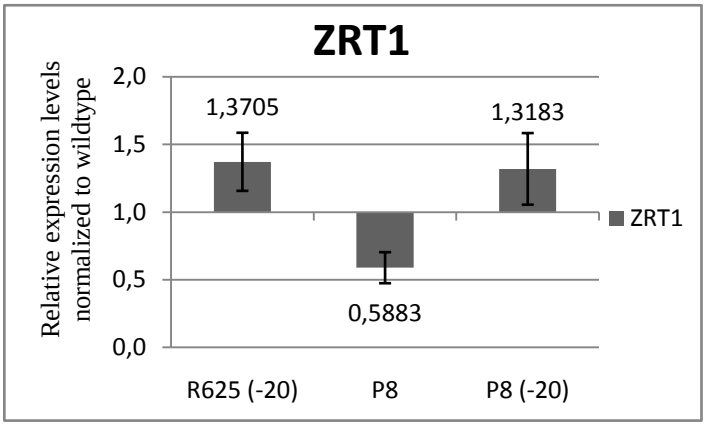

Figure 3.14: Expression levels of *ZRT1* gene in control and freeze-thaw stress conditions.

Table 3.2 Error and efficiency values for qRT-PCR in R625 and P8.

Genes Name	***HSP12***	***FMP45***	***HXK1***	***HSP26***	***PHO84***	***ZRT1***	***NSR1***
Error	0.03093	0.02626	0.02076	0.06316	0.03076	0.03963	0.04373
Efficiency	1.93733	1.973	1.994	1.871	1.95333	1.94233	1.94366

Efficiency and error values of the target genes are shown in Table 3.2. The values were in the acceptable limit range (Url-3). The efficiency values were between 1.8 and 2.0.

Table 3.3: Comparison of microarray data (F1) and qRT-PCR data (F1 and P8) for selected genes.

Gene Symbol	**[Microarray Results] Log FC ([F1] vs [WT]) (Yilmaz U., 2013)**	**[qRT-PCR results] F1**	**[qRT-PCR results] P8**
HSP12	8	3.8004	4.9032
FMP45	7.8	7.4948	4.3980
HXK1	7.8	0.7213	4.0752
HSP26	7.4	4.9872	0.1827
PHO84	-6.5	0.0488	4.8741
ZRT1	-5.8	0.3195	0.5883
NSR1	-4.8	2.3427	3.2160

The comparison of microarray data of F1 (Yilmaz U., 2013) with qRT-PCR data of F1 and P8 for selected genes is shown in Table 3.3.

4 DISCUSSION AND CONCLUSION

In this thesis project, to investigate freeze-thaw stress resistant *Saccharomyces cerevisiae* mutants, expression levels of their freeze-thaw stress-related genes were determined. qRT-PCR method was performed to determine expression levels of freeze-thaw stress-related genes during control and freeze-thaw stress conditions.

In previous studies in our laboratory, it was aimed to obtain freeze-thaw resistant mutant by using evolutionary engineering strategy. For this purpose, the wild type strains of the laboratory strain (905) and industrial strain (R625) were exposed to chemical mutagenesis (ethyl methane sulphonate, EMS). The freeze-thaw stress was applied ten times to the chemically mutagenized population of *Saccharomyces cerevisiae.*The mutant generations were obtained under this selective pressure. According to phenotypic, physiological and genetic analyses, mutant individual F1 (laboratory strain) and P8 were selected for detailed analysis.

Whole genome transcriptomic analysis of the freeze-thaw resistant mutant of the laboratory strain (F1) was performed by DNA microarray technology previously (Yilmaz U., 2013). According to microarray data, the most highly upregulated 4 genes and most highly downregulated 3 genes were chosen to be analysed by qRT-PCR in this study. According to microarray data, *HSP12*, *FMP45*, *HSP26* and *HXK1* were significantly upregulated in the freeze-thaw stress-resistant mutant individual F1. On the other hand, *PHO84*, *NSR1* and *ZRT1* were significantly downregulated in the freeze-thaw resistant mutant individual F1.

Heat shock proteins play a major role in stress response mechanism. HSPs prevent protein aggregation during temperature changes and various stress conditions (Arya *et al*., 2007). *HSP12* and *HSP26* encode heat shock proteins. They are significantly upregulated in the freeze-thaw resistant mutant of the laboratory strain (F1). According to microarray data, *HSP12* gene was upregulated in mutant individual up to 8-fold of the wild type. Hsp12 is a plasma membrane protein that preserves membrane organization under heat shock stress conditions. It acts as a cryoprotectant

like trehalose. In previous studies, it was observed that the upregulated gene related to membrane robustness provides resistance to freeze-thaw stress conditions (Park *et al*., 1997; Tanghe *et al*., 2002). It was also observed in this study by qRT-PCR that the expression levels of *HSP12* increased up to 3.8-fold in the freeze-thaw resistant mutant individual of laboratory strain, F1. Besides, the expression level of *HSP12* gene also increased up to 4.9032-fold in the freeze-thaw resistant mutant individual of industrial strain P8, according to the qRT-PCR results.

According to microarray data, *HSP26* gene was significantly upregulated in mutant individual F1, up to 7.4-fold of the wild type. It is known that Hsp26 is a cytosolic small heat-shock protein which is responsible for protein folding and stabilization (Takashi *et al*., 2009). The transcript levels of the *HSP26* could not be detectable in unstressed conditions. qRT-PCR results showed that the expression levels of *HSP26* increased up to 4.9872-fold in the freeze-thaw stress-resistant mutant individual of laboratory strain, F1. On the other hand, the expression level of *HSP26* gene decreased in the freeze-thaw stress-resistant mutant individual of the industrial strain, P8. The difference between expression levels of freeze-thaw stress-resistant individuals obtained from laboratory and industrial strains is significant.

The whole genome transcriptomic analysis results showed that the expression level of *FMP45* increased up to 7.8-fold in the mutant laboratory strain, F1 (Yilmaz U., 2013). Similarly, according to the qRT-PCR results, the expression levels of *FMP45* increased up to 7.495-fold in the freeze-thaw resistant mutant individual of laboratory strain F1, in control conditions. But when the wild type laboratory strain and the freeze-thaw stress-resistant mutant strain were exposed to freeze-thaw stress, the expression levels decreased, as compared to the expression level of '905' under control conditions. In the industrial strain, the expression level of *FMP45* decreased up to 4.398-fold in the freeze-thaw stress-resistant mutant strain, P8. But it was observed that in 905 and F1, the expression level of *FMP45* decreased during freeze-thaw stress conditions, compared to 905 under control conditions. Differently, the expression level of *FMP45* decreased when compared to freeze-thaw stress-resistant mutant 'P8', but when compared to 'R625' in control conditions, the expression level showed an increased profile. This observation implies that the laboratory strain and the industrial strain show different expression profiles during freeze-thaw stress conditions. Fmp45 is integral membrane protein localized to the mitochondria. It is required for sphingolipid metabolism and is responsible for cell wall organization. It

is known that cell wall mutations cause alteration in the transcriptomic profile, like induction of Environmental Stress Response (ESR) Genes. The increased gene expression level profiles of mutant individuals F1 and P8 are in line with previous findings (Lagorce *et al*., 2003).

Hxk1 is a protein that is responsible for carbohydrate metabolic process. It is responsible for catalyzing glucose phosphorylation during glucose metabolism. According to whole genome transcriptomic analysis data (Yilmaz U., 2013) the expression level of *HXK1* gene decreased up to 7.8-fold in mutant individual F1 in control conditions. Differently from these results, qRT-PCR results showed that the expression level of *HXK1* significantly decreased in control conditions for 'F1' and in freeze-thaw stress conditions for '905' and 'F1'. On the other hand, the expression level of *HXK1* in industrial freeze-thaw stress-resistant mutant individual increased in both wild type and the mutant individual during control and stress conditions. The difference between expression profiles could be related to the genetic complexity of the industrial strain that may result from its polyploidy.

DNA microarray results also showed a dramatic decrease in *PHO84* levels in F1 (Yilmaz U., 2013). Similarly, it was observed in qRT-PCR analysis that the expression levels of *PHO84* decreased in control conditions for mutant individual 'F1' and in freeze-thaw stress conditions for both wild type and mutant individual. But interestingly, the expression profile was completely different from 905 and F1 in the industrial strain R625 and its mutant individual 'P8'. The expression level increased in P8 up to 4.8741-fold during control conditions. The expression also increased in stress conditions for both wild type and the freeze-thaw stress-resistant mutant during stress condition. The expression level was slightly higher in the mutant individual 'P8' in control conditions, when compared to expression levels during freeze-thaw stress. It was reported that the *PHO84* gene was upregulated during phosphate starvation (Wykoff, 2001). It is known that the expression of *PHO84* depends on inorganic phosphate 'P_i', so it could be deduced that the expression level difference could be related to phosphate levels in the cells. In addition, the *PHO84* is an integral component of the plasma membrane and it is known that freeze-thaw stress causes damage to cell membrane. From this observation, it could be implied that the alteration of plasma membrane may cause a difference in the transcriptomic profile of the Pho84p in cell membrane.

Whole genome transcriptomic analysis data showed that the expression levels of *ZRT1* markedly decreased (Yilmaz U., 2013). The qRT-PCR data also supported these results. It was observed that the expression levels of *ZRT1* decreased in control and stress conditions of mutant individual 'F1'. However, the expression level increased in the wild type strain during control conditions. Similarly, the expression level decreased in industrial strain mutant individual 'P8' in control conditions, but the expression levels decreased in wild type and freeze-thaw resistant mutant individual during stress conditions. Zrt1 is an integral component of the plasma membrane, so it could be deduced that the cell membrane damage could have caused on expression difference during stress conditions.

Whole genome transcriptomic analysis results also revealed that the expression level of *NSR1* was 4.8-fold down-regulated (Yilmaz U., 2013). However, qRT-PCR results showed that the expression profile of *NSR1* gene was upregulated in freeze-thaw stress-resistant mutant individual 'F1' at control conditions, and '905' and 'F1' at stress conditions. Furthermore, the expression level of *NSR1* also increased in freeze-thaw stress-resistant mutant of industrial strain 'P8' at control condition and the industrial strain 'R625'. Different from this observation, the expression level decreased in 'P8' during stress conditions. In a previous study, it was also found that the expression level of *NSR1* increased up to 3-fold (Kondo *et al*., 1992). The qRT-PCR results are in line with this observation (Kondo *et al*., 1992).

To conclude, the expression level differences between the laboratory strain and the industrial strain could be explained by their differences in the number of gene sets. The cells, which have different ploidy type, show different morphological, physiological and developmental characteristics (Galitski et al., 1999). This difference can cause changes in genome organization and function. The gene redundancy and genetic robustness might have caused an adjuvant effect on mutations in polyploid industrial strain, compared to laboratory strain. In a polyploidy-dependent regulation study, it was observed that the genes show different expression patterns in a polyploid strain, compared to a haploid strain in *S. cerevisiae*. In that study, 17 ploidy-regulated genes were found. Ten genes were ploidy-induced and 7 genes were ploidy-reduced (Galitski *et al*., 1999).

Additionally, it is known that qRT-PCR is more sensitive, compared to microarray analysis. Although there is generally a good correlation between qRT-PCR and microarray data, the existing data indicated that there could be different results for

individual genes (Dallas *et al*., 2005). For this reason, the microarray data must be verified by another technology platform (Sinicropi et al., 2006).

To summarize, freeze-thaw stress causes significant differences in gene expression patterns in both laboratory and industrial strains of *S. cerevisiae*. In addition, there could be also some differences between gene expression levels determined by microarray and qRT-PCR methods. Thus, verification of the microarray data by a more sensitive method is crucial, particularly for critically important genes.

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APPENDICES

APPENDIX A: Microarray results of the upregulated genes (Yilmaz U., 2013)

ProbeName	FC (abs) ([F1] vs [WT])	FC ([F1] vs [WT])	Log FC ([F1] vs [WT])	Regulation ([F1] vs [WT])	GeneSymbol
A_06_P2839	263,59637	263,59637	8,042187	up	HSP12
A_06_P1957	235,10191	235,10191	7,8771424	up	FMP45
A_06_P2951	224,0665	224,0665	7,807783	up	HXK1
A_06_P1320	172,37367	172,37367	7,4293957	up	HSP26
A_06_P5966	146,30128	146,30128	7,1927986	up	YNL194C
A_06_P7208	133,5849	133,5849	7,061613	up	GPH1
A_06_P5144	112,39571	112,39571	6,8124433	up	TMA10
A_06_P5604	107,301704	107,301704	6,745529	up	ALD3
A_06_P5534	104,10143	104,10143	6,701846	up	PGM2
A_06_P6244	92,9783	92,9783	6,538822	up	DDR2
A_06_P6147	79,45069	79,45069	6,311988	up	YNR034W-A
A_06_P2840	77,464096	77,464096	6,275456	up	
A_06_P1085	73,9561	73,9561	6,208597	up	BDH2
A_06_P1368	68,787636	68,787636	6,1040773	up	
A_06_P2057	68,77427	68,77427	6,103797	up	FMP16
A_06_P2333	68,37984	68,37984	6,095499	up	HXT7
A_06_P2580	68,00499	68,00499	6,0875688	up	CYC7
A_06_P3464	67,20628	67,20628	6,070524	up	FMP43
A_06_P5391	63,016106	63,016106	5,9776487	up	TSL1
A_06_P2334	62,379963	62,379963	5,963011	up	HXT6
A_06_P1939	61,828705	61,828705	5,950205	up	RTN2
A_06_P5645	57,808434	57,808434	5,853208	up	YMR206W
A_06_P2736	57,329113	57,329113	5,841196	up	SSA4

A_06_P2698	52,719315	52,719315	5,7202597	up	YER067W
A_06_P1369	52,01474	52,01474	5,7008486	up	TKL2
A_06_P3469	51,35355	51,35355	5,682392	up	SOL4
A_06_P3671	50,674263	50,674263	5,6631813	up	HXT5
A_06_P3308	48,284515	48,284515	5,5934887	up	CTT1
A_06_P6276	45,49709	45,49709	5,5077024	up	PHM7
A_06_P6411	41,05222	41,05222	5,3593884	up	RSB1
A_06_P6548	39,06226	39,06226	5,2877035	up	YOR186W
A_06_P3363	36,454224	36,454224	5,188014	up	BTN2
A_06_P4865	33,390087	33,390087	5,061348	up	YLR053C
A_06_P6535	30,789804	30,789804	4,9443808	up	DCS2
A_06_P5066	30,374544	30,374544	4,924791	up	
A_06_P5688	29,07838	29,07838	4,861875	up	GAD1
A_06_P1749	27,646416	27,646416	4,7890205	up	GPM2
A_06_P6946	27,049719	27,049719	4,7575417	up	UIP4
A_06_P5392	26,78184	26,78184	4,743183	up	
A_06_P2247	26,523039	26,523039	4,729174	up	HSP78
A_06_P2838	26,176872	26,176872	4,710221	up	
A_06_P3662	25,664948	25,664948	4,6817274	up	RTC3
A_06_P5422	25,531118	25,531118	4,674185	up	MSC1
A_06_P6540	24,81259	24,81259	4,6330004	up	GAC1
A_06_P5967	24,268454	24,268454	4,6010103	up	YNL195C
A_06_P4992	24,145754	24,145754	4,5936975	up	TFS1
A_06_P2783	24,012003	24,012003	4,585684	up	SPI1
A_06_P3477	23,776533	23,776533	4,5714664	up	GND2
A_06_P2366	23,580488	23,580488	4,5595217	up	YDR374C

APPENDIX B: Microarray results of the downregulated genes (Yilmaz U., 2013)

ProbeName	FC (abs) ([F1] vs [WT])	FC ([F1] vs [WT])	Log FC ([F1] vs [WT])	Regulation ([F1] vs [WT])	GeneSymbol
A_06_P5417	93,883995	-93,883995	-6,5528073	down	PHO84
A_06_P3211	56,652176	-56,652176	-5,8240595	down	ZRT1
A_06_P3380	28,050575	-28,050575	-4,8099585	down	NSR1
A_06_P5883	20,626204	-20,626204	-4,3664064	down	DBP2
DCP_1_7	19,296618	-19,296618	-4,270276	down	
A_06_P4488	15,744709	-15,744709	-3,9767952	down	DHR2
A_06_P3466	14,92292	-14,92292	-3,899458	down	SDA1
A_06_P6924	14,840365	-14,840365	-3,8914547	down	SET6
DCP_1_4	14,52173	-14,52173	-3,8601415	down	
A_06_P5912	13,91414	-13,91414	-3,7984798	down	AAH1
A_06_P6802	13,85253	-13,85253	-3,7920775	down	NOP4
A_06_P1693	13,839871	-13,839871	-3,7907586	down	RSA4
A_06_P6166	13,831526	-13,831526	-3,7898884	down	NOG2
A_06_P6852	13,66951	-13,66951	-3,7728896	down	NOG1
A_06_P4779	13,5612955	-13,5612955	-3,761423	down	RIX7
A_06_P6272	13,50429	-13,50429	-3,7553458	down	REX4
A_06_P2022	13,107077	-13,107077	-3,712274	down	ARO3
A_06_P1047	12,28207	-12,28207	-3,6184819	down	MAK16
A_06_P2089	12,066789	-12,066789	-3,59297	down	ARX1
A_06_P1605	11,983451	-11,983451	-3,5829716	down	SPB1
A_06_P7159	11,897739	-11,897739	-3,5726156	down	MRD1
A_06_P6771	11,572682	-11,572682	-3,5326514	down	RRP12
A_06_P1901	11,328058	-11,328058	-3,5018287	down	NRP1
A_06_P4142	11,26691	-11,26691	-3,49402	down	ALB1
A_06_P5954	11,161398	-11,161398	-3,4804459	down	IPI3
A_06_P5559	11,109728	-11,109728	-3,4737515	down	ECM16
A_06_P2288	10,738331	-10,738331	-3,4246979	down	BFR2
A_06_P5036	10,525949	-10,525949	-3,3958783	down	UTP13
A_06_P3660	10,517619	-10,517619	-3,3947363	down	IPI1
A_06_P4031	10,456018	-10,456018	-3,3862617	down	NOP9
A_06_P1281	10,371873	-10,371873	-3,3746045	down	HMT1

A_06_P1129	10,266322	-10,266322	-3,3598475	down	
A_06_P1681	10,095533	-10,095533	-3,3356452	down	PWP2
A_06_P1327	10,044703	-10,044703	-3,328363	down	RPG1
A_06_P2684	10,006562	-10,006562	-3,3228745	down	FCY2
A_06_P4661	10,005197	-10,005197	-3,3226776	down	DBP7
A_06_P4585	9,971803	-9,971803	-3,3178544	down	EBP2
A_06_P2489	9,951245	-9,951245	-3,314877	down	PUF6
A_06_P5091	9,937391	-9,937391	-3,3128672	down	DBP9
A_06_P5227	9,920635	-9,920635	-3,3104324	down	UTP21
A_06_P5383	9,914491	-9,914491	-3,3095386	down	UTP14
A_06_P5268	9,843553	-9,843553	-3,299179	down	FPR4
A_06_P1767	9,566229	-9,566229	-3,2579503	down	PRM7
A_06_P2760	9,534473	-9,534473	-3,2531533	down	LCP5
A_06_P5903	9,516876	-9,516876	-3,250488	down	KRE33
A_06_P2071	9,484735	-9,484735	-3,2456074	down	RRP8
A_06_P4752	9,449851	-9,449851	-3,2402916	down	DRS1
A_06_P3625	9,366224	-9,366224	-3,2274675	down	CIC1
A_06_P7185	9,25516	-9,25516	-3,210258	down	RRP9

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