A SYSTEM BASED APPROACH TO UNDERSTAND HUMAN DISORDERS USING *SACCHAROMYCES CEREVISIAE* AS A MODEL ORGANISM

by

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ABSTRACT

A SYSTEM BASED APPROACH TO UNDERSTAND HUMAN DISORDERS USING *SACCHAROMYCES CEREVISIAE* AS A MODEL ORGANISM

Understanding the function of the genes is essential to elucidate the molecular mechanisms behind the Mendelian disorders. Conservation of the biochemical and molecular functions between organisms enables using model organisms to understand the molecular functions of disease associated genes. Studies performed with a model organism carrying a mutation in a conserved gene elucidate not only the molecular function of the gene but also the organism-level function of that gene. High degree of conservation of copper homeostasis pathway between yeast and human makes yeast an ideal model organism to study the copper-related disorders. The aim of this thesis was to provide a systems level understanding of the copper related disorders such as Wilson and Menkes diseases by making use of the yeast ortholog of the genes that were associated with these diseases. For this purpose, the genome-wide effects of the deletion of *CCC2* and that of *ATX1* were investigated using a systems based approach. The transcript levels were investigated in conjunction with interactome and regulome in order to further elucidate the pathways that were affected from the disturbance of copper homeostasis. The analysis of the transcriptional response to the deletion of *CCC2* gene in a copper level dependent manner revealed the disturbed mechanisms due to the absence of this gene under changing copper levels. The analysis also revealed that absence of *ATX1* gene and *CCC2* gene resulted in different transcriptional responses. Further analyses of the reference and *CCC2* deleted cells growing in copper deficient chemostat cultures engendered the differences between the effects of the short and long term exposure to high copper levels in both strains.

ÖZET

HASTALIKLARIN MAYADA SİSTEM BAZLI BİR YAKLAŞIM İLE İNCELENMESİ

Gen fonksiyolarının anlaşılması Mendeliyen hastalıkların altında yatan moleküler mekanizmaların aydınlatılması açısından büyük önem taşımaktadır. Biyokimyasal ve moleküler işlevlerin organizmalar arasında korunuyor olması, genlerin fonksiyonlarının anlaşılması için model organizmaların kullanılmasına olanak sağlamaktadır. Organizmalar arasında korunmakta olan bir genin mutasyonunu taşıyan bir model organizma ile yapılacak çalışma sadece o genin işlevinin değil ayrıca o genin organizma düzeyindeki işlevinin de ortaya çıkmasını sağlayacaktır. Maya ve insan arasında büyük ölçüde korunan bakır mekanizması, bu mekanizmadaki aksaklıklardan kaynaklanan hastalıkların çalışılması için mayayı ideal bir model organizma haline getirmektedir. Bu tezin amacı, Wilson ve Menkes hastalıklarıyla ilişkilendirilmiş olan genlerin mayada ortoloğunun bulunmasından yararlanılarak, sistem bazlı bir yaklaşımla bakır mekanizmasındaki bozukluklardan kaynaklanan hastalıkların anlaşılmasıdır. Bu amaçla, *CCC2* ve *ATX1* genlerinin yokluğunun genom düzeyindeki etkileri sistem bazlı bir yaklaşımla incelenmiştir. Bakır homeostazındaki bozuklukların etkilediği diğer yolizlerinin aydınlatılabilmesi için gen anlatım düzeyleri protein-protein ve protein-DNA etkileşim ağ yapıları ile birlikte incelenmiştir. Gen anlatım düzeyinde *CCC2* geninin delesyonuna karşı verilen tepkinin bakır derişimine bağlı olarak incelenmesi, bu genin yokluğundan kaynaklanan aksaklıkların, değişik bakır düzeylerinde ortaya çıkartılmasını sağlamıştır. Analizler ayrıca *ATX1* ve *CCC2* genlerinin yokluğuna karşı verilen gen anlatım düzeyindeki tepkinin farklılıklarını ortaya koymuştur. Kemostat kültüründe gerçekleştirilen deneyler sonucunda elde edilen veriler, hücrelerin uzun ya da kısa süre bakır eksikliğine maruz bırakılmalarının etkileri arasındaki farkın anlaşılmasını sağlamıştır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

Cu	Copper
g	Gram
h	Hour
L	Litre
min	Minute
mM	Millimolar
vol	Volume
wt	Weight
μ	Specific growth rate

LIST OF ACRONYMS/ ABBREVIATIONS

ANOVA	Analysis of variance
aRNA	Amplified ribonucleic acid
ATP	Adenosine triphosphate
cDNA	Complementary DNA
BLAST	Basic Local Alignment Search Tool
Corr.	Corrected
Cq	Quantification cycle
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
E	Efficiency
ER	Endoplasmic reticulum
FC	Fold change
FDR	False discovery rate
GO	Gene Ontology
HILIC	Hydrophilic interaction chromatography
ICC	Indian childhood cirrhosis
ICP-MS	Inductively coupled plasma mass spectrometry
ICT	Idiopathic copper toxicity
LCB	Lower confidence bound
LC-MS	Liquid chromatography–mass spectrometry
max	Maximum
MD	Menkes disease
MeV	Multi experiment viewer
MIQE	Minimum information for publication of quantitative real-time PCR experiments
MM	Miss match
mRNA	Messenger ribonucleic acid
OD	Optical density
ORF	Open reading frames

PCC	Pearson correlation coefficient
PCR	Polymerase chain reaction
PDR	Pleiotropic drug resistance
PM	Perfect Match
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SGD	*Saccharomyces* Genome Database
TCA	Tricarboxylic acid
TF	Transcription factor
TGN	Trans-Golgi network
TORC1	Target of rapamycin complex 1
tRNA	Transfer ribonucleic acid
WD	Wilson disease
$Y_{e/s}$	Ethanol Yield on Glucose
$Y_{gly/s}$	Yeast extract - peptone
YP	Yeast extract - peptone
YPD	Yeast extract - peptone - dextrose
YPG	Yeast extract - peptone - glycerol
$Y_{x/s}$	Biomass Yield on Glucose

1. INTRODUCTION

Today, more than 3600 genes with mutations causing Mendelian disorders have been discovered however there are still close to 3700 Mendelian or suspected-Mendelian diseases with unidentified molecular basis. Obviously not only the sequence information but also the function of the gene should be identified in order to understand the molecular mechanisms behind the disorders. Identification of orthologs of disease genes, which play equivalent roles in the model organism and human, brought about significant progress in the understanding of molecular mechanisms behind the genetic diseases.

Yeast is claimed to be a valuable model organism to study the molecular basis of human diseases [1]. Yeast is the first eukaryote of which the genome was sequenced and has served as a basis for all genomic studies. Although the human genome is 100 times larger, it contains only 3-5 times as many genes when compared to yeast. This difference stems from the presence of intervening sequences, non-coding regions and repeated sequences in the human genome [2]. To date, approximately 6,600 open reading frames have been annotated, with more than 80% functionally characterized. Approximately 40% of the yeast proteins have an ortologous protein in human and 20% of these ortholog proteins were associated with a disease [3]. Genomic homology explains the conservation of fundamental cell biological processes between yeast and mammalian cells. The development of system based integrative approaches and identification of co-expressed modules in yeast may not only provide valuable information and reduce the dimensionality but serves also as a basis for the construction of human disease associated networks consisting of human orthologues [2].

There are several features which make *Saccharomyces cerevisiae* an ideal model organism. Yeast is genetically well defined; its entire genome sequence is available. A collection of single deletion mutants is available for diploid cells and for non-essential genes of haploid cells. There are a large set of available high throughput data such as transcriptome, proteome, metabolome and interactome datasets. Moreover it is genetically tractable organism that exists both in haploid and diploid forms, which makes yeast amenable for the genetic modifications. Besides, it can be easily cultivated [4].

The aim of this study was to identify the molecular mechanisms underlying human genetic disorders related with copper metabolism using *S. cerevisiae* as the model organism. For this purpose the genome-wide effects of the absence of the yeast ortholog of *ATP7A, ATP7B* and that of *ATOX1* were investigated using a systems based approach in yeast.

In the first part of the study, the change in the transcript and metabolite levels in response to the deletion of *CCC2* gene, which is the yeast ortholog of Wilson and Menkes disease causing genes in human, were investigated under changing copper levels. The transcript levels were further analysed in conjunction with interactome and regulome in order to elucidate the pathways that were affected from these perturbations.

In the second part of the study, the effect of the absence of the yeast ortholog of the *ATOX1,* namely *ATX1,* that encodes a copper chaperone, which transports copper to Ccc2p, was investigated at transcriptional level. The change in the transcript levels, growth characteristic as well as the intra and extracellular iron and copper concentrations in response to the deletion of *ATX1* gene was examined under similar conditions with the *CCC2* deleted strain and the alteration in these two mutants were compared with each other.

In the third part of the study, a novel strategy was developed for selection and validation of reference genes to be used in dynamic real-time RT-qPCR expression profiling experiments. The proposed candidate reference gene set was validated to outperform the use of common reference genes in the determination of dynamic transcriptional response of the target genes under two different experimental conditions.

In the last part of the study, the dynamic transcriptional re-organisation of the high affinity, low affinity and siderophore iron transporters in response to a sudden relaxation of copper deficiency in the *CCC2* deleted and reference strains have been identified using real-time RT-qPCR. The candidate reference gene set that was identified in the third part was used for the determination of the suitable pool of reference genes for the calculation of the expression levels of the genes of interest. The transient changes in the iron transporters

in these two mutants were investigated in conjunction with the transient change in the intra and extracellular iron and copper levels.

2. TRANSCRIPTIONAL REMODELLING IN RESPONSE TO CHANGING COPPER LEVELS IN WILSON DISEASE MODEL OF *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae is an ideal model organism to study Wilson and Menkes diseases due to the high degree of conservation of copper and iron homeostatic pathways between yeast and human. In this part of the thesis, the genome-wide effect of the absence of *CCC2*, the yeast ortholog of Wilson and Menkes disease causing gene, was investigated. For this purpose the transcriptome and metabolome levels of the reference strain and the *CCC2* deleted strain were analysed under changing copper conditions. The significantly expressed genes were identified in order to elucidate the pathways and biological processes that were affected from the absence of *CCC2* gene or the change in the copper level. The transcriptome data was also integrated with interactome and with the yeast regulatory network to further elucidate the mechanisms that were affected from the applied perturbations. The intracellular and extracellular metabolite levels were used to support the findings obtained from the transcriptome analyses.

2.1. Background

Copper is a trace element, which is required as a cofactor for many proteins and enzymes. The enzymes that require copper for proper functioning have roles in mitochondrial respiration, connective tissue formation, pigmentation, iron oxidation, neurotransmitter processing, and antioxidant defence [4, 5]. The ability of copper ions to be available both at oxidized (Cu(II)) and reduced (Cu(I)) forms makes it a suitable cofactor but this property also makes it toxic when present in excess [6, 7]. The copper concentrations above the adequate levels lead to formation of reactive oxidative species, which cause damages in nucleic acids, proteins and lipids [6, 8]. Copper toxicity might also lead to protein misfolding [7]. Thus the copper levels must be maintained at adequate levels both to prevent the damages due to excessive amounts of copper and also to prevent defects in the proper functioning of cuproenzymes due to copper deficiency.

Specialized homeostatic mechanism to maintain copper homoeostasis are available virtually in all organisms. In human, the mutations or deletion in the genes that play role in the maintenance of copper homeostasis were associated with some hereditary disorders. These diseases can be associated either with copper deficiency (Menkes Disease (MD) and occipital horn syndrome (OHS)) or with excessive copper accumulation (Wilson disease (WD), Indian childhood cirrhosis (ICC), endemic Tyrolean infantile cirrhosis, and idiopathic copper toxicity (ICT)) [10].

Menkes disease is an X-linked recessive disorder resulting in neurological symptoms including mental retardation, seizures, hypothermia, feeding difficulties and decreased muscle tone. This disease is associated with mutations in *ATP7A*, which is expressed in almost all tissue types. Inactivation of *ATP7A* results impaired copper export from enterocytes into the blood, which results in restricted copper supply to other tissues [11]. In Menkes disease, copper transfer across placenta, gastrointestinal tract and blood-brain barrier was impaired and this leads to copper deficiency in developing fetus due to deficient activity of essential cuproenzymes [12].

Wilson disease is autosomal recessive hepatic disease with neurological symptoms. This disease is associated with mutations in *ATP7B*, which is expressed predominantly in the liver. *ATP7B* transports copper from cytosol into the lumen of the trans-Golgi network (TGN) for subsequent incorporation of into ceruloplasmin and excretion into bile when the cytosolic copper exceeds a certain threshold [13]. In Wilson disease, copper accumulation is observed due to the impaired copper delivery and excretion. Since *ATP7B* is predominantly expressed in liver, copper accumulation leads to chronic hepatitis and cirrhosis and eventually liver failure. Deposition of copper in the basal ganglia results in cavitary degeneration, gliosis and neuronal loss, which lead to neurologic features such as Parkinsonian symptoms, tremors and dystonia [14]. Although most of the symptoms observed in Wilson disease patients are associated with ability of excess copper to induce formation of reactive oxygen species, transcriptome analyses performed with the samples collected from $Atp7b^{-/-}$ mice revealed that elevated copper up-regulated genes associated with cell cycle and chromatin structure and down-regulated lipid metabolism, whereas the genes associated with oxidative stress were not observed to be significantly expressed [15]. More than 380 mutations have been identified in Wilson disease patients and disease

phenotypes show diversity. The diversity and complexity of the Wilson disease phenotypes suggest that improper functioning of *ATP7B* has extensive consequences, which can only be identified by "omics" studies [13].

Conservation of the homeostatic mechanisms from prokaryotes to humans enables the use of model organisms to better understand these diseases. The baker's yeast *Saccharomyces cerevisiae* is one of the well-understood eukaryotic model organisms. The relatively high degree of conservation of copper and iron homeostatic pathways between yeast and human makes yeast an ideal model organism to study these mechanisms [7, 8, 13].

Copper transport inside the cells is mediated by high affinity (Ctr1p and Ctr3p) and low affinity (Fet4p and Smf1p) permeases in yeast. Copper should be reduced by Fre1p and Fre2p plasma membrane reductases before it is transported inside the cell. When the intracellular copper levels increases, copper binds to Cup1p and Crs5p, which are metallothioneins that are required for copper resistance [16]. The expression level of *CUP1* and *CRS5* are induced in response to high level of copper by the transcription factor *ACE1* [6]. There are three identified copper chaperones which delivers copper to target molecules and organelles in yeast. Lys7p, which is the yeast ortholog of CCS in human, delivers copper to antioxidant Cu, Zn-superoxide dismutase. The second chaperone Cox17p, which delivers copper to Sco1p and Cox11p for eventual delivery to cytochrome c oxidase, is the yeast ortholog of human hCox7p [17]. The third copper chaperone, Atx1p, which is the yeast ortholog of *ATOX1* in human, delivers copper to Ccc2p, a copper ATPase and ortholog of *ATP7A* and *ATP7B* [18]. *CCC2* encodes a protein, which transfers copper across the membrane into lumen of the TGN. apoFET3 which obtains copper transported by Ccc2p is a member of the Fet3p/Ftr1p complex. This complex translocates in response to iron availability and requires copper incorporation to traffic to plasma membrane in response to iron deficiency [19].

Yeast can use reductive or non-reductive mechanisms to transport iron into the cell. In the reductive mechanism iron should be reduced before it is transported by Fre1p and Fre2p plasma membrane reductases. Then the reduced iron can be transported either with low affinity non-specific iron transporters Fet4p and Smf1p, which can also transport

copper, or with high affinity iron transport system consisting of multi-copper oxidase Fet3p and high affinity iron transport Ftr1p [13, 19]. Iron can also be transported with a non-reductive mechanism by siderophores. Although yeast cannot produce siderophores, it can express the facilitators (Arn1p, Arn2p, Arn3p and Arn4p) for their uptake and cell wall mannoprotein (Fit1p, Fit2p and Fit3p) to retain siderophore-bound iron in the cell wall [20].

There are four known transcription factors which are affected by the availability of copper and iron. Mac1p and Ace1p are specifically affected by copper status, whereas Aft1p is an iron responsive transcription factor. Aft2p is another iron responsive transcription factor which is the paralog of Aft1p and regulates the targets of Aft1p, redundantly [21]. Mac1p is activated under copper deficient conditions and activates copper uptake genes (*CTR1*, *CTR3* and *FRE1*) [6], whereas Ace1p is activated under copper overload conditions and induces genes (*CRS5*, *CUP1* and *SOD1*) that buffer copper ion concentration [17, 22]. Aft1p regulates reductive (*FET3*, *FTR1*) and non-reductive iron transporters (*ARN1-ARN4* and *FIT1-FIT3*), ferric reductases (*FRE1-FER6*) and also cytosolic copper chaperones (*CCC2*, *ATX1*) [23]. Although Aft2p have partially redundant activity with Aft1p, it has also some distinct roles in regulation of the genes involved in vacuolar and mitochondrial iron homeostasis [14]. Under copper deficient conditions, iron uptake genes, which are regulated by Aft1p/Aft2p, were observed to be up-regulated indicating a secondary iron deficiency due to the reduction in copper-dependent reductive iron transport [23] .

The requirement of Fet3p to be loaded with copper by the copper ATPase, Ccc2p, for proper iron import links copper and iron metabolisms. ATPase-mediated transport of copper to secretory pathways was first identified in yeast *Saccharomyces cerevisiae* [24]. Ccc2p, the yeast ortholog of *ATP7A* and *ATP7B*, which are known as Menkes and Wilson disease genes, is located in the late Golgi compartment. It transports copper from the cytosol to the Golgi lumen for biosynthetic incorporation into Fet3p, which is the yeast ortholog of ceruloplasmin. Existence of a yeast ortholog of copper ATPases in yeast makes it an excellent model organism to study Wilson and Menkes diseases. *CCC2* deletion mutant shows defects in iron uptake [18] and respiration but these defects can be restored by supplementing the growth medium with copper or, in some cases, iron [24]. Deletion of

CCC2 gene also results in deficient inositolphosphorylceramide D (IPC-D) production, possibly due to failure to deliver copper to an unknown enzyme [25]. The expression level of *CCC2* gene was induced in response to iron starvation [26].

The aim of this present study is to investigate the genome-wide effect of the absence of copper ATPase using *CCC2* deletion strain of yeast as the model organism. For this purpose, transcriptional and metabolic responses of the yeast cells to the absence of *CCC2* gene were investigated in a copper level dependent manner. Taking the advantage of the wealth of information on protein-protein and protein-DNA interaction in yeast, transcriptome data was analysed in conjunction with the interactome and regulome in order to elucidate the mechanisms that were affected from the absence of *CCC2* gene and the disturbance in the copper homeostasis.

2.2. Materials and Methods

2.2.1. Strains and Growth Media

Homozygous *ho*Δ*/ho*Δ and *ccc2*Δ*/ccc2*Δ strains of *S. cerevisiae* from a genetic background of BY4743 (*MAT*a*/MAT*Δ *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/l*eu2*Δ*0 lys2*Δ*0*/+ *met15*Δ*0*/+ *ura3*Δ*0*/*ura3*Δ*0*), purchased from the European *Saccharomyces cerevisiae* Archive for Functional Analysis [27], was kindly provided by Prof. Stephen G. Oliver. The absence of the deleted gene was verified using PCR-based methods.

The preculture was incubated in YPD medium (2% [wt/vol] D-glucose, 2% [wt/vol] peptone, 1% [wt/vol] yeast extract) at 30°C and 180 rpm in an orbital shaker. Synthetic defined medium without $CuSO_4$ (copper deficient condition), with standard copper concentration (0.04 μM) (low or adequate copper level) and with 0.5 mM $CuSO_4$ supplementation (high copper level) was used in the fermentations.

2.2.2. Experimental Design and Sample Description

Batch fermentations were performed using B-Braun Biostat B plus fermenters with 1.5L working volume at three different copper levels of synthetic defined medium in

duplicates. The temperature was controlled at 30^{o}C and the agitation was set to 800 rpm. The pH was controlled at 5.5 with 1M NaOH and HCl. The air flow into the fermenters was adjusted at 1.5 L/min to keep dissolved oxygen saturation above 80% throughout the experiment. RNA, metabolite and biomass samples were collected at mid-exponential phase at an OD range of 0.7-0.8. The samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until further processing. Biomass concentration was determined using gravimetric methods.

For the determination of intracellular iron levels, the reference and the *CCC2* deleted cells were cultivated in the Erlenmeyer flasks under copper deficient, low copper and high copper conditions in duplicates. Samples were collected at mid-exponential phase.

2.2.3. RNA Extraction and Microarray Hybridization

RNA was isolated from samples with the Quigen RNeasy mini kit (Cat no: 74106) using the "RNeasy protocol for extracting yeast via enzymatic lysis" using robotic workstation, QIAcube (Quiagen, USA), modified by the manufacturer for yeast applications. β-mercaptoethanol (Cat no: 444203) was purchased from Merck. The nucleic acid concentrations were determined and the purity of the RNA (A_{260}/A_{280}) was confirmed using NanoDrop (ND-1000, Thermo Scientific). The observed yield was ensured to be at least 90 % of the expected yield. RNA integrity was determined using Bioanalyzer 2100 (Agilent Technologies, USA) using RNA6000 Nanokit (Agilent Technologies, USA). The samples with RIN values 7-10 were processed.

First-strand cDNA was synthesized and then converted into a double-stranded DNA initially from 100ng of total RNA using GeneChip® 3' IVT Express Kit (Affymetrix Inc., U.S.A). This double stranded cDNA was used as a template for *in vitro* transcription and synthesis of biotin-labelled aRNA. The final product was purified and quantified using the Nanodrop spectrophotometer before fragmentation. The purification and fragmentation steps were carried out using GeneChip reagents. Fragmented aRNA was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Affymetrix Yeast 2.0 arrays were prepared for hybridization using the reagents supplied in the GeneChip® Hybridization, Wash, and Stain Kit. A total of 5μg of aRNA was loaded onto 169 format

arrays and hybridized for 16 hours. The chips were then loaded into a fluidics station for washing and staining using Affymetrix Command Console® Software (AGCC) 3.0.1 Fluidics Control Module with Mini_euk2v3. Finally, the chips were loaded onto the Affymetrix GeneChip Scanner 3000. All applications were performed as described in the Affymetrix GeneChip®Expression Analysis Technical Manual.

2.2.4. Metabolite Extraction

Extraction of extra and intracellular metabolites were carried out as described previously [28]. Samples were subjected to methanol quenching and immediate centrifugation. The supernatant was used for the analysis of extracellular metabolites. Boiling ethanol procedure [29] was used in order to obtain intracellular metabolites. The extracts were lyophilised and stored at −20 °C until further analysis.

2.2.5. Metabolome Analysis

Chromatographic separation was performed using an Agilent 1260 Infinity UPLC (USA) with a 4.6 x 100 hydrophilic interaction chromatography (HILIC) column (Zorbax Rapid Resolution, particle size 3,5 µM). The initial composition of the mobile phases were 15 % (A) containing 40 mM ammonium formate with 2% formic acid and 85 % (B) acetonitrile followed by a linear gradient to 85% A and 15% B in 13 minutes with a 2 minutes hold and 4 minutes of re-equilibration of the column to its initial conditions prior to the next run, thus a total assay time for each assay was 19 minutes. The flow rate was 0.4 µL/min with a 2 µL sample injection.

The amino acids standards were obtained from Sigma-Aldrich, Inc. (USA). The optimal MS/MS MRM transitions, fragmentation patterns and the retention time behaviours of each amino acid were determined using Agilent 6430 Triple Quadropole LC-MS (USA). The MS parameters were as follows: gas temperature at 300^{o}C, gas flow 10 L/min, nebulizer pressure 40 psi, the capillary current at 3750 V. The ESI probe was operated at positive ion mode.

The quantification of the samples was acquired using MassHunter Quantitative Analysis Software, version B.05.00 (Agilent Tech. Inc., USA) against calibration curves generated with standard solutions.

Microsoft Excel Analysis ToolPak Add-in was used to calculate the significance of change in the metabolite levels by 2-way ANOVA analysis.

2.2.6. Intracellular Iron Determination and Sample Preparation

Samples (24 ml) were collected from the cell cultures that were grown until mid-exponential phase in the Erlenmeyer flasks under copper deficient, low copper and high copper conditions. The samples were centrifuged at 400g for 3 minutes at 4 ^{o}C. The cell pellets were washed with 1mM KCN and centrifuges at 400g for 3 min. After digestion in 5 ml of nitric acid at 100°C for 2 h, the samples were centrifuged at 10000g for 2 min and diluted with 5 ml of water.

The intracellular iron concentrations were kindly determined by Redokslab Analytical Systems Inc., *Istanbul, Turkey*, using iCap Q ICP-MS (Thermo Scientific Inc., USA).

2.2.7. Microarray Data Acquisition and Analysis

The cell files generated by Microarray Suite v5.0 were pre-processed with dChip software [30] using the perfect match (PM)-miss match (MM) difference model to obtain the expression levels of transcripts. These values were normalized by a baseline array with a median overall intensity. The array corresponding to reference strain with low/adequate copper condition was selected as the baseline array. The presence of single, array and probe outliers was checked, and no outlier chips were identified.

Hierarchical Clustering Explorer (HCE) 3.0 [31] was used for hierarchical clustering of the conditions. Euclidian distance was used as the similarity measure and average linkage was used as the linkage method in hierarchical clustering analysis.

In order to identify the significantly expressed genes, two-way Analyses of Variance (ANOVA) analyses were performed using Matlab statistics toolbox. In order to identify the differentially expressed genes, fold change analyses were performed using dChip sample comparison. P-value threshold was selected as 0.05. The fold change threshold was set as 1.5 with a 90% lower confidence bound (LCB) and intensity difference greater than 50. The significantly expressed genes were determined as the genes, which satisfied both p-value and fold change thresholds.

These significantly expressed genes were analysed in three groups. The first group, G1, included the genes that were significantly expressed only in response to deletion of *CCC*2 gene. The second group, G2, contained the genes, which were significantly expressed only in response to copper level. The last group, G3 compose of the genes, which were affected from the interactive effect of gene deletion and copper level. For the genes in G1, fold changes were considered between reference strain and *CCC*2 deleted strain at any copper level. The genes in G2 satisfied the fold change threshold between changing copper levels in any strain. The genes in G3 showed a fold change above the threshold either between two strains at any copper level or between changing copper levels at any strain. The significantly enriched process ontology terms of the each group were determined by BINGO [32], which is a Cytoscape plug-in [33]. The Benjamini-Hochberg method was used for the calculation of false discovery rates and 0.05 was selected as the cut-off threshold for p-values.

2.2.8. Identification of reporter proteins and transcription factors

For the integration of interactome and regulome with transcriptome, Reporter Features algorithm was used [33, 34]. The physical protein-protein interactions that were reported in BioGrid database (Release 3.1.81) [36] were used in the re-construction of the yeast protein-protein interaction network. Transcription factors in consensus list described in Yeastract database [37–39] and the genes, which were described as “regulatory activator”, “regulatory repressor” or “transcription factor” in SGD database were considered for the construction of regulatory network in yeast. The transcription factor (TF)-protein interactions with direct evidence in Yeastract database were used for Reporter TF analyses.

2.3. Results

The aim of this study was to provide a further insight into the copper homeostasis in *Saccharomyces cerevisiae* as well as to provide a systems level understanding of the copper related disorders such as Wilson and Menkes diseases. A 3 x 2 factorial design was used to elucidate the genome-wide transcriptional response to the deletion of yeast ortholog of Wilson and Menkes disease causing gene; *CCC2*, at changing copper levels. Homozygous deletion mutant of *CCC2*, which encodes Cu^{+2} transporting P-type ATPase required to export copper from the cytosol into the extracytosolic compartment, and the reference strain were cultivated in fully controlled fermenters in duplicates in glucose-rich defined medium containing three different levels of copper. The three different copper concentrations were selected such that; copper deficient condition, which was prepared by excluding the $CuSO_4.7H_2O$ from the defined medium, low copper or adequate copper concentration, which is the standard amount of copper in defined medium (0.04 μM) and high copper concentration (0.5 mM), which was able to restore respiration deficiency in *ccc2*Δ/*ccc2*Δ strain. Global adaptive transcriptional and metabolomic response of these cells to different extracellular copper levels were identified and compared in the samples which were collected in the mid-exponential phase (A_{600} of 0.7-0.9). Deletion mutant of *HO* was used as the reference strain.

2.3.1. Global Transcriptional Response of Δ*ccc2*/Δ*ccc2* and Reference Strain to Different Extracellular Copper Levels

Genome-wide gene expression analysis in reference strain and Δ*ccc2*/Δ*ccc2* strain, which were cultivated in media containing different copper levels, were carried out with the samples collected at mid-exponential phase. This experimental setup enabled the identification of both the transcriptional response of the cells to the deletion of *CCC2* gene at different copper levels and the adaptive transcriptional response of each strain to different extracellular copper levels.

2.3.1.1. Hierarchical Clustering of Conditions. Hierarchically clustering of the conditions using the normalized microarray data resulted in the clustering of the conditions into two major clusters according to the copper level (Figure 2.1). Biological replicates (1 and 2)

were clustered together for each strain under similar conditions. At high copper conditions, both strains were clustered together in a separate cluster from copper deficient or low copper conditions indicating a similar transcriptional response of both strains under this condition. On the other hand, for the copper deficient and low copper conditions, the key determinant for the arrangement of different clusters seemed to be the deletion of *CCC2* gene. For the reference strain, copper deficient and low copper conditions were clustered together indicating a less pronounced effect of a small change in copper level at transcriptional level of this strain. The addition of a low level of copper led the *CCC2* deleted strain to be clustered together with reference strain. However under copper deficient conditions, deletion of *CCC2* gene caused this strain to be clustered separately. This observation indicated that the *CCC2* deleted strain has a different behaviour under low (adequate) or copper deficient condition when compared to reference strain.

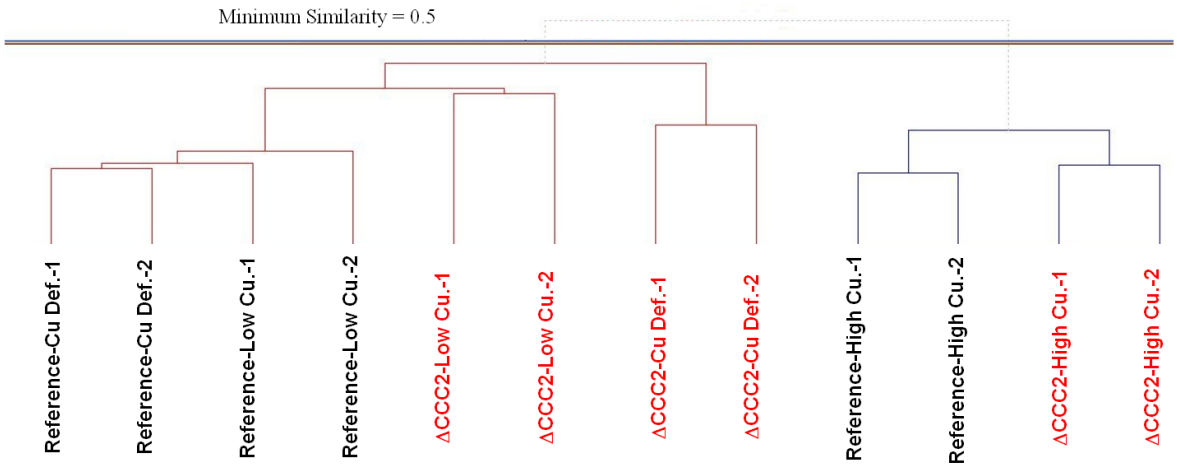


Figure 2.1. Hierarchical clustering of conditions.

2.3.1.2. Analysis of the Transcriptional Response. The normalized dataset was then examined by two-way Analyses of Variance (2-way ANOVA) and fold change analysis. Two-way ANOVA analysis assessed the significance of change (p-value < 0.05) at transcriptional level according to three different criteria; the effect of gene deletion (Factor 1), effect of change in the copper level (Factor 2) and the interaction effect between gene deletion and copper level (Factor 1 x Factor 2). The expression levels of 1260 and 2115 genes were significantly affected by gene deletion and change in the copper level without a significant interaction effect, respectively. On the other hand, 1429 genes were affected from the interaction of these factors. The genes that showed a change more than 1.5 fold in

their expression level with a lower 90 % confidence interval and intensity difference greater than 50 were defined as differentially expressed for those specific conditions.

Using the intersection of the differentially and significantly expressed genes, enabled the identification of the genes that were significantly up or down regulated in response to either gene deletion, or change in the copper level, or interacting effect of those two factors.

The intersection of the genes that were identified to be significantly expressed in response to gene deletion (without an interaction effect) and that were differentially expressed between two strains at any copper level were defined as the gene set 1 (G1).This group contains 31 genes, which were significantly expressed in response to gene deletion. Similarly, the intersection of the genes that were identified to be significantly expressed in response to a change in the copper level (without an interaction effect) and that were differentially expressed between different copper levels in any strain were defined as the gene set 2 (G2) and this group is comprised of 310 genes, which were significantly expressed in response to changing copper levels.

The intersection of the genes that were identified to be significantly expressed in response to interaction effect and that were differentially expressed either between two strains at any copper level or between changing copper levels at any strain were defined as the gene set 3 (G3). G3 contains 305 genes, for which the gene deletion and copper level made an interactive effect, in other words the effect of the changing copper levels depended on the presence or absence of *CCC2* gene. The number of significantly up- or down-regulated genes in each distinct group and the GO-biological process terms associated with each group were summarized in Figure 2.2, Figure 2.3, Table A.1, Table A.2 and Table A.3.

The numbers of the significantly up- or down-regulated genes were represented for each group separately. Blue arrows indicate the comparison between strains and purple arrows indicate the comparison between conditions, while the green and red arrows indicate the up or down regulated genes, respectively.

The transcriptional responses to the deletion of *CCC2* gene at changing copper levels were further analysed by integrating the transcriptome data with the protein-protein interaction network using Reporter Feature algorithm [33, 34]. This analysis identified the proteins, whose surrounding genes were significantly expressed in response to a perturbation in the extracellular copper levels or to the deletion of *CCC2* gene or to the interactive effect of both perturbations. Yeast protein-protein interaction network was constructed using all physical interactions reported in BioGRID database [36] and this network was integrated with the transcriptome data. Three sets of reporter proteins (p-value < 0.05), whose neighbour proteins were significantly changed in response to either change in the copper level or to the deletion of *CCC2* gene or to the interaction effect of gene deletion and change in the copper level, were identified.

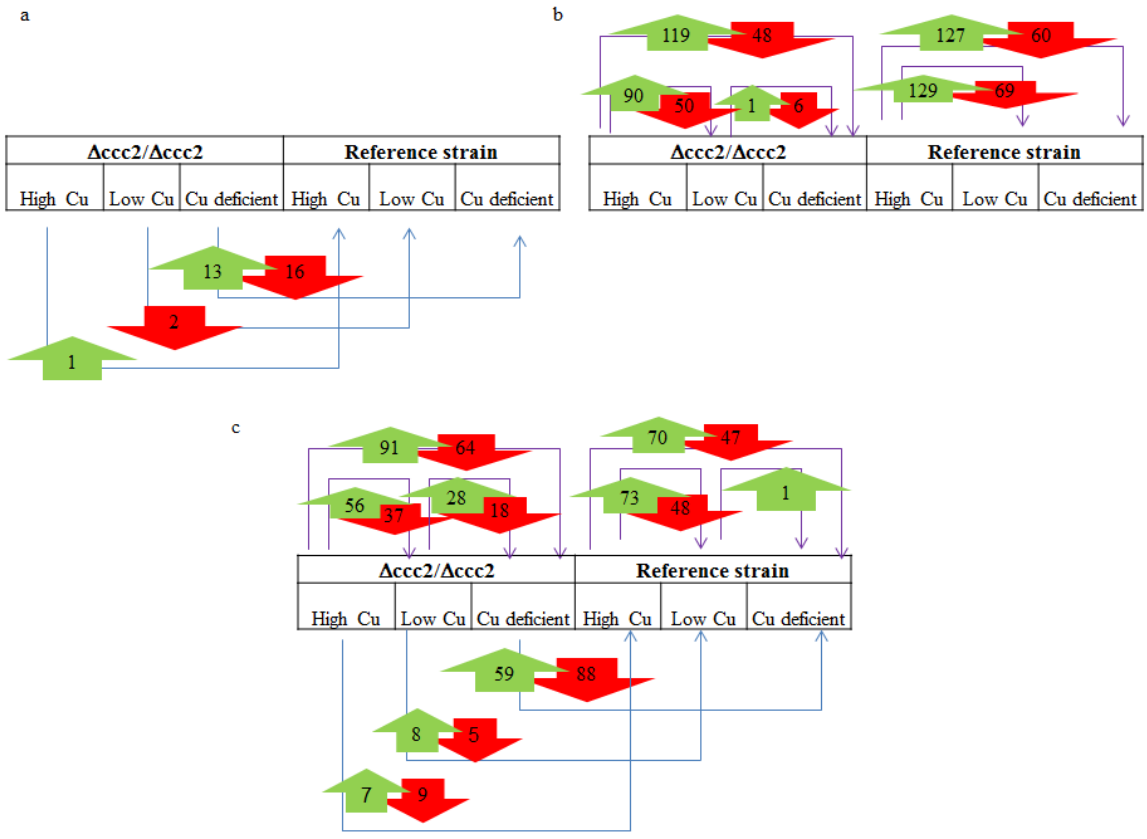


Figure 2.2. Number of the significantly expressed genes which are up and down regulated under different conditions in G1 (a), G2 (b) and G3 (c).

2.3.1.3. Transcriptional Response to the Deletion of *CCC2* Gene. Expression levels of 1260 genes were significantly changed due to deletion of *CCC2* gene irrespective of the

copper level and only 31 of them showed differential expression between two strains. 16 of these genes were differentially down-regulated and 13 genes were differentially up-regulated under copper deficient conditions. These genes were not significantly enriched with any process term (Figure 2.3).

GRX4 and *FRE3*, which are involved in metal ion homeostasis, *YPC1*, *LSC2*, *PDR12*, *GCV1* and *DUR3*, which are involved in cellular catabolic process, *MEP2*, which encodes ammonium permease, *TAT1*, which encodes amino acid transporter for valine, leucine, isoleucine and tyrosine and low-affinity tryptophan and histidine transporter and *TPT1*, which encodes tRNA 2'-phosphotransferase were induced in a response to the deletion of *CCC2* under copper deficient condition.

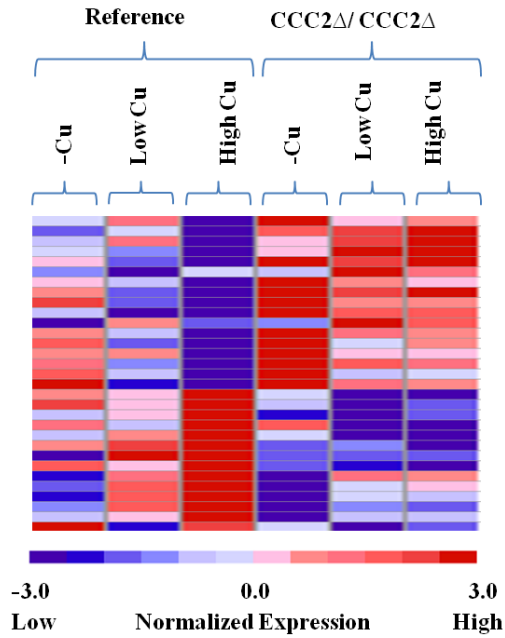


Figure 2.3. Heat map representation of significantly and differentially expressed genes in response to deletion of *CCC2*.

STF1 and *PBI2*, which are associated with negative regulation of hydrolase activity, *GPH1*, *GLO2*, *TDH1* and *AMS1* that are involved in carbohydrate metabolic process, *PDC6* and *RPO21*, which have roles in nitrogen compound metabolic process, were down-regulated in the *CCC2* deleted strain. *GPH1*, which encodes glycogen phosphorylase, *IGD1*, which encodes cytoplasmic protein that inhibits Gdb1p glycogen debranching activity and *MF(ALPHA)1*, which encodes mating pheromone alpha-factor were also

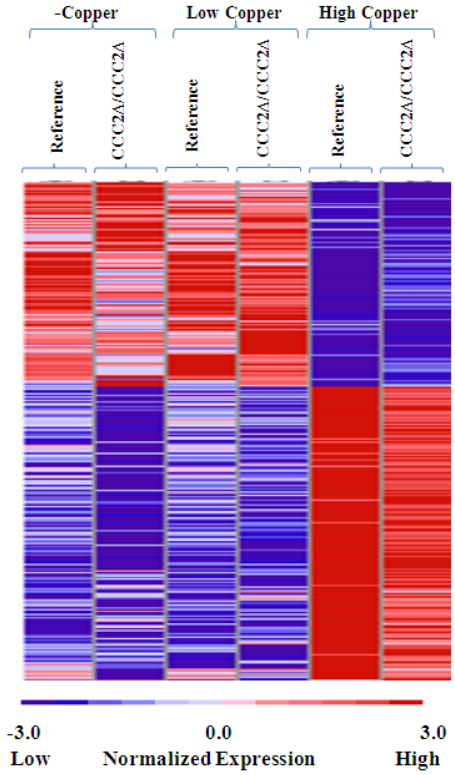
identified among the down regulated genes in a response to *CCC2* deletion under copper deficiency.

Three up-regulated (*YLR264C-A*, *YDR524W-A* and *YJL213W*) and three down regulated genes (*YKR045C*, *YMR196W*, *GIS3*) under copper deficient conditions encode proteins with unknown function.

In the presence of adequate or low level of extracellular copper, 2 genes *MF(ALPHA)1* and *CAF40*, which encodes a protein with unknown function, were observed to be down regulated in the *CCC2* deleted strain. In the presence of high extracellular copper level, *PUT1*, proline oxidase, which is involved in the utilization of proline as a sole nitrogen source, was identified to be differentially up-regulated in the absence of *CCC2* gene.

A total of 270 reporter proteins (p-value < 0.05), around which the transcriptional responses to the deletion of *CCC2* significantly concentrated were identified as reporter proteins. These proteins were enriched with proteolysis, ubiquitin-dependent protein catabolic process, phytosteroid biosynthetic process, response to stress and cellular catabolic process terms (p-value < 0.01). In order to further analyse the directionality of change around the reporter proteins, the reporter feature analysis was conducted for the genes, which were induced or repressed by the deletion of *CCC2* gene under all conditions. This analysis revealed that the reporter proteins, which were enriched with proteolysis, cellular catabolic process, phytosteroid biosynthetic process and ubiquitin-dependent protein catabolic process term, were interacting with the down-regulated genes in the *CCC2* deleted strain. The subunits of proteasome regulatory particle (Rpt5p, Rpn10p, Rpn11p and Rpn1p) ranked among the top scoring ten proteins. Bre5p, which is involved in the co-regulation of anterograde and retrograde transport between the endoplasmic reticulum and Golgi compartments and Atp4p, which encodes a subunit of mitochondrial F1-F0 ATP synthase involved in the ATP synthesis, Igo1p which prevents degradation of nutrient regulated mRNAs and Rad10p involved in the nucleotide excision repair were also identified among the top scoring reporter proteins (Table 2.1).

2.3.1.4. Transcriptional Response to Changing Copper Conditions. When high copper condition was compared to copper deficient condition, comparable number of genes was differentially expressed in both strains. 187 genes (60 down, 127 up) in the reference strain and 167 genes (48 down, 119 up) in the *CCC2* deleted strain were identified to be significantly and differentially expressed. The up-regulated genes were enriched with actin cortical patch assembly in *CCC2* deleted cells whereas in the reference strain the up-regulated genes were not significantly enriched with any process term. In the *CCC2* deleted strain the genes that are involved in copper ion import, oxidation-reduction process, and iron ion homeostasis were observed to be down-regulated in response to high copper in comparison to copper deficient conditions (p-value < 0.05). In the reference strain the down-regulated genes were enriched with ribosome biogenesis term (Figure 2.2, Figure 2.4, Table A.1).



		Enriched GO-Biological Process Terms	
EFFECT OF COPPER LEVEL		CCC2Δ/ CCC2Δ	Reference strain
UP	High Cu vs Low Cu		
	High Cu vs Cu Def	Actin cortical patch assembly	
DOWN	High Cu vs Low Cu		Ribosome biogenesis Maturation of SSU-rRNA process Copper ion import
	High Cu vs Cu Def	Copper ion import Oxidation-reduction process Iron ion homeostasis	Ribosome biogenesis
	Low Cu vs Cu Def	Copper ion import	

Figure 2.4. Heat map representation and enriched GO process terms among the significantly and differentially expressed genes in response to copper effect.

When high copper condition was compared to low copper condition, 198 genes (69 down, 129 up) in the reference strain and 140 genes (50 down, 90 up) in the *CCC2* deleted strain were differentially expressed. The up- or down- regulated genes were not

significantly associated with any process term in the *CCC2* deleted strain. In the reference strain, the down-regulated genes were found to be significantly associated with ribosome biogenesis, maturation of SSU-rRNA and copper ion import process terms (p-value < 0.01) but up-regulated genes were not significantly enriched with any process term.

When low copper and copper deficient conditions were compared in the *CCC2* deleted strain, the transcriptional responses of 7 (6 down and 1 up) genes significantly changed. The down regulated genes were enriched with copper import process. No gene was differentially expressed between copper deficient and low copper conditions in the reference strain (Figure 2.4).

The integration of the transcriptional responses with interactome identified 162 reporter proteins (p-value < 0.05), around which the transcriptional responses to a change in the copper level were significantly concentrated. These proteins were enriched (p-value < 0.01) with phytosteroid biosynthetic process, cortical actin cytoskeleton organization and transport related biological process terms including vacuolar transport and vesicle-mediated transport (Table 2.1). In order to further analyse the directionality of change around the reporter proteins, the reporter feature analysis was conducted for the genes, which were induced or repressed by high copper in both strains. This further analysis revealed that, the genes that were repressed by high copper levels were concentrated around the reporter proteins, which were enriched with phytosteroid biosynthetic process term. The reporter proteins, which were enriched with actin cytoskeleton organization, endocytosis and vacuolar transport terms, were interacting with the genes that were up-regulated by high levels of copper. Tor1p and Tor2p, which are involved in the regulation of cell growth as a response to nutrient availability, cellular stresses and endocytosis [40] and a mitochondrial membrane protein, Fmp45p required for sporulation and maintaining sphingolipid content were identified as the most significant reporter proteins in response to the change in the copper level. Syp1p with a putative role in actin cytoskelatal organization, She1p which is a mitotic spindle protein, Chc1p which is a subunit of the major coat protein involved in intracellular protein transport and endocytosis were also among the most significant top ten reporter proteins. The significant transcriptional variations were also observed around the gene encoding a multifunctional E3 ubiquitin ligase, Rps5p,which is involved in the regulation of many processes including endocytosis,

multivesicular body sorting, RNA export, transcription, lipid biosynthesis, mitochondrial inheritance and protein catabolism and the gene encoding a metalloprotease subunit of 19S regulatory particle of proteasome, Rpn11p. Rpn11p is reported to be involved in fission of mitochondria and peroxisomes [41]. Sro9p, which is involved in heme regulation of Hap1p as a component of high-molecular-weight complex (HMC) was also identified within the top ten reporter proteins, around which important transcriptional changes in a response to change in the copper level, was concentrated.

Table. 2.1. Reporter proteins identified in response to change in the copper level, deletion of *CCC2* gene and their interacting effect.

Effector	**# of Reporter Proteins (p-value<0.05)**	**Enriched Biological Process terms (p-value< 0.01)**	**TOP 10 Reporter Proteins**
Mutant Effect	270	proteolysis response to stress cellular catabolic process phytosteroid biosynthetic process macromolecule catabolic process ubiquitin-dependent protein catabolic process	RPT5 RPN10 RPN1 RPN11 HEK2 IGO1 BRE5 RAD10 ATP4 NPA3
Copper Effect	162	phytosteroid biosynthetic process cortical actin cytoskeleton organization nitrogen compound transport negative regulation of catabolic process actin filament-based process endocytosis vacuolar transport vesicle-mediated transport hydrogen transport ion transport	TOR2 TOR1 FMP45 SYP1 SHE2 RSP5 CHC1 RPN11 SPS1 SRO9

Table. 2.1. Reporter proteins identified in response to change in the copper level, deletion of *CCC2* gene and their interacting effect (continued).

Interaction Effect	193	ubiquitin-dependent protein catabolic process protein complex biogenesis macromolecule catabolic process vitamin B6 biosynthetic process cytoplasmic translation ATP biosynthetic process proton transport hydrogen transport heterocycle biosynthetic process aromatic compound biosynthetic process cellular nitrogen compound biosynthetic process nucleotide metabolic process anti-apoptosis cellular macromolecule metabolic process	VTC1 ATP4 RPF2 PET9 ATP6 RPT6 RPT5 HSM3 FCJ1 MAP1

2.3.1.5. The Interaction Effect of Gene Deletion and Copper Level. Expression levels of 305 genes showed significant change in response to the interactive effect of gene deletion and copper level. The number of significantly up- or down-regulated genes between different conditions in each strain as well as between two strains under similar conditions was presented in Figure 2.2. Further analysis of these genes revealed the biological processes that were up- or down-regulated as a response to an interactive effect of the deletion of *CCC2* and changing copper concentrations (Figure 2.5, Table A.2 and Table A.3).

When high copper condition was compared to copper deficient condition, 91 genes were up-regulated in the *CCC2* deleted strain and they were enriched with ATP synthesis coupled electron transport. Seventy genes, which were up-regulated under similar conditions in the reference strain, were significantly enriched with iron homeostasis, siderophore transport, *de novo* NAD^+ biosynthetic process from tryptophan, *de novo* UMP biosynthetic process, response to copper ion and cell wall organization and biogenesis process terms. In the *CCC2* deleted strain, 64 down-regulated genes were found to be significantly enriched with iron homeostasis and siderophore transport terms (p-value < 0.01). 47 genes, which were down-regulated in the reference strain between these

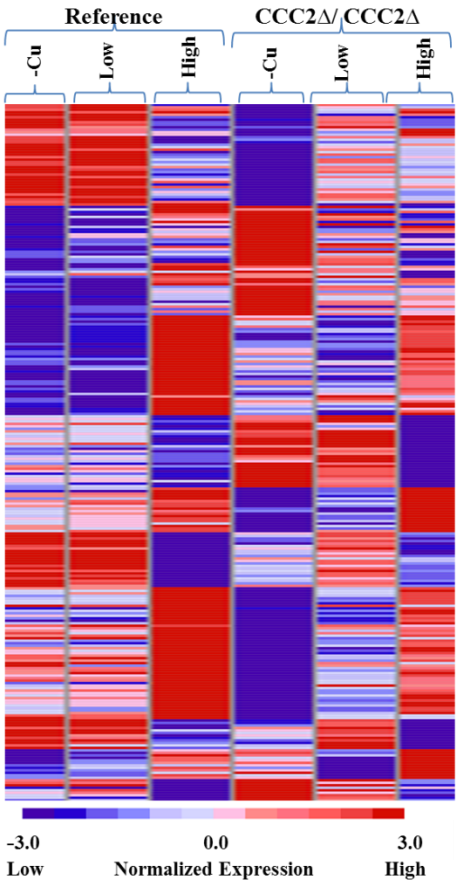
conditions, were not found to be significantly enriched with any particular GO biological process term.

When high copper condition was compared to low copper condition, 56 genes were up regulated in the *CCC2* deleted strain and they were significantly associated with carboxylic acid and arginine metabolic process term (p-value < 0.01). A total of 73 genes, which were observed to be up-regulated under similar conditions in the reference strain, were significantly associated with iron ion homeostasis and siderophore transport process terms (p-value < 0.05). On the other hand, 37 and 48 genes were differentially down-regulated when high copper condition was compared to low copper condition in the *CCC2* deleted and reference strain, respectively. However these groups were not found to be significantly enriched with any biological process term.

When low copper condition was compared to the copper deficient condition, 28 genes were up-regulated and 18 genes were down-regulated in the *CCC2* deleted strain. The down-regulated genes were enriched with arginine, ornithine, urea, cellular ketone metabolic processes, drug transmembrane transport and *de novo* UMP biosynthetic process terms (p-value < 0.01). The up-regulated genes were not significantly associated with any biological process term. In the reference strain, only a single gene; *BNA2*, which encodes putative tryptophan 2,3-dioxygenase or indoleamine 2,3-dioxygenase, required for de novo biosynthesis of NAD^+ from tryptophan via kynurenine, was found to up-regulated.

When differentially expressed genes in the CCC2 deleted strain in comparison to reference strain, were investigated under changing copper levels, 59 genes were identified as differentially up-regulated under copper deficient conditions and they were found to be enriched with iron homeostasis, siderophore transport, arginine and cellular ketone metabolic processes, drug membrane transport, ornithine and urea metabolic process, de novo NAD+ process from tryptophan ,de novo UMP process, allantoin and pyridoxal phosphate metabolic process terms. In the *CCC2* deleted cells, 88 genes were down regulated under this condition and they were significantly enriched with glucose transport, phosphate metabolic process and ribosome biogenesis.

In the presence of high levels of extracellular copper, 9 genes, which were significantly enriched with iron ion homeostasis, siderophore transport, de novo NAD^+ biosynthetic process from tryptophan, ATP metabolism and oxidation-reduction process terms were down-regulated in the *CCC2* deleted strain. The up-regulated genes under this condition and the genes that were up- or down-regulated under low copper conditions in the *CCC2* deleted cells were not found to be significantly enriched with any particular GO biological process term.



		Enriched GO-Biological Process Terms	
INTERACTION EFFECT		CCC2Δ/ CCC2Δ	Reference strain
UP	High Cu vs Low Cu	Arginine metabolic process Carboxylic acid metbaolic process	Iron ion homeostasis Siderophore transport
UP	High Cu vs Cu Def	ATP synthesis coupled electron transport	Iron ion homeostasis Siderophore transport "de novo" NAD biosynthetic process from tryptophan Response to copper ion Cellular cell wall organization or biogenesis Regulation of sporulation
DOWN	High Cu vs Cu Def	Iron ion homeostasis Siderophore transport	
DOWN	Low Cu vs Cu Def	Arginine metabolic process Drug membrane transport	

		Enriched GO-Biological Process Terms	
INTERACTION EFFECT		Copper Deficient Conditions	High Copper Conditions
UP	CCC2Δ/ CCC2Δ vs Reference strain	Iron ion homeostasis Siderophore transport Arginine metabolic process "de novo" NAD biosynthetic process from tryptophan pyridoxal phosphate metabolic process	
DOWN		Glucose transport Polyphosphate metabolic process Ribosome biogenesis	Iron ion homeostasis Siderophore transport "de novo" NAD biosynthetic process from tryptophan Oxidation reduction process

Figure 2.5. Heat map representation and enriched GO process terms among the significantly and differentially expressed genes in response to interaction effect.

A total of 193 proteins were identified as reporter proteins (p-value < 0.05), around which the transcriptional changes in response to interacting effect of changing copper levels and deletion of *CCC2* gene were concentrated. These proteins were enriched with several metabolic process terms including ubiquitin-dependent protein catabolic process, ATP biosynthetic process, vitamin B6 biosynthetic process, proton and hydrogen transport,

anti-apoptosis process terms. *VTC1*, which encodes a subunit of the vacuolar transporter chaperone (VTC) complex, *ATP4* and *ATP6* which encode mitochondrial subunits of ATP synthase, *PET9*, which encodes a major ADP/ATP carrier of the inner mitochondrial membrane and heme transporter, *RPT5*, *RPT6* and *HSM3*, which are involved in assembly of proteasome regulatory particle and *RCJ1*, which encodes a mitochondrial inner membrane protein involved in the import of intermembrane space (IMS) proteins, probably by positioning Mia40p relative to the TOM complex to receive incoming proteins, were among the ten top ranking reporter proteins identified in response to interacting effect of deletion of *CCC2* gene and change in the copper level.

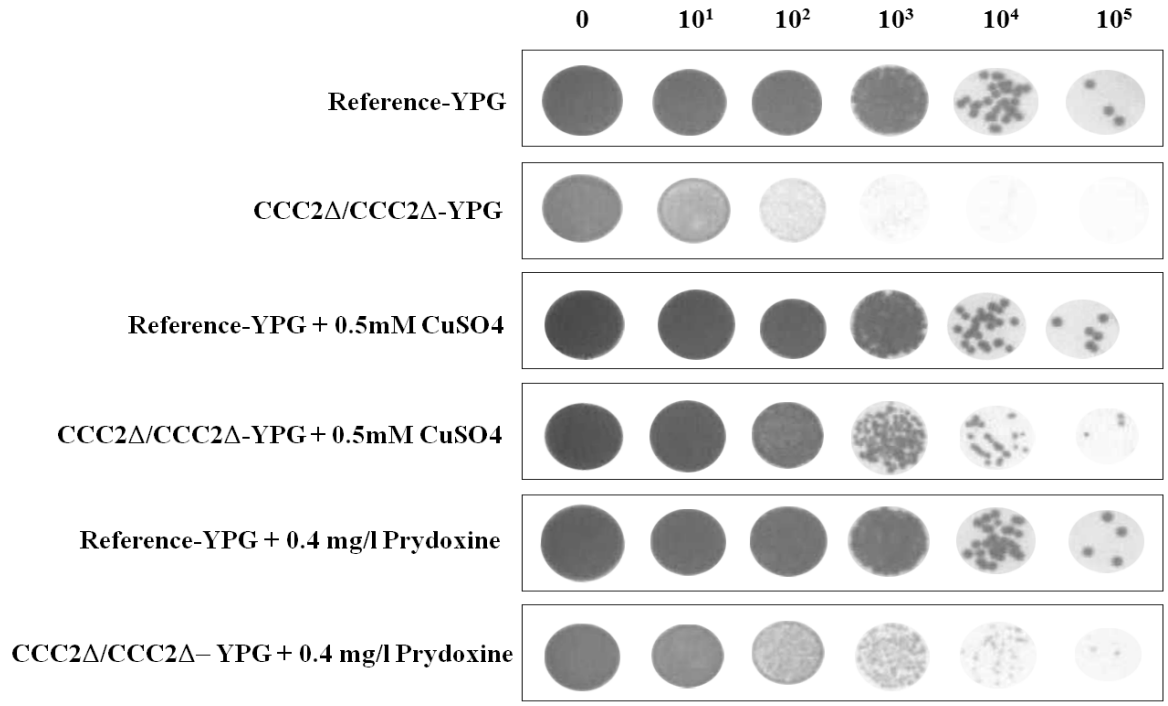


Figure 2.6. Analyses of respiratory capacity of the reference and *CCC2* deleted strain using spot assay.

Identification of pyridoxine biosynthetic pathway to be affected from the deletion of *CCC2* gene in a copper level dependent manner and also the up-regulation of *SNZ1*, which encodes a protein involved in vitamin B6 biosynthesis, in *CCC2* deleted cells under copper deficient conditions rise the question that whether absence of *CCC2* gene might be leading to pyridoxine deficiency, which also leads to iron deficiency [42]. The analyses of the significantly expressed genes and also the intracellular iron levels under copper deficient conditions revealed that absence of *CCC2* gene leads to iron deficiency, which results in

respiratory deficiency [24]. This respiratory and iron deficiency could be restored by addition of copper. In order to test whether the respiratory deficiency in the *CCC2* deleted cells could also be restored by supplementing pyridoxine to the growth medium, cells were grown on pyridoxine containing YPG plates. It was observed that addition of pyridoxine resulted in an improvement in the respiratory capacity of the *CCC2* deleted cells although its effect was not pronounced as that of copper supplementation (Figure 2.6).

2.3.1.6. Analysis of Reporter Transcription Factors. In order to identify the regulatory pathways, which were affected in response to changing copper levels or in response to deletion of *CCC2* gene or in response to the interacting effect of these two factors, Reporter feature analysis were conducted using yeast regulatory network. For the construction of regulatory network in yeast, the transcription factor (TF)-protein interactions with direct evidence in Yeastract database were curated [37–39]. In order to obtain the list of the transcription factors in yeast, the TF consensus list described in Yeastract database and the genes, which were described as "regulatory activator", "regulatory repressor" or "transcription factor" in SGD database were used. A total of 148 transcription factors were identified and only for 127 TFs, at least one TF-protein interaction with direct evidence was reported in Yeastract database. The reconstructed yeast regulatory network comprise of 24702 TF-protein interactions.

This analysis identified 20, 24 and 24 top-scoring (p-value < 0.01) transcription factors, whose interacting partners were significantly changed in response to either change in the copper level or deletion of *CCC2* gene or the interaction effect of gene deletion and change in the copper level, respectively (Figure 2.7).

Ten of these transcription factors were commonly identified among the top scoring TFs in response to both copper effect, mutant effect and interaction effect indicating that these transcription factors play a major role in the transcriptional organization in response to a perturbation in iron and copper homeostasis pathway. Hap1p and Hap5p, which are involved in the regulation of cellular respiration, Flo8p, Ste12p, Mga1p, Tec1p, which are associated with filamentous growth, Sko1p, Swi4p, Pho4p and Skn7p, which regulate transcription in response to stress, were the commonly identified reporter transcription factors.

Cad1p, Cup2p, Ino2p, Rgt1p and Rlm1p were identified as reporter transcription factors specifically in response to change in the copper level. Cad1p and Cup2p are involved in iron and copper metabolism, respectively. Ino2p, which is required for derepression of phospholipid biosynthetic genes, Rgt1p, which is a glucose responsive transcription factor that regulates the expression of several glucose transporter genes and Rlm1p, which is involved in the maintenance of cell integrity were also among the reporter transcription factors that were identified only in response change in the copper level.

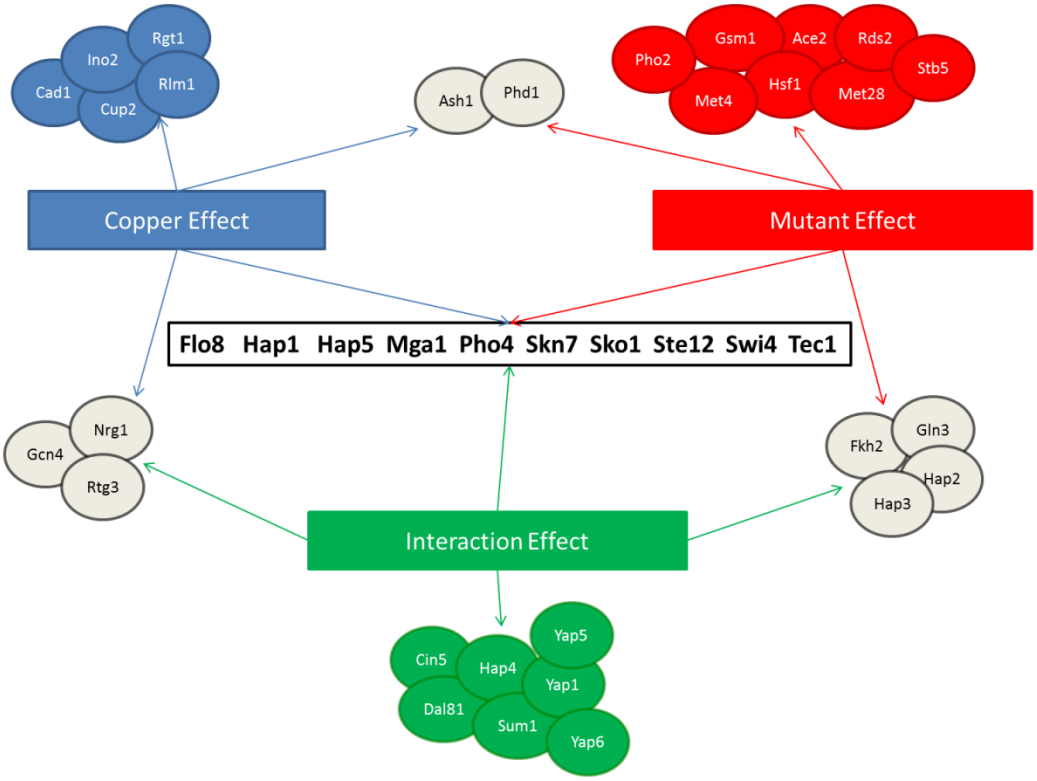


Figure 2.7. Reporter transcription factors in response to the change in the copper level, deletion of *CCC2* gene or their interacting effect.

Rtg3p, Gcn4p and Nrg1p were identified as key transcription factors responsive to copper and interactive effect of copper and *CCC2* deletion. Rtg3p is involved in the activation of the retrograde (RTG) and TOR pathways and Nrg1p is a transcriptional repressor which recruits the Cyc8p-Tup1p complex to the promoters to mediate glucose repression. Gcn4p is a transcriptional activator of amino acid biosynthetic genes and is also involved in purine biosynthesis, organelle biosynthesis, autophagy, glycogen homeostasis and multiple stress responses [43].

Eight transcription factors were identified as reporter transcription factors only in response to deletion of *CCC2* gene. Ace2p, which is involved in the cell cycle and plays a role in the basal-level expression of *CUP1* [44], Hsf1p, whose target genes are involved in protein folding, detoxification, energy generation, carbohydrate metabolism, and cell wall organization, Pho2p, which regulates genes involved in phosphate metabolism and Rds2p, which is involved in regulating gluconeogenesis and glyoxylate cycle genes, Hsf1p, whose target genes are involved in protein folding, detoxification, energy generation, carbohydrate metabolism, and cell wall organization, were among the key reporter TFs that were identified only in response to deletion of *CCC2* gene. Hsf1p was also reported to be involved in the activation of yeast metallothionein gene, *CUP1*, in a response to oxidative stress [45]. Met4p and Met28p, which are involved in the biosynthesis of sulfur-containing amino acids, glutathione metabolism and heavy metal response, Stb5p, which is involved in regulating multidrug response and oxidative stress response, Gsm1p, which is proposed to be involved in the regulation of energy metabolism were also identified as the reporter transcription factors, around which important transcriptional changes occurred in response to the deletion of *CCC2* in yeast cells.

There were 2 transcription factors, which were identified among the key transcription factors both in response to gene deletion and change in the copper level. These transcription factors were, Phd1p, which is the transcriptional activator of the genes involved in pseudohyphal growth, and Ash1p, which is required for the activation of *FLO11* during pseudohyphal growth.

Seven transcription factors were identified only when the significantly expressed genes in response to the interaction effect of copper level and gene deletion was considered in reporter TF analyses. Although they are not significantly expressed, members of YAP family; Yap1p, which is the major oxidative stress regulator and regulator of iron metabolism, Yap5p, which is also involved in the regulation of vacuolar iron storage [46], Yap6p and its paralog Cin5p, which are involved in osmotic stress, were identified as reporter transcription factors [47]. These results indicated that the stress response activated in response to both the deletion of *CCC2* gene and changing copper levels were further regulated by a HSF-independent mechanism involving *YAP* transcription factors. The stress factors Yap6p, Cin5p, Sko1p and Skn7p, which were identified as key transcription

factors in response to the interaction effect of the deletion of *CCC2* and change in the copper levels, were also reported to physically interact with Tup1p and/or Ssn6 and direct this complex to their targets [48]. Tup1-Ssn6 complex is involved in the repression of genes that are activated in response to environmental changes and cellular stress. Hap4p, which a transcriptional activator and global regulator of respiratory gene expression, Dal81p, which is positive regulator of genes in multiple nitrogen degradation pathways and Sum1p, which is a transcriptional repressor required for mitotic repression of middle sporulation-specific genes, were also among the transcription factors identified in response to interaction effect.

The other members of HAP family, namely Hap2p and Hap3p, which are members of Hap complex acting as global regulator of respiratory gene expression, were identified as reporter transcription factors both in response to interaction effect and mutant effect. HAP complex was reported as the key regulator of the mitochondrial function and involved in the control of nuclear genes implicated in several mitochondrial processes such as import and mitochondrial division [49]. Gln3p, which up-regulates the genes involved in the nitrogen catabolite repression under nitrogen limited conditions and Fkh2p, which regulates the expression of G2/M phase genes were also identified among the key transcription factors both in response to gene deletion and interaction effect.

2.3.2. The Effect of Deletion of *CCC2* Gene and Changing Copper Levels on Metabolite Profiles

In order to further investigate the effect of changing extracellular copper levels and deletion of *CCC2* gene on metabolic pathways, intracellular and extracellular amino acid levels were determined using LC-MS-MS-MS. Metabolome analyses in reference and *CCC2* deleted cells were conducted also using the samples collected at mid-exponential phase in order to observe whether there is a correlation between the transcript and metabolite levels. The metabolites, for which reproducible results were obtained, were further investigated (Figure 2.8). Since the number of the identified metabolites was relatively low when compared to transcriptome and interactome, this data was not used in the integrative analysis. The metabolome data was only used to support the analysis of the transcriptional response.

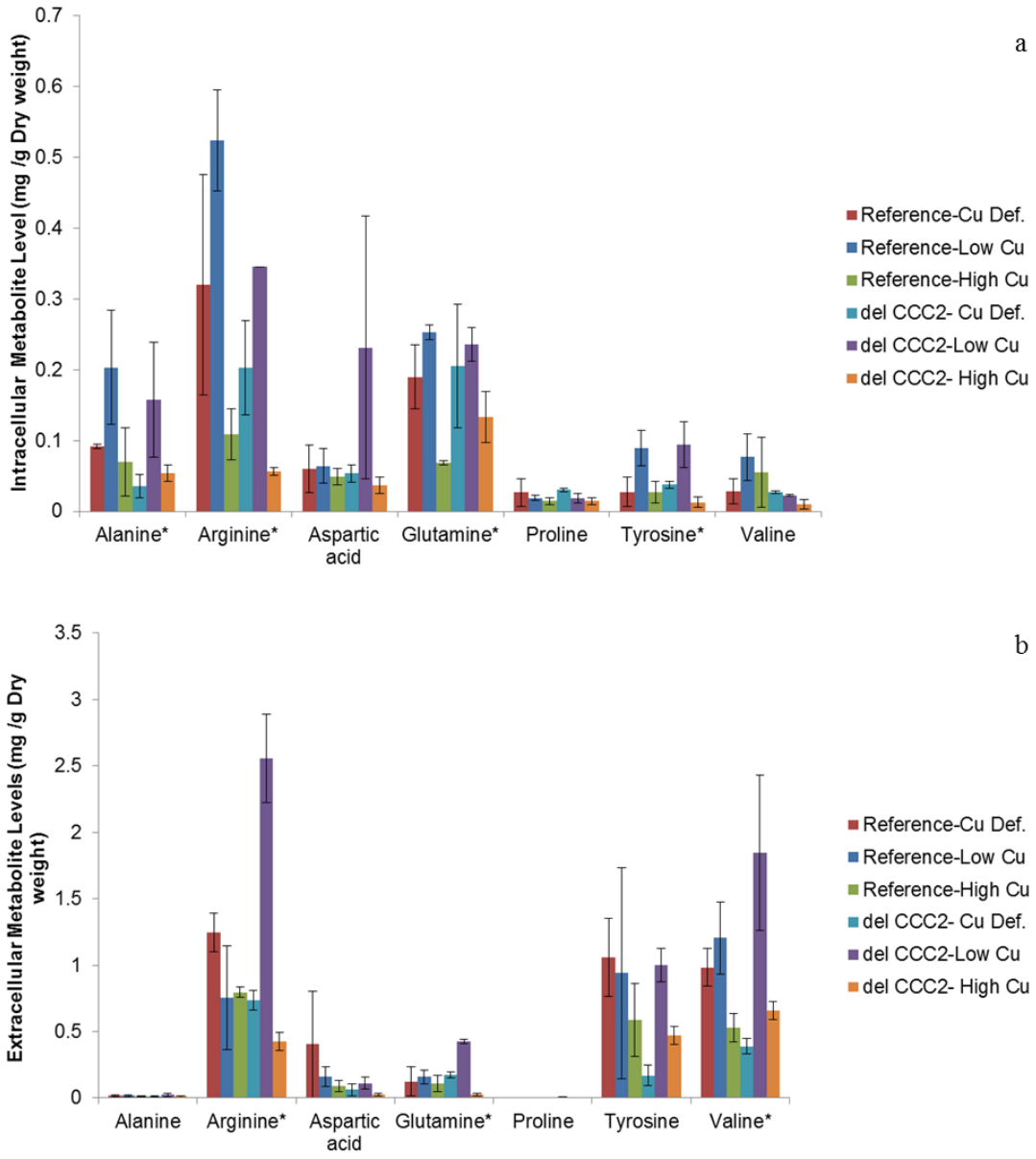


Figure 2.8. Intracellular (a) and extracellular (b) metabolite levels in response to changing copper levels and deletion of *CCC2* gene. * indicates p-value < 0.05.

Intracellular levels of alanine, arginine, glutamine and tyrosine were observed to be significantly affected from the deletion of *CCC2* gene and the presence of different extracellular copper or from both perturbations (p-value < 0.05). Under adequate level of copper containing conditions, the intracellular levels of these metabolites were higher in comparison to copper deficient or high copper conditions. Lowest levels of arginine, glutamine and tyrosine were observed under high copper conditions for both strains. Intracellular alanine levels in the *CCC2* deleted strain under copper deficient condition

were slightly lower than that of under high copper conditions, whereas in the reference strain, the lowest alanine levels were identified under high copper conditions.

When the intracellular metabolite levels in these two strains were compared, it was observed that alanine and arginine levels were lower in the *CCC2* deleted strain under all conditions. Glutamine levels were observed to be higher in the *CCC2* deleted strain under copper deficient and high copper conditions. Tyrosine levels were observed to be not affected from the deletion of *CCC2* gene under low or copper deficient conditions however, under high copper conditions tyrosine levels in this strain showed a 2-fold decrease.

When the extracellular metabolite levels were analysed, it was observed that arginine, glutamine and valine concentrations were significantly changed. In the *CCC2* deleted strain, these metabolites showed a sharp increase under low copper condition. The extracellular arginine and glutamine levels under copper deficient conditions were relatively higher than that were observed under high copper conditions for both strains. The extracellular valine concentration were lower under high copper condition in comparison to copper deficient conditions in reference strain, whereas in *CCC2* deleted strain higher concentration was obtained under high copper condition.

When the extracellular metabolite levels of these two strains were compared, arginine levels were observed to be lower under copper deficient and high copper conditions in the case of *CCC2* deleted strain in comparison to reference strain under same conditions. However, under adequate level of copper containing condition, arginine level was much higher in the case of *CCC2* deleted cells. The change in the extracellular glutamine concentrations between two strains was highly dependent on the copper levels. Under copper deficient conditions the difference was not pronounced, however adequate copper levels led to an increase and high levels of copper led to a notable decreased in the extracellular glutamine concentration in the case of *CCC2* deleted strain in comparison to reference strain.

2.3.3. The Effect of Deletion of *CCC2* Gene and Changing Copper Levels on Intracellular Iron Levels

In order to observe the effect of the deletion of the *CCC2* gene and also changing copper levels on the iron pools, intracellular iron levels of the reference and *CCC2* deleted cells were determined using ICP-MS using the samples collected at mid-exponential phase in order to analyse the transcript levels in conjunction with iron levels (Figure 2.9).

This analysis revealed that cellular iron levels were lower in the *CCC2* deleted strain under copper deficient and low copper conditions in comparison to the reference strain. On the other hand, higher extracellular copper concentration resulted in elevated intracellular iron levels in this strain and this intracellular accumulation was observed to be higher than even that in the case of the reference strain.

On the other hand, iron levels did not change in response to changing copper levels in the reference strain when cells were cultivated in batch cultures.

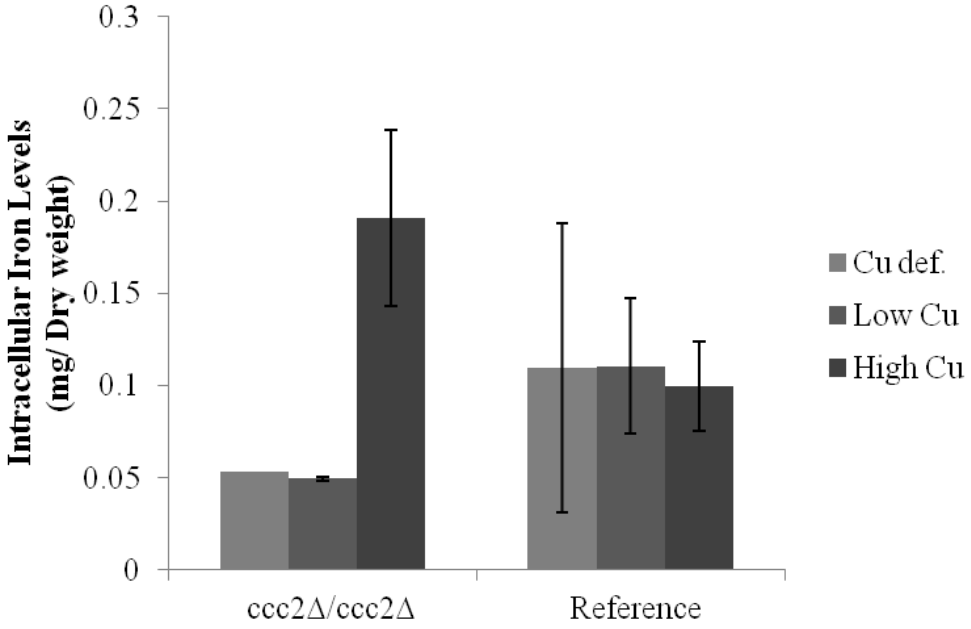


Figure 2.9. Intracellular iron levels in response to changing copper levels and deletion of *CCC2* gene.

2.4. DISCUSSION

2.4.1. Experimental Design

In the present study a systems biology approach was used to investigate the transcriptional response of yeast cells to the deletion of *CCC2* gene, the yeast ortholog of Wilson and Menkes disease causing genes, under different conditions. The 3x2 factorial design applied in this study enabled the observation of the effect of *CCC2* deletion in a copper level dependent manner.

The three different copper levels, under which the transcriptional response to the deletion of *CCC2* gene was investigated, were selected such that cells were grown under either copper deficient, or adequate level of copper, or copper enriched conditions. Copper deficient conditions were generated simply by excluding the $CuSO_4.7H_2O$ from the defined medium instead of using copper chelating agent in order to avoid the unspecific chelation of other metals [50] and also unpredictable side effects of the chemicals on the transcriptional response of yeast cells. Induction of the *CTR1* gene under copper deficient conditions in comparison to both high copper and low (adequate) copper conditions confirmed that copper deficient condition could be mimicked by excluding the $CuSO_4.7H_2O$ from the defined medium. High copper concentration was selected in such a way that it was sufficient to restore the respiration deficiency in *CCC2* deleted cells.

The significance of the change in the expression level of each gene in response to each factor could be identified by two-way ANOVA analyses. Integration of the two-way ANOVA and fold change analyses enabled the identification of significantly up or down regulated genes in response to gene deletion or change in the copper level and more importantly interactive effect of gene deletion and copper level.

2.4.2. The Global Transcriptional Reprogramming in Response to *CCC2* Deletion

Analysis of the global transcriptomic and metabolic response of yeast cells to the deletion of *CCC2* and/or changing copper levels in comparison to the control indicated that several metabolic processes including copper and iron uptake and homeostasis, ATP

synthesis coupled electron transfer, actin cortical patch assembly, ribosome biogenesis, NAD^+ metabolism, amino acid metabolism, allantoin and pyridoxal phosphate biosynthesis and glucose and drug transport mechanisms were affected. Integrative analysis of transcriptome interactome and regulome provided additional supportive information as well as pointing out several affected processes, which were missed out when GO enrichment analysis alone was conducted.

2.4.2.1. Copper and Iron Uptake and Homeostasis. The genes, which are involved in copper ion import process, were down regulated in response to high copper in both strains. The down-regulation of the copper ion import genes was consistent with the finding that the copper overload may decrease copper uptake [19]. *CTR1*; high affinity copper transporter, *FRE1* and *FRE7*; cubric and ferric reductases were also significantly down-regulated in the *CCC2* deleted strain under adequate level of extracellular copper in comparison to copper deficient conditions. In the reference strain, down-regulation of *CTR1* and *FRE1* was observed only in response to high copper levels. *FET4*, which encodes a low affinity iron (Fe^{+2}) and copper transporter was repressed in both strain in the presence of high extracellular copper levels. These results are in good concordance with the previous reports stating the induction of *CTR1*, *FRE1* and *FRE7* in response to copper deficiency in yeast [51]. The fact that copper import is repressed even in the presence of adequate levels of copper in *CCC2* deleted strain indicated the higher sensitivity of this strain to copper when compared to reference strain.

The genes encoding two soluble Cu(I)-binding metallochaperons, namely *ATX1*, *CCS1* and *COX17*, which transport the copper ions to the sites of utilization, did not display any differential expression in both strains in response to increasing extracellular copper levels or copper deficiency. The expression levels of *CUP1* and *CRS5*, which encode the proteins to sequester the intracellular copper, were up-regulated in response to high amounts of extracellular copper in both strains. Interestingly no significant change could be observed in the expression levels of *CTR2* and *CCC1*, which are involved in the vacuolar transportation of copper in any condition investigated within the framework of the present study. The expression level of *CCC2* gene was observed to be up-regulated when compared to condition containing adequate level of copper or copper deficient condition in the reference strain.

Copper and iron metabolisms are closely linked through Fet3p, which is a high affinity iron transporter and should be loaded with copper by Ccc2p to transfer iron [19, 51]. The presence of high amount of extracellular copper resulted in the induction of *FET3* in comparison to copper deficient condition both in the *CCC2* deleted and reference strains.

The genes associated with siderophore transport (*FIT1*, *FIT2*, *FIT3*, *ARN4* and *ARN2*) were observed to be significantly and differentially expressed in response to both the deletion of *CCC2* gene and change in the copper level, thus indicating that both perturbation affected iron homeostasis. These genes were observed to be up-regulated in the *CCC2* deleted strain under copper deficient conditions in comparison to the reference strain and also in comparison to high copper condition. However, some of these genes, *ARN2* and *FIT2*, were observed to be down regulated in comparison to the reference strain under high copper conditions. *FIT1*, *FIT2* and *FIT3*, which encode a family of proteins involved in the retention of siderophore iron in the cell wall and a set of transporters (*ARN1*, *ARN2*, *ARN3* and *ARN4*) that specifically recognizes siderophore-iron chelators were reported to be induced under iron deprived conditions [20]. The up-regulation of these non-reductive iron transport genes, which do not require copper to transport iron, in the *CCC2* deleted strain under copper deprived conditions when compared to the reference strain might indicate a more defective high affinity iron transport due to copper deficiency in this strain. Moreover down-regulation of these genes in the *CCC2* deleted strain under high copper condition indicated that supplementation of high levels of copper restored the defective ferrous iron transport as well as the respiratory deficiency observed under copper deficient condition. The up-regulation of the electron transport chain (*QCR10*, *SDH4*, *SDH2*, *QCR8* and *QCR2*) in the *CCC2* deleted strain in the presence of high copper levels also supported the observation that the presence of high copper restored the respiratory deficiency in this strain. The analyses of the intracellular iron levels showed also the presence of lower levels of intracellular iron under adequate copper or copper deficient conditions when compared to high copper condition in the *CCC2* deleted strain and also in comparison to the reference strain.

Siderophore transport genes, namely *FIT1*, *FIT2*, *ARN1* and *ARN2*, were also responsive to changing copper levels in the reference strain. These siderophore transport

genes as well as *HMX1* and *CCC2*, which are involved in iron homeostasis, and *FET3* and *FTR1*, which encodes proteins involved in high affinity iron transport, were observed to be up-regulated under high copper conditions when compared with the copper deficient or low copper conditions. The up-regulation of siderophore transport genes together with the genes that play a role in the maintenance of iron ion homeostasis and transport in response to high copper level in the reference strain indicated that copper overload induces iron deficiency in yeast in the presence of *CCC2* gene. A possible explanation for the induction of the iron transporters in response to high copper was proposed to be diminution of cellular iron pools. However, quantitation of the intracellular iron levels did not support this postulate [6]. Also the analyses of the intracellular iron levels conducted in the framework of this study revealed that, the decrease in the iron pools with high copper was not more than 10% in comparison to copper deficient conditions. Another possible explanation for this observation might be enhanced requirement for iron as a cofactor to decompose H_2O_2 in response to increased oxidative stress by high levels of copper [53]. Induction of iron transporters in response to high copper in the reference strain but not in the *CCC2* deleted strain might indicate that, Ccc2p plays a role in the cellular defence mechanism in response to high copper, with a similar mechanism observed in higher eukaryotes [8].

Moreover *JAC1*, which is involved in Fe-S cluster biosynthesis in mitochondria, was down-regulated in the *CCC2* deleted strain with respect to reference strain under copper deficient condition and also even in the presence of adequate level of copper. This finding might indicate the deficiency of this strain in the mitochondrial Fe-S clusters biosynthesis when the intracellular iron concentrations were low.

In addition to these genes, *ERV1*, which is also associated with iron ion homeostasis, was found to be up-regulated under copper deficient condition in the *CCC2* deleted strain when compared to the reference strain. The Mia40/Erv1 disulphide relay system was reported to be involved in the transportation of Ccs1p, which is a molecular chaperon of Cu,Zn superoxide dismutase, Sod1p, to the mitochondria. Ccs1p delivers copper to Sod1p, which is also involved in the activation of Mac1p and the transcriptional response of yeast to copper deficiency [54]. The localization of Cox17p to the cytoplasm and mitochondrial IMS was also mediated via Mia40p. Cox17p delivers copper to Sco1p and Cox11p, which

are Cu binding IM–associated proteins required for the metallation of cytochrome oxidase (CCO) [53]. The up-regulation of *ERV1* under copper deficiency in the *CCC2* deleted strain might indicate a possible defect in the transportation of Ccs1p and Cox17p to mitochondria and consequently in the maintenance mitochondrial copper homeostasis.

Additionally, Yap5p, which is an iron responsive transcriptional activator that regulates vacuolar iron storage in yeast, was identified as reporter transcription factor in a response to the interactive effect of *CCC2* deletion and copper. This result also indicated that the effect of copper on the iron pools dependent on the absence or presence of *CCC2* gene.

2.4.2.2. NAD^+ Homeostasis. NAD^+ metabolism was also observed to be affected both from the deletion of *CCC2* gene and changing copper levels. Although salvage pathways were not affected, the genes associated with *de novo* NAD^+ biosynthetic process from tryptophan (*BNA2* and *BNA4*) were induced under copper deficient conditions in the *CCC2* deleted strain in comparison to the reference strain. These genes were observed to be down-regulated under high copper conditions, where respiratory deficiency was restored. The expression level of NAD^+ dependent glycerol 3-phosphate dehydrogenase, *GPD2*, which is involved in the oxidation of NADH, was repressed in the *CCC2* deleted cells under copper deficient condition and also even in the presence of adequate levels of copper. These results indicated a disturbance in NAD^+ homeostasis under copper deficiency in the absence of *CCC2*. In contrast, *BNA2* and *BNA4* were induced in the reference strain in response to an increase in the copper level indicating a disturbance in NAD^+ metabolism with copper overload. This observation also provided further support and explanation for the observation of tryptophan requirement for yeast growth under copper overload conditions [8].

NAD^+ is an essential cofactor involved several important biochemical processes. Mitochondrial dysfunction, which is associated with a large number of disorders including type II diabetes, cancer, Alzheimer's disease and neurological disorders, is closely related to the maintenance of NAD^+/NADH ratio [55]. Mitochondrial dysfunction was reported to be strictly associated with aging [56] and NAD^+ was also reported to be associated with life expansion through regulation of NAD^+ dependent longevity factors, including the Sir2

family proteins [57]. These proteins are the down-stream targets of calorie restriction under which a decrease in the levels of copper and iron [58] was reported [59].

Cycline dependent kinase inhibitor (Pho81p), repressible phosphatases (Pho5p and Pho8p) and a high affinity phosphate transporter (Pho84p), which are members of phosphate responsive signalling (PHO) were observed to be repressed significantly in the *CCC2* deleted strain under copper deficient conditions with respect to the reference strain. PHO pathway was recently identified as novel component of NAD^+ metabolism in yeast and Pho8p, which is a phosphate regulated vacuolar phosphatase, is a potential production factor of nicotineamide riboside (NmR), which plays an important role in the maintenance of NAD^+ pool [60]. NmR was recently reported as an endogenous precursor for NAD^+ synthesis and the salvage of endogenous NmR is important in the homeostasis and life span [60, 61]. Nicotinic acid mononucleotide (NaMN), which is an intermediate produced from tryptophan during the synthesis of NAD^+, were also produced from nicotinamide (Nam) and nicotinic acid (NA) generated intracellularly or taken from environment [59]. Supplementation of NA but not Nam or tryptophan resulted in an improvement in the respiratory capacity of the *CCC2* deleted cells (Figure 2.10). Observation of a positive effect of NA supplementation, but not Nam or tryptophan, on the respiratory capacity of the *CCC2* deleted cells might indicate that the disturbed homeostasis between these intermediate products might be the reason for the up-regulation of *de novo* NAD^+ biosynthetic pathway. However, further analyses on the intracellular levels of these metabolites are required in order to elucidate the effect of absence of *CCC2* on NAD^+ homeostasis.

Two subunits of the vacuolar transporter chaperon complex, *VTC3* and *VTC4*, involved in membrane trafficking, vacuolar polyphosphate accumulation and non-autophagic vacuolar fusion and *PHO84*, which is the inorganic phosphate transporter, were down-regulated in the *CCC2* deleted cells under copper deficiency. In addition the expression level of *PHM6* and *PHM8*, which were reported to be regulated by phosphate availability, were observed to be down-regulated in the *CCC2* deleted strain under copper deficient conditions. The expression of *PHM6* was also observed to be regulated by copper levels. Identification of the genes associated with polyphosphate metabolic process as

significantly expressed together with the genes associated with NAD^+ metabolism supported the connection between these two pathways.

Although several factors, including; insulin signalling, energy intake, aging were reported to influence the mitochondrial NAD^+/NADH ratio to date, [63–65] copper was identified for the first time as a disturbing factor of NAD^+ metabolism within the framework of the present study. However further studies are required to elucidate the probability that this observed disturbance in NAD^+ metabolism may be a secondary effect of the disturbance in iron metabolism and the respiratory deficiency of the *CCC2* deleted strain might be the result of the disturbance in NAD^+/NADH ratio.

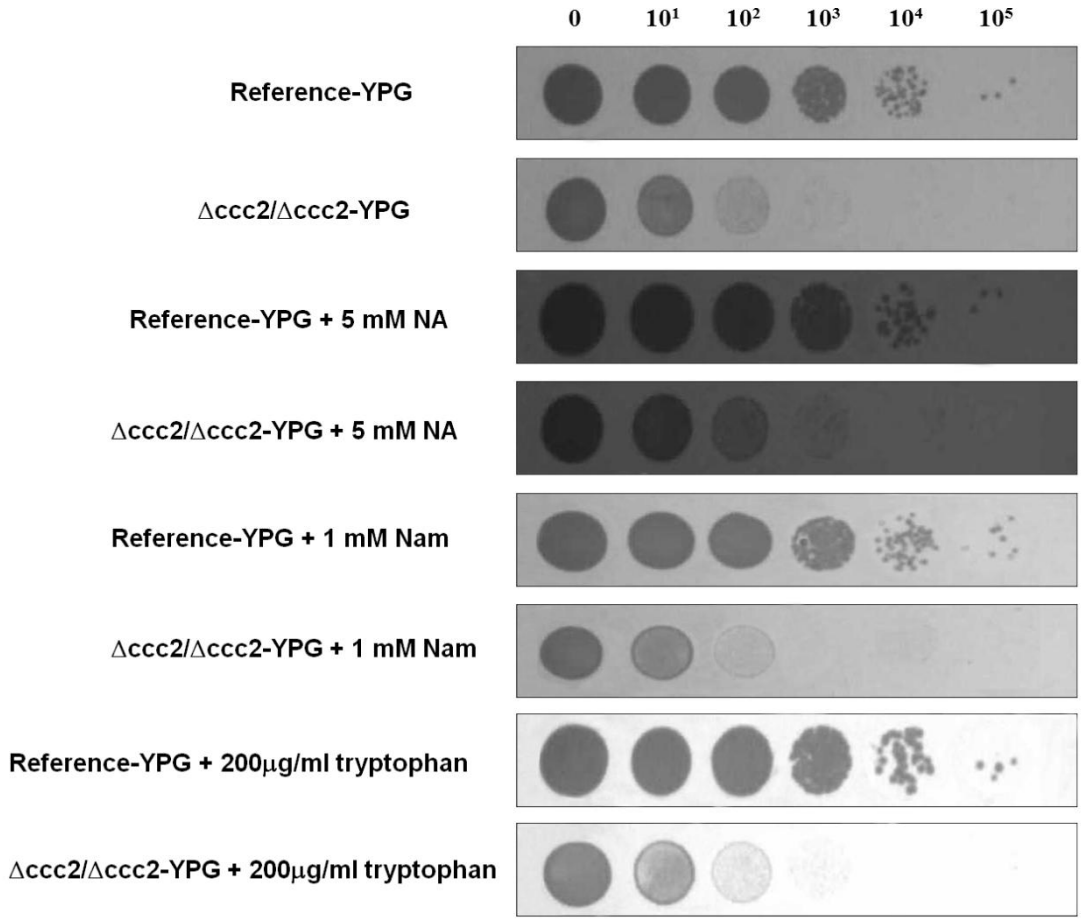


Figure 2.10. Respiratory capacity of the reference and *CCC2* deleted strains on NA, Nam and tryptophan supplemented medium.

2.4.2.3. Arginine Metabolism and Nitrogen Utilization. The genes associated with arginine metabolic process term (*ARG4*, *DUR1*,*2*, *ARG7* and *CAR1*) were up-regulated in

CCC2 deletion strain in comparison to the reference strain under copper deficient condition. Arginine, urea and ornithine metabolic processes were also detected to be up-regulated in the *CCC2* deleted cells under copper deficient conditions in comparison to adequate level of copper containing conditions. The expression levels of *ARG4*, *DUR1,2* and *ARG7* were also significantly and differentially up-regulated under high copper conditions in comparison to adequate level of copper containing condition in this strain. These results indicated that either deficiency or presence of high amounts of copper affected amino acid metabolism in the absence of *CCC2* gene.

Quantitative analyses of intracellular and extracellular arginine levels also indicated that arginine levels were affected from deletion of *CCC2* and copper level. Intracellular arginine levels were found to be copper dependent in both strains and slightly lower in the *CCC2* deleted strain when compared to the reference strain. In both strains, arginine levels were higher under adequate copper level in comparison to copper deficient and high copper conditions. Extracellular arginine levels were also lower in the *CCC2* deleted strain in comparison to the reference strain under copper deficient or high copper conditions. However, under adequate copper levels, under which the expression levels of the genes associated with arginine metabolic process (*ARG4*, *DUR1,2* and *ARG7*) were down-regulated in the *CCC2* deleted cells, extracellular arginine levels were higher in *CCC2* deleted strain.

When optimal sources of nitrogen are unavailable, *S. cerevisiae* is able to utilize arginine as its sole nitrogen source. Arginine catabolism begins in the cytosol with the hydrolysis of arginine by the Car1p to form urea and ornithine. Iron depletion was reported to activate genes involved in the utilization of alternative nitrogen sources and the metabolism of amino acids. Expression levels of *CAR1*; an arginase and *DUR3*; an urea permease, *MEP2*; ammonium permease and *DAL3*; ureidoglycolate hydrolase were reported to be up-regulated under low iron conditions [66]. The up-regulation of these genes in Δccc2/Δccc2 cells under copper deficient conditions might also be explained by the disruption of iron homeostasis in these cells. However, *DUR3*, *MEP2* and *PUT1* were identified to be significantly up-regulated in the *CCC2* deleted cells in response to mutant effect indicating that this response was independent of copper level. Expression of these genes were also up-regulated in the *CCC2* deletion mutant under adequate level of copper

and high copper conditions although this response was not as pronounced as it was under the copper deficient conditions. This finding together with the identification of genes associated with arginine metabolic process term (*ARG4*, *DUR1,2* and *ARG7*) to be up-regulated also in response to high copper might indicate that the effect of *CCC2* deletion on amino acid metabolism was not directly associated with the disturbance in the iron homeostasis.

Glutamate syntheses, Glt1p, which syntheses glutamate from glutamine and alpha-ketoglutarate, require an iron-sulphur cluster for activity. Expression level of *GLT1* was reported to be repressed under low iron conditions [66]. Although *GLT1* was not identified among the differentially expressed genes between *ccc2Δ/ccc2Δ* and reference strain under copper deficient conditions due to the threshold limits, analyses of the mRNA levels of *GLT1* by real-time RT-qPCR revealed that its expression was repressed by 1.8 fold in the absence of *CCC2* gene under copper deficient conditions (Figure 2.11). The decrease in the intracellular and extracellular levels of glutamine under high copper conditions in comparison to low copper or copper deficient conditions in *CCC2* deleted strain could be explained by increased activity of glutamate synthase with increasing iron levels [66].

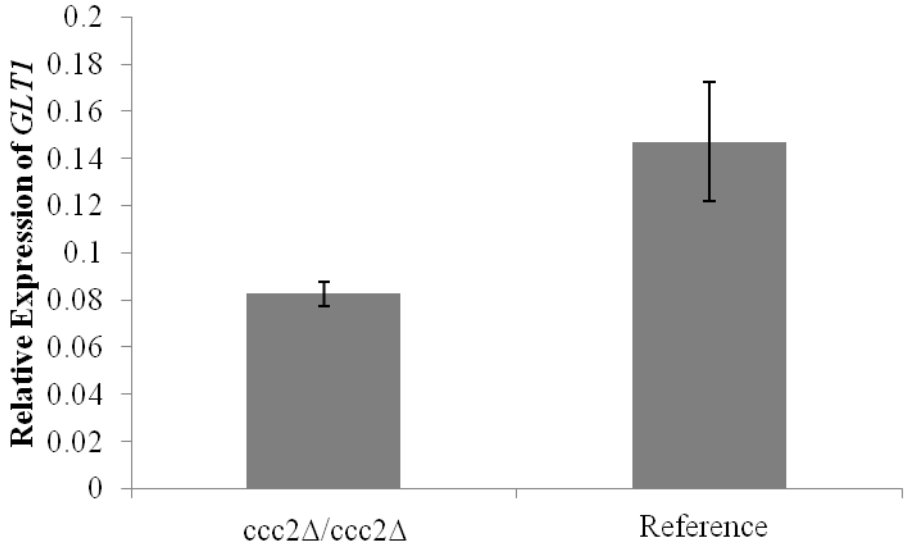


Figure 2.11. Relative expression of *GLT1* under copper deficient conditions.

Integration of transcriptome with regulome also revealed that, the genes, which were affected from the deletion of *CCC2* gene in a copper level dependent manner were the

targets of the transcription factors that were involved in the regulatory pathways related with amino acid availability. Identification of the transcriptional regulators involved in filamentous growth and amino acid starvation among the key transcription factors in response to the interaction effect of gene deletion and change in the copper level also indicated that the effect of copper level on the amino-acid metabolism was dependent on the presence or absence of *CCC2* gene. Moreover, Nrg2, which is involved in the negative regulation of filamentous growth, was down-regulated in the *CCC2* deleted cells under copper deficient conditions.

2.4.2.4. Glucose Transport. Glucose and hexose transport mechanisms were observed to be affected from both the deletion of *CCC2* gene and changing copper levels. The expression of *HXT2* and *HXT5* (high and medium affinity glucose transporters) were up-regulated and that of *HXT1* (low affinity glucose transporter) were down-regulated by high copper levels in both strains. The induction of high affinity transporters together with the repression of low affinity transporters is known as a transcriptional response to the glucose limitation [67]. A similar transcriptional re-organisation was also observed in response to iron deficiency and thus iron deficiency was suggested to result in a secondary glucose starvation [68]. The present findings might also indicate that high copper levels mimicked the glucose starvation independent of the presence or absence of *CCC2* gene.

Moreover, *CAT8*, which is a transcriptional activator that mediates derepression of several genes during the diauxic shift and *GSM1*, which is a putative glucose starvation modulator were induced both in the reference and *CCC2* deleted cells in the presence of high copper levels. The expression levels of *NRG1* and *ADR1*, which are required for the transcription of glucose repressed genes, were also up-regulated in response to higher copper levels in both strains. These results also provided additional support for the fact that a glucose starvation-like response was induced by high copper levels.

Identification of the Rgt1p, which is glucose responsive transcription factor, among the key transcription factors in response to copper effect also supported the transcriptional regulation of glucose transporters by copper availability.

On the other hand, glucose transport mechanism was also affected from the deletion of *CCC2* under copper deficient conditions. *HXT2* and *HXT7*, which are the high affinity glucose transporters, *MTH1*, which is negative regulator of the glucose-sensing signal transduction pathway and *SKS1*, which is putative serine/threonine protein kinase; involved in the adaptation to low concentrations of glucose, were down-regulated in the absence of *CCC2* gene in comparison to the reference strain. These results indicated that the transcriptional response of the glucose transporters to the deletion of *CCC2* gene under copper deficient condition coincided with their transcriptional responses to high glucose levels. On the other hand, under high copper conditions, these genes were not differentially expressed between two strains. The repression of the high affinity glucose transporters in the *CCC2* deleted strain in comparison to the reference strain under copper deficient conditions might indicate that the disturbance in the intracellular copper transport mechanism resulted in a defect in glucose utilization. On the other hand, observation of this response only under copper deficient but not high copper conditions might also indicate that the down-regulation of the high affinity glucose transporters was related with the respiratory deficiency in this strain. The increased fermentative capacity of this strain, which is usually observed under high glucose conditions, might lead to a transcriptional re-organisation of glucose transporters mimicking glucose enriched conditions. Defective phosphate, urate, amino acids and glucose transports were previously reported in Wilson's disease patients [69]. The unexplained disturbance in the regulation of glucose transporters in the absence of *CCC2* gene under copper deficient conditions might be an indication of a defect in the transport of materials in Wilson's disease model.

Integration of the transcriptome with regulome also highlighted the glucose responsive transcriptional factors, around which the transcriptional change were concentrated in response to the change in the copper level or interaction effect of gene deletion and copper level. Identification of Rgt1p among the key transcription factors in response to copper effect might indicate that transcriptional changes in the hexose transporters in response to high copper level were regulated by this transcription factor. Nrg1p, which mediates glucose repression, was identified among the key transcription factors in response to change in the copper level and also in response to interaction effect. This might indicate that, the transcriptional re-organisation in the *CCC2* deleted strain

under copper deficient condition, which mimicked high glucose condition, was under the control of Nrg1p.

2.4.2.5. Pyridoxal Phosphate Biosynthetic Process. Pyridoxal phosphate metabolic process was up-regulated in the *CCC2* deleted strain when compared to the reference strain under copper deficient condition. *VHR1* encoding a transcription factor, which mediates the induction of *VHT1* and *BIO5* that are involved in the transport of vitamin H and biotin biosynthesis intermediates, respectively, was up-regulated in the *CCC2* deleted cells when high copper condition was compared with low copper condition. Vitamin B metabolic process term was also enriched among the key reporter proteins, which were identified in response to the interaction effect of gene deletion and change in the copper level. Heme biosynthesis begins in the mitochondrion with the condensation of glycine and succinyl-CoA to form delta-amino levulinic acid (ALA) and pyridoxal phosphate is a cofactor in this reaction [70]. The human ortholog of *HEM1* gene that encodes the enzyme 5-aminolevulinate synthase, which catalyzes the first step in heme biosynthesis, is associated with sideroblastic anemia. *HEM1* gene also regulates expression of iron and copper transporter genes. Due to the requirement for pyridoxine as a cofactor in the biosynthesis of heme, pyridoxine deficiency can lead to a sideroblastic anemia. Deletion of *CCC2* gene also leads to iron deficiency and this deficiency could be related with the defect in respiration observed in this mutant [24]. The present finding, which was reported for the first time in this study, showed the fact that pyridoxine supplementation lead to an improvement in the respiratory capacity of the *CCC2* deleted cells. This finding together with the previous reports indicating the similarity between the symptoms observed between copper and vitamin B deficiency [71], highlight the importance of maintenance of vitamin B at adequate levels in Wilson disease patients.

2.5. Concluding Remarks

The quantification of the changes in the transcriptome in response to the deletion of *CCC2* gene under changing copper levels and the integration of this data with interactome, regulome and metabolome revealed the genome-wide effects of both the absence of yeast ortholog of Wilson and Menkes disease causing gene and the changing copper levels in yeast.

The 3x2 factorial design applied in this study enabled to identify the pathways that were affected in the same way from high copper levels in both strains or from the deletion *CCC2* gene under all conditions and also the pathways, for which the effect of copper level depends on the presence or absence of *CCC2* gene. The identification of the significantly expressed genes by two-way ANOVA analyses revealed that the transcriptional response to the deletion of *CCC2* gene highly depended on the copper level.

Iron homeostasis was found to be affected from the absence of *CCC2* gene in a copper level dependent manner. Under copper deficient conditions, deletion of *CCC2* gene resulted in iron deficiency, which caused an increased expression of siderophore iron transporters. Quantification of the intracellular iron levels also supported this prospect. High level of copper was observed to restore the iron deficiency as well as the respiratory deficiency in this strain. On the other hand, high copper levels lead to an iron deficient-like response in reference strain, although the iron pools was not decreased in response to high level of copper. This result demonstrated and provided further evidence for the increased requirement of iron under high copper conditions and also indicated that Ccc2p might play a role in this response. Moreover, up-regulation of *CCC2* gene in response to high copper levels in the reference strain also supported this prospect.

NAD^+ metabolism was also found to be affected from the absence of *CCC2* gene and change in the copper level. Under copper deficient conditions, NAD^+ biosynthesis from tryptophan was induced in the *CCC2* deleted cells when compared to the reference strain. This observation might be interpreted as a transcriptional re-organisation of the respiratory deficient *CCC2* deleted cells to maintain NAD^+/NADH ratio. Moreover, the observation of a positive effect of nicotinic acid supplementation, which is a precursor of NAD^+ synthesis, but not Nam or tryptophan, on the respiratory capacity of the *CCC2* deleted cells also indicated the disturbed homeostasis in NAD^+ metabolism. On the other hand, high copper level was also identified to be affecting the NAD^+ metabolism in the reference strain and leading to the induction of NAD^+ biosynthesis from tryptophan pathway in comparison to both copper deficient condition and the *CCC2* deleted strain. However further studies are required to elucidate whether this observation is a secondary effect of the disturbance in iron metabolism or copper itself is a disturbing factor of NAD^+ homeostasis.

The absence of *CCC2* gene resulted in a nitrogen starvation-like response in a copper level dependent manner. Under copper deficient conditions, arginine metabolic process was induced in comparison to reference strain. Moreover, both the copper deficient and high copper conditions resulted in the induction of arginine metabolic process in comparison to conditions containing adequate level of copper in the *CCC2* deleted cells. Metabolome analyses of amino acids also complemented the transcriptome data and revealed that the intracellular arginine, alanine, glutamine and tyrosine levels were significantly lower under copper deficient and high copper conditions in comparison to conditions containing adequate level of copper. Alanine and arginine levels were also lower in the *CCC2* deleted strain in comparison to the reference strain under all conditions. Integration of regulome with transcriptome also provided additional support for this observation by pointing out the fact that the genes, which were significantly affected from the interaction effect of gene deletion and copper level were concentrated around the transcription factors that were involved in the regulatory pathways related with amino acid availability. Since this nitrogen starvation like response of the *CCC2* deleted cells was also observed under high copper conditions, which was shown to restore the iron deficiency in this strain, this response seems to be a direct effect of the absence of *CCC2* gene, rather than being a secondary effect of iron deficiency.

Glucose transport mechanism was identified to be affected from high levels of copper and also from the deletion of *CCC2* gene. High copper levels led to a glucose starvation-like response in both strains. On the other hand, the deletion of *CCC2* gene under copper deficient conditions resulted in an opposite response indicating a defective glucose transport mechanism in this strain under copper deprived conditions, under which the *CCC2* deleted cells were respiratory deficient. The disturbance in the regulation of glucose transporters in the absence of *CCC2* gene under copper deficient conditions might be an indication of a defect in the transport of materials in Wilson's disease model.

The analysis of the significantly expressed genes and also integration of the interactome and transcriptome highlighted that pyridoxine biosynthetic pathway was affected from the absence of *CCC2* gene under copper deficient conditions. Pyridoxal phosphate is a cofactor in the biosynthesis of heme and deficiency of pyridoxine leads to iron deficiency. The present study revealed that, supplementation of pyridoxine led to

improvement in the respiratory capacity of the *CCC2* deleted cells. This observation together with the induction of pyridoxal phosphate metabolic process under copper deficient conditions in the *CCC2* deleted strain indicated that absence of *CCC2* gene might lead to pyridoxine deficiency, which might manifest the iron and respiratory deficiency.

Integration of the transcriptome and regulome revealed the regulatory mechanisms that were affected from the deletion of *CCC2* gene and changing copper levels. This integrative analysis highlighted the changes in the respiratory and amino-acid metabolisms. In addition, it was identified that both perturbations and also their interacting effect induced stress response. Sko1p, Skn7p, Cin5p, Yap1p and Yap6p, which were oxidative stress regulators were identified among the key transcription factors identified in response to interacting effect of change in the copper level and deletion of *CCC2*.

3. TRANSCRIPTIONAL REMODELLING IN RESPONSE TO THE DELETION OF *ATX1* UNDER CHANGING COPPER LEVELS

This chapter of the study is concern with how yeast responses to the absence *ATX1* gene, which is the yeast ortholog of *ATOX1* gene in human. The function of Atox1p is to deliver copper to the proteins encoded by *ATP7A* and *ATP7B*, which are the Menkes and Wilson disease causing genes. In yeast Atx1p is also responsible from the transport of copper ions to Ccc2p on a post-Golgi vesicle for eventual targeting to high-affinity iron uptake protein, Fet3p. Since Atx1p and Ccc2p have consecutive functions in the intracellular transport of copper, the absence of *ATX1* might be expected to result in a similar response with that of absence of *CCC2* gene. In order to test this hypothesis, the genome-wide effect of the absence of *ATX1* gene on the transcriptional profiles was investigated under copper deficient and high copper conditions. The transcript levels and also the growth characteristics of the *ATX1* deleted strain were investigated in comparison to the reference and *CCC2* deleted strains in order to elucidate the differences and similarities between the *ATX1* and *CCC2* deletion mutants.

3.1. Background

Maintenance of copper homeostasis is essential not only to prevent copper-dependent damage and to maintain proper functioning of cuproenzymes but also for the maintenance of iron homeostasis. The interdependent connections between copper and iron homeostasis have been shown in yeast and also in mammalian cells [72].

In yeast Fet3p receives iron(II) ions from cell-surface iron reductases (Fre1p and Fre2p) and passes iron(III) ions to the iron permease Ftr1p [73]. Copper is required for the oxidase activity of Fet3p [52]. The requirement of copper for the trafficking of Fet3p/Ftr1p complex to the plasma membrane under iron deprived conditions represents the importance of copper for the maintenance of iron homeostasis in yeast [19].

The copper delivery pathway that mediates the transport of copper to Fet3p includes Ctr1p, Atx1p and Ccc2p [18]. Ctr1p is the high affinity copper transporter, which transfers

the reduced copper by membrane reductases Fre1p and Fre2p. The expression of *CTR1* is regulated by copper level through Mac1p. It is induced under low copper conditions and repressed by high copper levels to reduce copper uptake [19]. Ctr1p is also involved in the transport of copper ions to secretory pathway. Copper is transported to cytosolic copper chaperone Atx1p, which delivers copper to Ccc2p. Atx1p was first identified as multi-copy suppressor of oxygen toxicity in superoxide dismutase (SOD) deficient cells [74]. However this observation was shown to be not valid when the cells were treated with copper chelator. The decreased resistance to oxygen under copper deprived conditions were reversed by addition of copper. Copper dependent bypass of SOD deficiency and also the presence of copper ion binding domains in Atx1p indicated that this protein is involved in the intracellular transport and sequestration of copper [74].

ATX1 deleted cell exhibited no growth on iron deficient medium similar to the *CTR1* and *CCC2* deletion mutant, which are defective in high affinity iron uptake. Measurement of ferrous iron uptake of *ATX1* deleted cells also showed a reduced iron uptake, which could be restored by copper supplementation. These results indicated that Atx1p operates in the same way as Ctr1p and Ccc2p to deliver copper to Fet3p. Studies also represented that overexpression of *CCC2* can suppress the effect of absence of *ATX1* gene but the overexpression of *ATX1* gene did not rescue the iron deficiency in *CCC2* deleted strains. This finding revealed that Ccc2p functions downstream of Atx1p and there is a Atx1p-independent pathway that can reverse the effect of absence of *ATX1* when *CCC2* is overexpressed [18]. Observation of a more severe growth defect in the temperature sensitive double deletion mutant of *ATX1* and *END3*, absence of which blocks endocytosis, on iron deficient medium indicated that endocytosis might be the Atx1p-independent pathway that delivers copper to intracellular locations [18].

ATOX1 is the human ortholog of *ATX1* and has a similar function in human. Atox1p directly interacts with the proteins encoded by *ATP7A* and *ATP7B*, which are the Menkes and Wilson disease causing genes and delivers copper to these proteins [75]. Studies performed with Atox1 deficient mice cells demonstrated that absence of *ATOX1* leads to copper accumulation due to impaired copper efflux [76]. Although no disease mutations have been reported in *ATOX1* [77], Menkes disease like phenotype was observed in *ATOX1* knockout mice [76].

In this study, the genome-wide transcriptional response to the absence of *ATX1* gene and the change in the intracellular and extracellular ion concentrations were investigated in a copper level dependent manner. Comparison of the growth characteristics and transcriptional profiles of the deletion mutants of *ATX1* and *CCC2* genes revealed the similarities and discrepancies between the genome-wide effects of the absence of these genes.

3.2. Materials and Methods

3.2.1. Strains and Growth Media

Homozygous *ho*Δ*/ho*Δ and *atx1*Δ*/atx1*Δ strains of *S. cerevisiae* from a genetic background of BY4743 (*MAT*a*/MAT*Δ *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/l*eu2*Δ*0 lys2*Δ*0*/+ *met15*Δ*0*/+ *ura3*Δ*0*/*ura3*Δ*0*) were purchased from the European *Saccharomyces cerevisiae* Archive for Functional Analysis. The preparation of the preculture was performed as described in Section 2.2.1. Synthetic defined medium without $CuSO_4$ (copper deficient condition) and with 0.5 mM $CuSO_4$ supplementation (high copper level) was used in the fermentations.

3.2.2. Batch Culture, Experimental Design and Sample Description

The fermentation experiments were carried out in triplicates as described in Section 2.2.2. RNA, metabolite and biomass samples were collected at mid-exponential phase at an OD range of 0.7-0.8. The samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until further processing. Biomass concentration was determined using gravimetric methods.

3.2.3. Metabolite Analysis

Extracellular ethanol, glucose and glycerol concentrations were determined using the enzymatic analysis kits supplied by Boehringer –Mannheim, Germany. The necessary dilutions of supernatants were carried out as indicated in the protocols prior to the analyses. The kits were used as described by the manufacturers.

3.2.4. Intracellular and Extracellular Ion Determination and Sample Preparation

The samples were centrifuged at 400g for 3 minutes at 4 ^{o}C. The supernatants that contain the extracellular metabolites were directly used. Preparation of intracellular samples for the determination of intracellular ion concentrations were carried out as described in Section 2.2.6. The intracellular and extracellular iron, copper and zinc concentrations were kindly determined by Redokslab Analytical Systems Inc., *Istanbul, Turkey*, using iCap Q ICP-MS (Thermo Scientific Inc., USA).

3.2.5. RNA Extraction and Microarray Hybridization

RNA extraction and microarray hybridization were performed as described in Section 2.3.3.

3.2.6. Microarray Data Acquisition and Analysis

The cell files generated by Microarray Suite v5.0 were pre-processed with dChip software [30] using the perfect match (PM)-miss match (MS) difference model to obtain the expression levels of transcripts. These values were normalized by a baseline array with a median overall intensity. The array corresponding to reference strain with high copper condition was selected as the baseline array. The presence of single, array and probe outliers was checked and no outlier chips were identified.

Multi Experiment Viewer (MeV) [78, 79] was used for the statistical analyses of the microarray data. The significantly expressed genes in response to the deletion of *ATX1* gene under changing copper levels were identified by two-way Analyses of Variance (ANOVA). These significantly expressed genes were analysed in four groups. The first group, G1, included the genes that were significantly expressed only in response to the deletion of *ATX1* gene. The second group, G2, contained the genes, which were significantly expressed only in response to copper level. The third group, G3, included the genes, which were significantly expressed both in response to gene deletion and change in the copper level in an additive manner. The last group, G4 compose of the genes, which were affected from the interactive effect of gene deletion and copper level. The

significantly enriched process ontology terms of the each group were determined by BINGO [32], which is a Cytoscape plug-in [33]. The Benjamini-Hochberg method was used for the calculation of false discovery rates and 0.05 was selected as the cut-off threshold for p-values. Integration of interactome and regulome with transcriptome data was performed as described in Section 3.2.5.

In order to identify the significantly expressed genes across the three group of samples, namely, *ATX1* deleted strain, *CCC2* deleted strain and the reference strain, one-way Analyses of Variance (ANOVA) was performed using MeV for copper deficient and high copper conditions, separately. GeneCluster 2.0 [80] was used for the clustering of the significantly expressed genes via self-organising maps. The significantly enriched process ontology terms of the genes falling into the same cluster were determined as described previously.

3.2.7. Identification of Reporter Proteins and Transcription Factors

The reporter proteins and transcription factors were determined as described in Section 2.2.8.

3.3. Results

In order to provide further inside into the role of *ATX1* in the copper homeostasis, the genome-wide transcriptional response to the deletion of *ATX1* gene was investigated in a 2x2 factorial design. *ATX1* double deletion mutant was cultivated in the fully controlled fermenters in triplicates in glucose-rich defined medium containing two different levels of copper. Global adaptive transcriptional response to the deletion of *ATX1* gene and also to different extracellular copper levels were investigated under copper deficient or high copper conditions (0.5 mM) in comparison to the reference strain (*HO* deletion mutant), which was also cultivated under similar conditions in triplicates. The high copper concentration was selected as 0.5 mM in order to compare the transcriptional response of the *ATX1* deletion mutant with that of the *CCC2* deletion mutant, which had been cultivated also under similar conditions. This level of extracellular copper was also shown to restore the decreased respiratory capacity of the *ATX1* deleted strain (Figure 3.1).

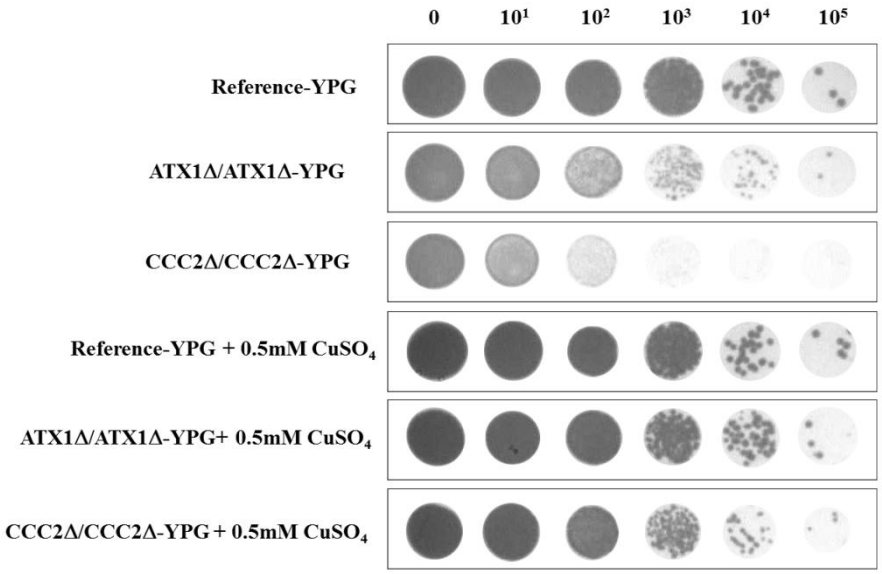


Figure 3.1. Restored respiratory deficiency in *ATX1* and *CCC2* deleted cells.

3.3.1. Global Transcriptional Response of Δ*atx1*/Δ*atx1* and Reference Strain to Different Extracellular Copper Levels

Genome-wide gene expression analysis in the reference and *ATX1* deleted strains, which were cultivated under copper deficient or high copper conditions, were carried out with the samples collected at mid-exponential phase (A_{600} of 0.7-0.9). This experimental setup enabled the identification of the transcriptional response of the cells to the deletion of *ATX1* gene at different copper levels and also the adaptive transcriptional response of each strain to different extracellular copper levels. The change in the transcript levels were also analysed in conjunction with the protein-protein interaction and regulatory network of yeast.

3.3.1.1. Identification of Significantly Expressed Genes. Significantly expressed genes were identified by two-way Analyses of Variance (2-way ANOVA). Two-way ANOVA analysis assessed the significance of change (p-value < 0.01) at transcriptional level according to three different criteria; the effect of gene deletion (Factor 1), effect of change in the copper level (Factor 2) and the interaction effect between gene deletion and copper level (Factor 1 x Factor 2). The expression levels of 173 and 2255 genes were significantly affected only by gene deletion or only by change in the copper level without a significant interaction effect, respectively. A total of 150 transcripts were observed to be significantly

expressed both in response to gene deletion and change in the copper level without a significant interaction effect. On the other hand, 79 genes were affected from the interaction of these factors.

The genes that were significantly expressed only in response to gene deletion were defined as the gene set 1 (G1). This group contained the genes, which were either up-regulated (44 transcripts) or down-regulated (129 transcripts) in the *ATX1* deleted strain under both copper deficient and high copper conditions. The genes, which were significantly expressed in response to change in the copper level, were defined as the gene set 2 (G2). These genes were observed to be either up-regulated (1244 transcripts) or down-regulated (1011 transcripts) in response to high copper levels in both strains. The genes that were significantly expressed in response to both factors without displaying a significant interaction effect were defined as the gene set 3 (G3). The genes in this set can be clustered into four groups according to their transcriptional responses; (1) genes, which were up-regulated both in response to high copper and gene deletion (17 transcripts), or (2) the genes, which were induced by high copper but repressed by gene deletion (51 transcripts), or (3) the gene, which were repressed by high copper but induced by gene deletion (16 transcripts), or (4) the genes that were repressed by both gene deletion and high copper levels (66 transcripts). Lastly, the 79 genes, which were significantly expressed in response to the interaction effect of gene deletion and change in the copper level, were identified as the gene set 4 (G4). For the genes in this set, the effect of the changing copper levels depends on the presence or absence of *ATX1* gene or in other words, the effect of deletion of *ATX1* gene depends on the copper level. The enriched GO-biological process terms associated with each group were summarized in Figure 3.3, Figure 3.4, Figure 3.5 and Figure 3.6.

3.3.1.2. Integration of Protein-Protein Interaction Network with Transcriptome Data. In order to integrate the protein-protein interaction network of yeast with the transcriptional response to the deletion of *ATX1* gene under copper deficient and high copper conditions, Reporter Feature algorithm was utilized [33, 34]. This analysis identified the proteins, whose surrounding genes were significantly expressed in response to the applied perturbations.

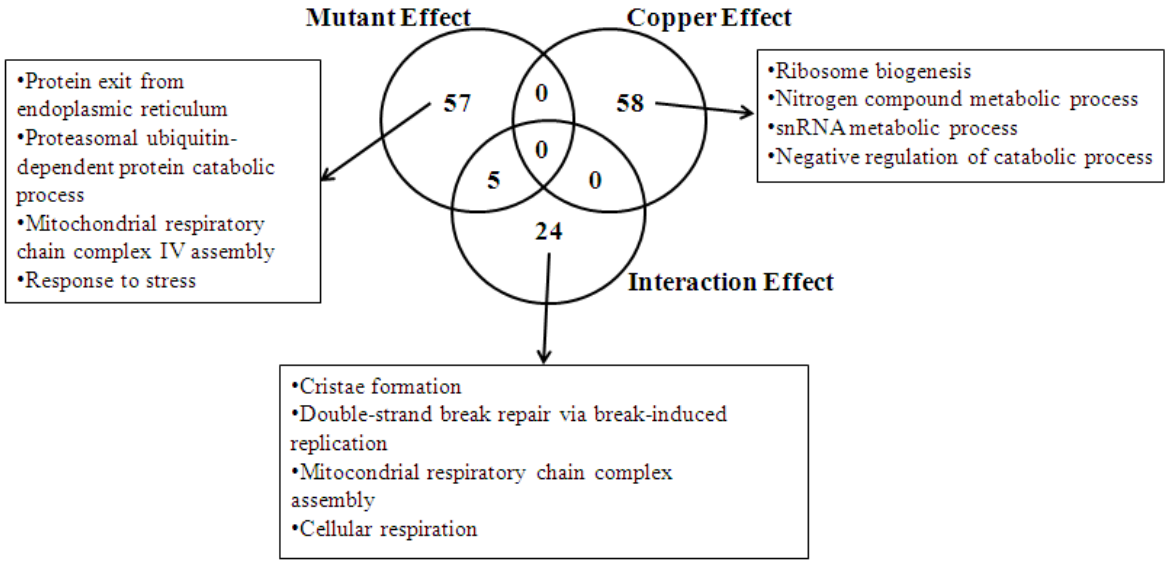


Figure 3.2. Reporter proteins identified in response to the deletion of *ATX1* gene, change in the copper level or interacting effect of both factors.

Three sets of reporter proteins (p-value < 0.01), whose neighbour proteins were significantly expressed in response to either change in the copper level or the deletion of *ATX1* gene or the interaction effect of gene deletion and change in the copper level, were identified. In response to the deletion of *ATX1* or change in the copper level or interacting effect of both factors, 62, 58 and 29 proteins were identified as reporter proteins, respectively. There were 5 proteins, which were identified among the reporter proteins both in response to deletion and interaction effects. The enriched GO-biological process terms associated with each group of proteins were summarized in Figure 3.3.

3.3.1.3. Transcriptional Response to the Deletion of *ATX1* Gene. The expression levels of 173 transcripts were observed to be significantly expressed in response to the deletion of *ATX1* gene independent of copper level. Among these 173 genes, 44 transcripts were up-regulated in the *ATX1* deleted cells both under high copper and copper deficient conditions however; these transcripts were not significantly enriched with any process term. A total of 129 genes were observed to be repressed in the *ATX1* deleted cells under both conditions. These genes were enriched with regulation of phospholipid biosynthetic process term (p-val < 0.03) (Figure 3.3, Table B.1).

When the proteins, around which the transcriptional response to the deletion of *ATX1* gene was concentrated, were analysed , it was observed that these proteins were enriched with protein exit from endoplasmic reticulum, proteasomal ubiquitin-dependent protein catabolic process, mitochondrial respiratory chain complex IV assembly and response to stress biological process terms (p-val < 0.05) (Figure 3.2).

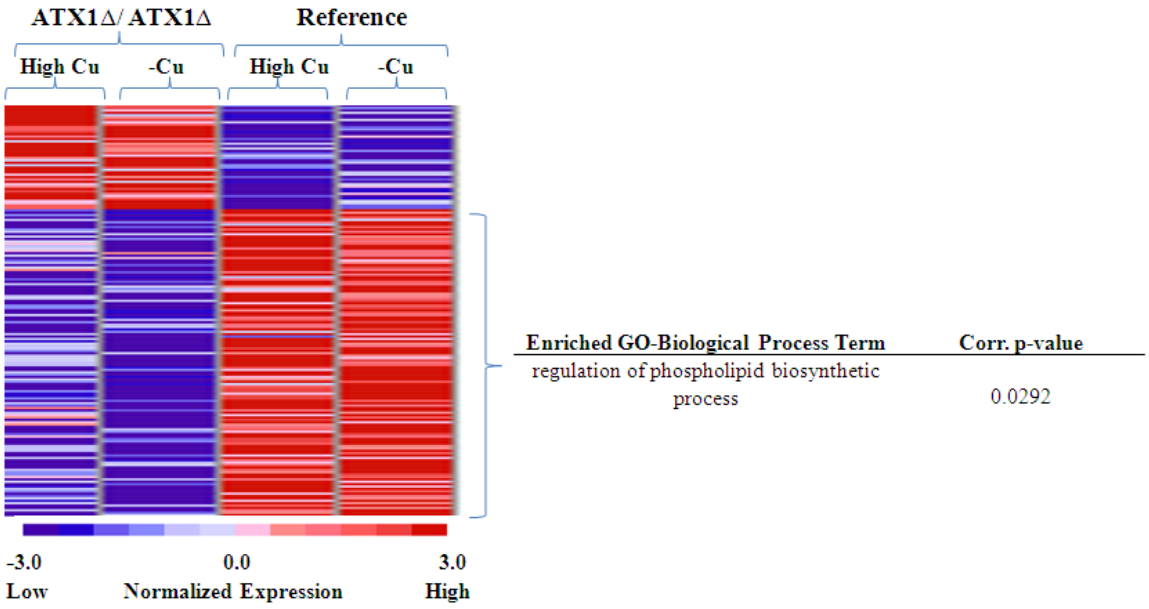
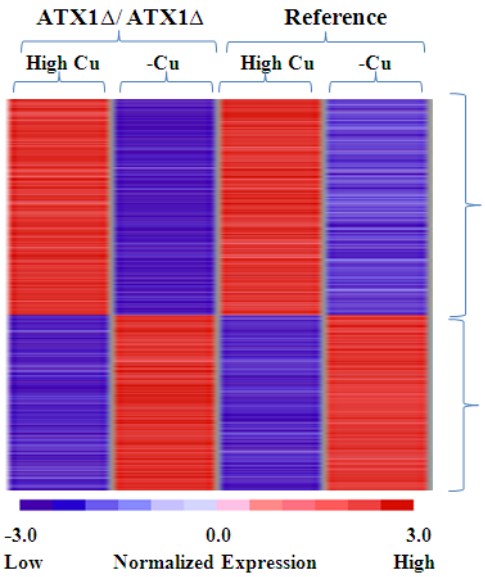


Figure 3.3. Enriched GO biological process terms among the significantly expressed genes in response to deletion of *ATX1*.

3.3.1.4. Transcriptional Response to Changing Copper Conditions. A total of 2255 transcripts were identified to be significantly expressed between high copper and copper deficient conditions independent of gene deletion. Among these transcripts, 1244 transcripts were significantly up-regulated. These genes were significantly associated with actin cytoskeleton organization, cell wall organization, endocytosis, *de novo* NAD^+ biosynthesis process from tryptophan, cellular amino acid metabolic process, response to starvation, autophagy, sporulation, iron ion homeostasis and TOR signalling cascade (Figure 3.4, Table B.2). 1011 genes were observed to be down-regulated and they were significantly enriched with processes related with ribosome biogenesis process term (Figure 3.4, Table B.3).

The transcriptional response to a change in the copper level was concentrated around the proteins, which were enriched with ribosome biogenesis, nitrogen compound metabolic

process, snRNA metabolic process and negative regulation catabolic process terms (p-value < 0.05).

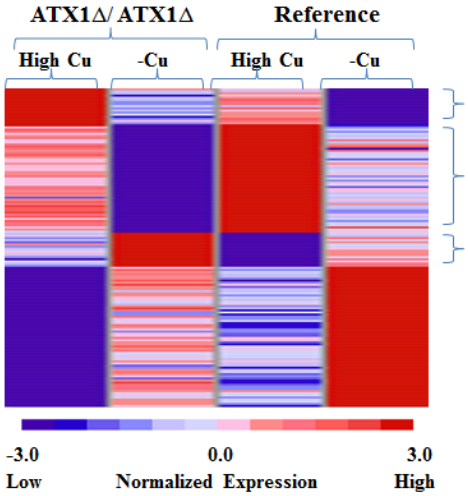


Enriched GO-Biological Process Terms	Corr. p-value
actin cytoskeleton organization	4.79E-06
cell wall organization	2.43E-05
endocytosis	9.63E-05
response to starvation	7.78E-04
autophagy	1.14E-03
de novo NAD biosynthetic process from tryptophan	1.06E-02
cellular amino acid metabolic process	1.74E-02
sporulation	3.84E-02
iron ion homeostasis	4.07E-02
TOR signaling cascade	4.39E-02

Enriched GO-Biological Process Term	Corr. p-value
ribosome biogenesis	9.26E-49

Figure 3.4. Enriched GO biological process terms among the significantly expressed genes in response to change in the copper level.

3.3.1.5. The Additive Effect of Copper Level and Gene Deletion. A total of 150 transcripts were observed to be significantly expressed both in response to a change in the copper level and the deletion of *ATX1* gene. There were 17 transcripts, which were up-regulated both in response to high copper and deletion of *ATX1* gene and these genes were enriched with carnitine metabolic process term (Figure 3.5, Table B.4). 51 transcripts were down-regulated under copper deficient conditions in comparison to high copper conditions and also in the *ATX1* deleted strain in comparison to the reference strain. These transcripts were enriched with mitochondrial ATP synthesis coupled electron transport process term (Figure 3.5, Table B.5). 16 transcripts were observed to be up-regulated under copper deficient conditions and also in the *ATX1* deleted strain and these transcripts were significantly enriched with post replication repair process term (Figure 3.5, Table B.6). There were 66 transcripts, which were down-regulated under high copper conditions in comparison to copper deficient conditions and also in the *ATX1* deleted strain in comparison to reference strain but these transcripts were not significantly enriched with any process term (Figure 3.5).



Enriched GO-Biological Process Term	Corr. p-value
carnitine metabolic process	2.83E-03

Enriched GO-Biological Process Term	Corr. p-value
mitochondrial ATP synthesis coupled electron transport	7.45E-04

Enriched GO-Biological Process Term	Corr. p-value
postreplication repair	5.49E-03

Figure 3.5. Enriched GO biological process terms among the significantly expressed genes in response to additive effect.

3.3.1.6. The Interaction Effect of Copper Level and Gene Deletion. Expression levels of 79 transcripts were observed to be affected from the interacting effect of the change in the copper level and the deletion of *ATX1* gene. The transcriptional responses of these genes were investigated in two groups. Firstly, the genes, which were affected from the deletion of *ATX1* gene in a copper level dependent manner, were investigated. Expression levels of 48 genes were up-regulated under high copper conditions and down-regulated under copper deficient conditions in the *ATX1* deletion mutant in comparison to the reference strain. These genes were significantly enriched with vesicle targeting process term (Figure 3.6, Table B.8). There were 20 genes, which were down-regulated under high copper conditions and up-regulated under copper deficient conditions in the *ATX1* deletion mutant in comparison to the reference strain. These genes were significantly enriched with telomere maintenance and '*de novo'* UMP biosynthetic process terms (Figure 3.6, Table B.9). The second group contains the genes, which gave opposite responses to high copper levels in the *ATX1* deleted strain and the reference strain. There were 25 transcripts, which were up-regulated by high copper levels in the *ATX1* deleted strain and down-regulated in the reference strain. These genes were enriched with golgi vesicle budding and retrograde vesicle mediated transport, Golgi to ER process terms (Figure 3.6, Table B.7). There were also 7 transcripts that were down-regulated by high copper levels in the *ATX1* deleted strain and up-regulated in the reference strain but these genes were not significantly enriched with any process term.

Integration of interactome with the transcriptional responses to the interaction effect of the gene deletion and change in the copper level revealed that the transcriptional response was concentrated around the 29 proteins. These reporter proteins were enriched with cristae formation, double-strand break repair via break-induced replication, mitochondrial respiratory chain complex assembly and cellular respiration process terms (p-value < 0.05).

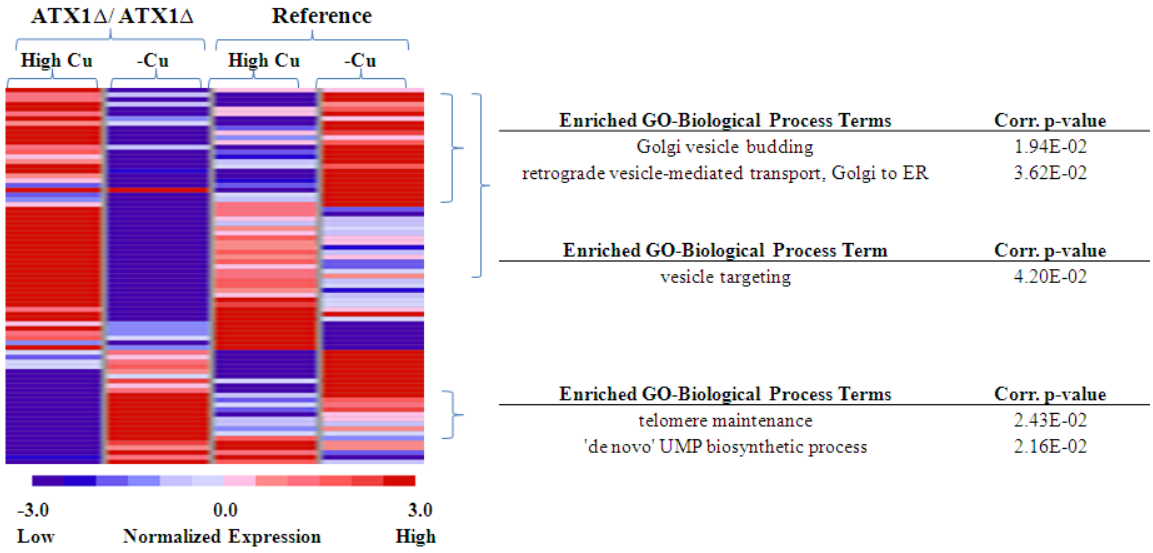


Figure 3.6. Enriched GO biological process terms among the significantly expressed genes in response to interaction effect.

3.3.1.7. Integration of Regulatory Network with Transcriptome Data. In order to identify the regulatory pathways, which were affected in response to changing copper levels, deletion of *ATX1* gene or the interacting effect of these two factors, Reporter feature analysis was performed using yeast regulatory network.

The transcriptional response to the deletion of *ATX1* gene was observed to be concentrated around 11 transcription factors (p-value < 0.05). One of the high scoring transcription factors; Hac1p is involved in the unfolded protein response, which is triggered by mutations in secretory proteins. Three of the key transcription factors, Mbp1p, Ace2p and Fkh2p, were involved in the regulation of cell cycle progression. Met4p, which is responsible for the regulation of sulphur amino acid pathway, Rds2p, which is involved in the regulation of gluconeogenesis and glyoxylate cycle genes, Msn1p, which is involved in the regulation of glucoamylase expression, pseudohyphal differentiation, iron uptake

and response to osmotic stress and Gcn4p, which is the transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation, were also among the key transcription factors.

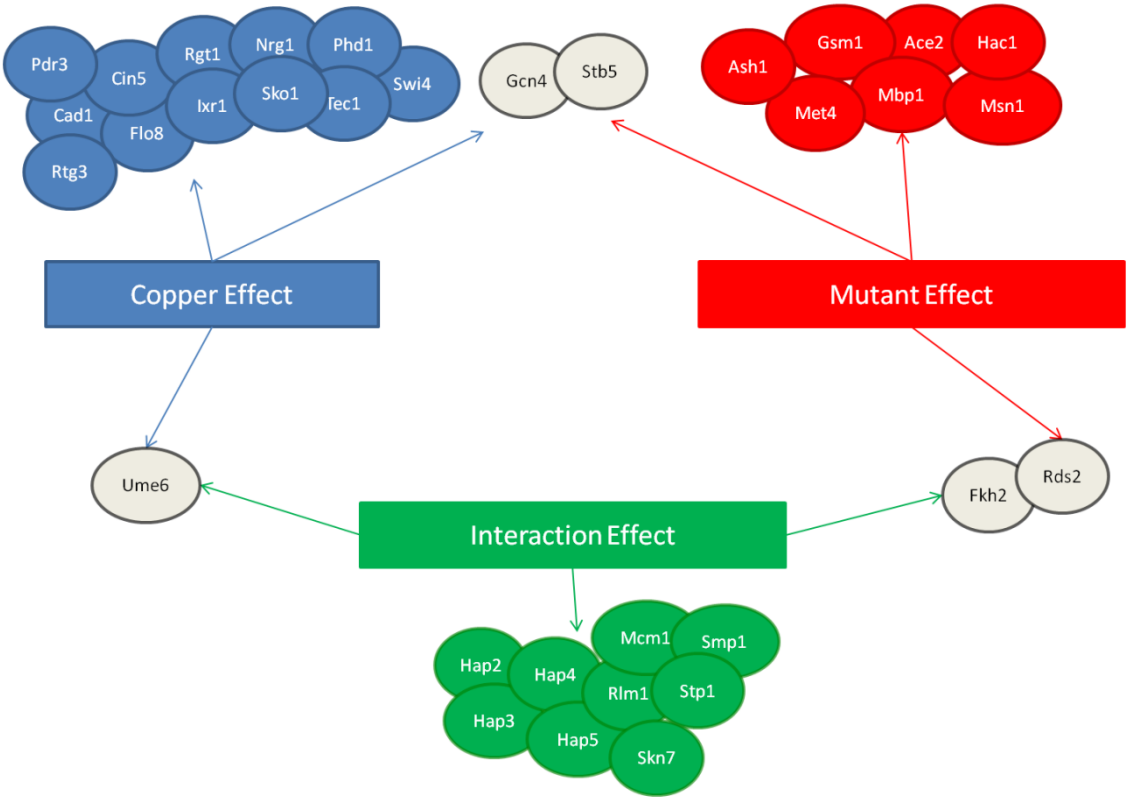


Figure 3.7. Reporter transcription factors in response to change in the copper level, deletion of *ATX1* gene or their interacting effect.

Fifteen transcription factors were identified as top scoring reporter transcription factors (p-value < 0.05) in response to change in the copper level. Two of these transcription factors, Gcn4p, Stb5p were also identified among the top scoring TFs in response to mutant effect and Ume6p was commonly identified among the significant TFs both in response to copper effect and interaction effect. The remaining 12 transcription factors were unique for copper response. Four of these transcription factors; Cad1p, Pdr3p, Cin5p and Stb5p were involved in pleiotropic drug resistance. Both the PDR pathway and the high copper concentrations were reported to affect plasma membrane composition [81, 82]. Identification of regulator proteins involved pleiotropic drug resistance in response to change in the copper level could be associated with the effect of copper level on membrane permeabilization. Five of the transcription factors; Tec1p, Nrg1p, Ume6p, Flo8p and

Phd1p were involved in pseudohyphal growth. Rtg3p, which activates retrograde (RTG) and TOR pathway, Gcn4p, which activates amino acid biosynthetic genes in response to amino acid starvation and Rgt1, which is glucose responsive transcription factor, were also among the reporter TFs identified in response to change in the copper level. Swi4p and Tec1p, which regulate the transcription of G1 cyclin genes [83, 84] were other transcription factors, around which the response to change in the copper level was concentrated. High copper levels were reported to have an inhibitory effect on yeast filamentous growth [85]. Identification of regulators of G1 cyclin genes and also TFs associated with pseudohyphal growth could also be an indicator of the possible effect of copper level on cell cycle progression. Additionally, TORC1 signalling pathway was reported to regulate Tec1p to proper control of cell division and cellular development in response to nutrient [84].

When the key transcription factors, around which the transcriptional response to the interacting effect of gene deletion and change in the copper level was concentrated, were identified, it was observed that Hap2p, Hap3p, Hap4p and Hap5p were among the top scoring transcription factors. HAP complex was reported as the key regulator of the mitochondrial function and involved in the control of nuclear genes implicated in several mitochondrial processes such as import and mitochondrial division [49]. Rlm1p, which is involved in the maintenance of cell integrity, Stp1p, which activates transcription of amino acid permease genes, Skn7p, which is involved in oxidative stress response, Mcm1p, which is involved in pheromone response and Smp1p, which is involved in regulating the response to osmotic stress were also among the key transcription factors in response to interaction effect of deletion of *ATX1* and change in the copper level.

3.3.2. Differences and Similarities between the *ATX1* and *CCC2* Deleted Strains

In order to elucidate the similarities and differences between the *ATX1* and *CCC2* deleted strains, the growth characteristics, transcripts levels, intracellular and extracellular ion concentrations of these two strains were compared.

3.3.2.1. Comparison of the Growth Characteristics of the *ATX1* and *CCC2* Deleted Cells.

The maximum specific growth rates that were calculated under high copper and copper

deficient conditions for each strain were compared in order to elucidate the differences between the growth characteristics of these mutants. Under copper deficient conditions, the maximum specific growth rate (μ_{max}) of the reference strain was highest and that of the *CCC2* deleted strain was the lowest. High copper levels resulted in a significant decrease in the μ_{max} of the reference strain (p-value <0.001). The maximum specific growth rate of the *ATX1* deleted strain also showed a slight but not significant decrease (p-value = 0.054) by high levels of copper but no change was observed in the *CCC2* deleted strain.

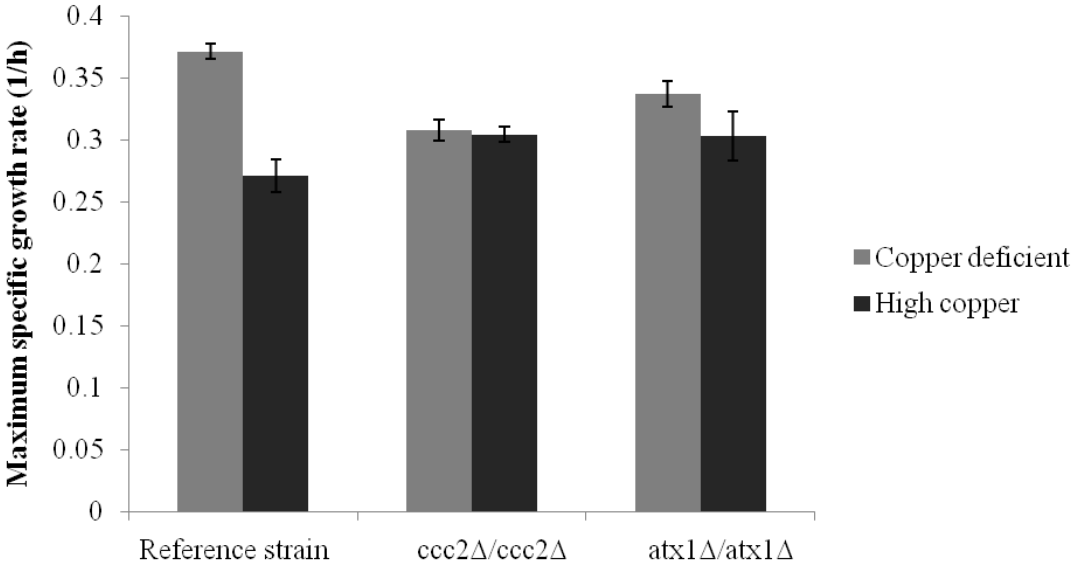


Figure 3.8. Maximum specific growth rates under copper deficient and high copper conditions.

Extracellular ethanol, glucose and glycerol concentrations and also biomass amounts were also determined for each strain under high copper and copper deficient conditions. These metabolite concentrations and biomass amounts were used to calculate the ethanol, glycerol and biomass yields on glucose.

Under copper deficient conditions, the ethanol yield on glucose was highest in the *CCC2* deleted strain and lowest in the reference strain. This result indicated that the respiratory deficiency in the *CCC2* deleted strain resulted in an increase in ethanol production in comparison to the reference strain. On the other hand, under high copper condition, the lowest ethanol yield on glucose was attained in the *CCC2* deleted strain. High copper levels increased the ethanol yield of the reference and the *ATX1* deleted strain

but decreased that of the *CCC2* deleted strain. The reduction of the ethanol yield in the *CCC2* deleted strain by high level of copper could be as a result of the increased respiratory capacity of this strain under high copper conditions. However, the increase in the ethanol yield of the *ATX1* deleted strain, which also has an increased respiratory capacity under high copper levels, was an interesting and unexpected result. When the glycerol production yields of these strains were compared under copper deficient conditions, similar to the ethanol yields, the highest glycerol yield was attained in the *CCC2* deleted strain. The glycerol yields of the reference strain and also that of the *CCC2* deleted strain decreased with high level of copper, whereas a slight increase was observed in the *ATX1* deleted strain. Glycerol is produced by fermentative mechanism in yeast and it is used for the reoxidation of NADH. Observation of higher glycerol yield under copper deficient condition in *CCC2* deleted strain provided additional support for the increased fermentative growth of this strain under copper deficient conditions. On the other hand, the increased glycerol yield of *ATX1* deleted strain, together with the increased ethanol yield, under high copper conditions when compared to copper deficient conditions, indicated that this strain preferred to show a fermentative behaviour under high copper conditions. Comparison of the biomass yields revealed that, high copper led to an increase in the biomass production in each strain. The biomass yield of the *ATX1* deleted strain was lower than that of the other strains under both conditions.

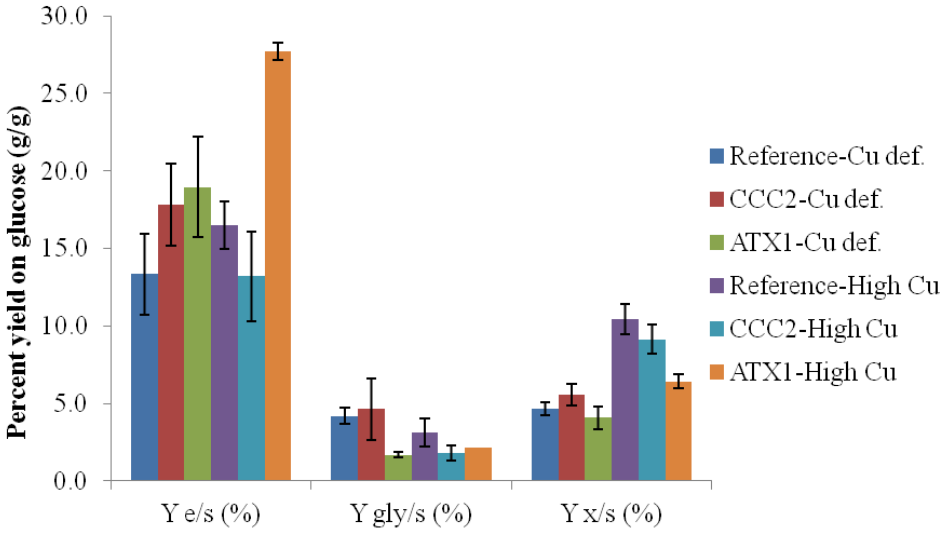


Figure 3.9. Ethanol, glycerol and biomass yields on glucose.

3.3.2.2. Comparison of Transcriptional Responses of the *ATX1* and *CCC2* Deleted Cells. In order to compare the transcriptional responses to the deletion of *ATX1* and *CCC2* genes, transcripts levels were investigated by one-way ANOVA analyses for copper deficient and high copper condition, separately.

Under copper deficient conditions, 429 genes were identified to be significantly expressed (p-value < 0.01) either in response to deletion of *ATX1* or *CCC2* gene. The expression levels of these transcripts were clustered into 5 clusters using Self Organizing Maps (SOM) [86] and the enriched GO Biological processes among the clusters were investigated (Figure 3.10a). The genes, which were induced slightly in the *ATX1* deletion mutant and showed a more pronounced induction in the *CCC2* deleted strain, were clustered in Cluster 0 and these genes were enriched with arginine metabolic process and cellular amino acid catabolic process terms (p-value < 0.05). The genes that were slightly down-regulated in the *ATX1* deleted strain and up-regulated in the *CCC2* deleted strain in comparison to the reference strain, were clustered in Cluster 1 and these genes were enriched with sporulation process term (p-value < 0.01). The genes, which were repressed both in the absence of *ATX1* and *CCC2* (Cluster 2), were enriched with tricarboxylic acid cycle and response to pheromone process terms (p-val< 0.05). The genes associated with ATP synthesis coupled proton transport term were clustered in Cluster 3 and these genes displayed a slight repression in the *ATX1* deleted strain and a more severe repression in the *CCC2* deleted strain. The down-regulation of these respiration related processes in the deletion mutants of *CCC2* and *ATX1* under copper deficient conditions were in accordance with the decreased respiratory capacity of these strains [87]. The genes, which were down-regulated in response to deletion of the *CCC2* gene but not differentially expressed in response to the *ATX1* deletion were clustered in Cluster 4 and these genes were enriched with ribosome biogenesis process term (p-val < 0.05).

Under high copper conditions, 89 genes were identified to be significantly expressed (p-value < 0.01) either in response to the deletion of the *ATX1* or *CCC2* gene. The expression levels of these transcripts were clustered into 4 clusters using Self Organizing Maps (SOM) [86] and the enriched GO Biological processes among the clusters were investigated (Figure 3.10b). The genes, which were repressed both in the *CCC2* and *ATX1* deletion mutants in comparison to the reference strain, were clustered in Cluster 1 and

these genes were enriched with regulation of lipid metabolic process term (p-value < 0.05). Other clusters, which included the genes that were up-regulated in the absence of *CCC2* gene but repressed in response to the deletion of *ATX1* gene (cluster 0), or the genes, which were up-regulated in the *AXT1* deleted cells both in comparison to the reference strain and the *CCC2* deleted strain (Cluster 2), or the genes, which were up-regulated in the *ATX1* deleted strain but repressed in the absence of *CCC2* gene (Cluster 3) were not significantly enriched (p-value < 0.05) with any process term.

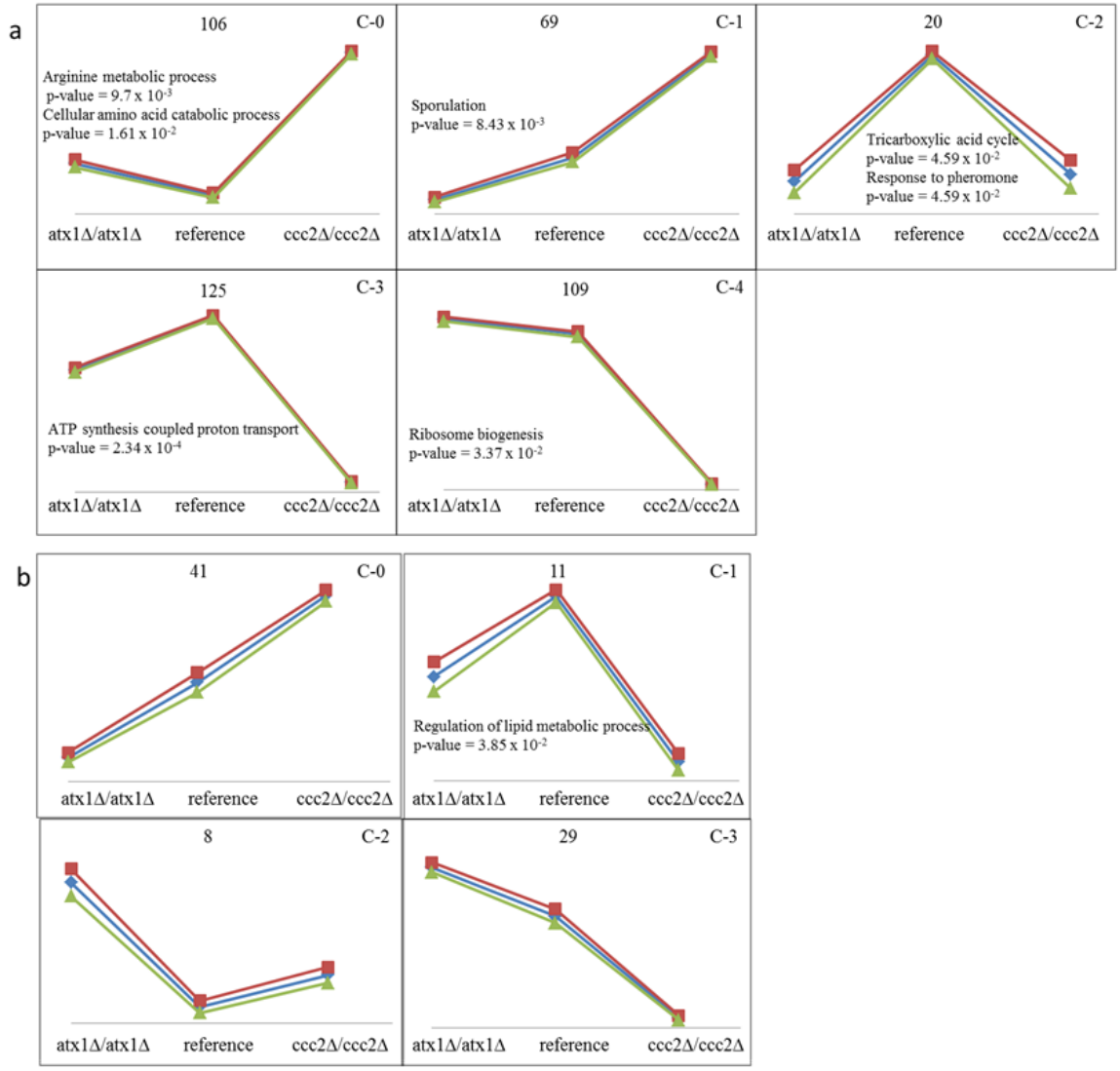


Figure 3.10. Clustering of significantly and differentially expressed genes in response to the deletion of *ATX1* and *CCC2* gene under copper deficient (a) and high copper (b) condition. The enclosing lines (■, ▲) indicate the confidence interval around the centroids.

3.3.2.3. Intracellular and Extracellular Ion Concentrations in Response to the Deletion of *ATX1* and *CCC2* under High Copper and Copper Deficient Conditions. In order to observe the effect of absence of the *CCC2* and *ATX1* gene on the cellular ion homeostasis, intracellular and extracellular iron, copper and zinc concentrations were determined in the deletion mutants of *ATX1* and *CCC2* and also in reference strain under high copper and copper deficient conditions. The intracellular and extracellular iron, copper and zinc ion concentrations in the *ATX1* and *CCC2* deleted strains were investigated in comparison to concentrations obtained in the reference strain under similar conditions. The intracellular and extracellular ion concentrations were expressed as ratio in Figure 3.11a and Figure 3.11b, respectively.

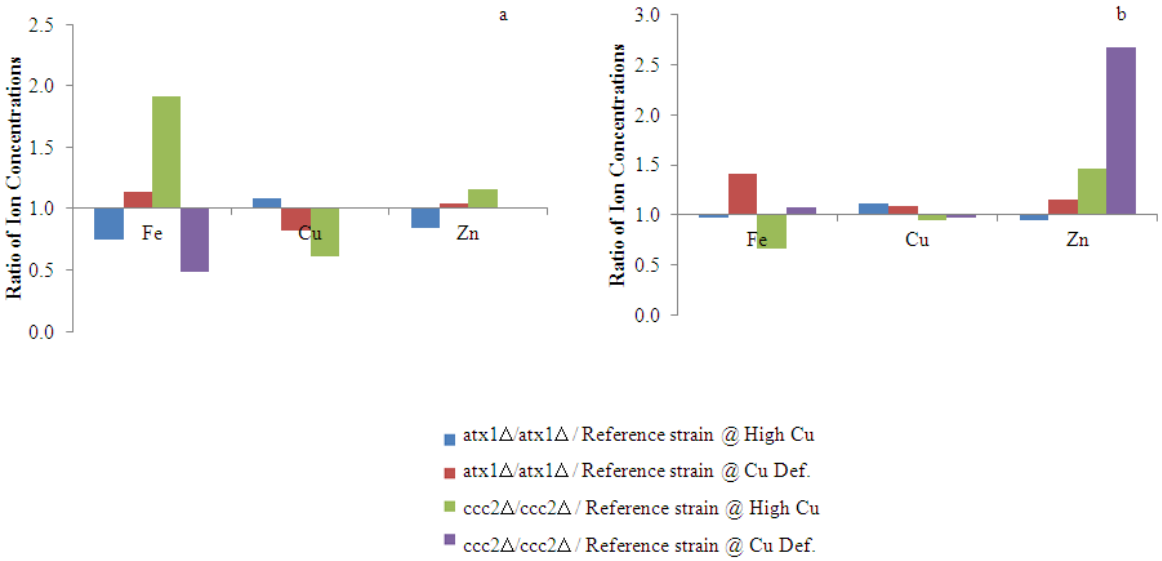


Figure 3.11. Intracellular (a) and extracellular (b) iron, copper and zinc concentrations.

Comparison of the iron concentrations in the *ATX1* deleted strain with the reference strain revealed that neither intracellular nor the extracellular iron levels showed more than 1.5 fold change between these two strains under any condition. On the other hand, in the *CCC2* deleted strain, intracellular iron levels showed approximately 2 fold increase under high copper conditions and 2 fold decrease under copper deficient conditions in comparison to the reference strain. When the extracellular iron levels were compared, a 1.5 fold reduction was observed in the *CCC2* deleted strain in comparison to the reference strain, under high copper conditions, under which the intracellular iron levels were high. On the other hand, under copper deficient conditions, extracellular iron levels did not

display a differential change in the *CCC2* deleted strain when compared to the reference strain.

When copper concentrations were investigated, it was observed that intracellular and extracellular copper concentrations did not display a differential change in the *ATX1* deleted strain in comparison to the reference strain (FC < 1.5). In the *CCC2* deleted strain, intracellular copper levels were lower under high copper conditions when compared to reference strain (FC = 0.6) but extracellular copper levels were almost same with the reference strain under both conditions.

Intracellular zinc ion concentrations did not display any differential change either in the *ATX1* or *CCC2* deleted strain under any conditions (FC < 1.5). When the extracellular zinc concentration were compared, no change was observed in the *ATX1* deleted strain in comparison to the reference strain but in the *CCC2* deleted strain, extracellular levels of zinc ion increased (FC > 1.5) both under copper deficient and high copper conditions.

3.4. Discussion

In this study, transcriptional response to the deletion of *ATX1* gene was investigated under copper deficient and high copper conditions in order to observe the effect of absence of the *ATX1* gene in a copper level dependent manner and also in order to compare the effect of absence of this gene with that of *CCC2* under similar conditions. The high copper condition, which was obtained by addition of 0.5 mM of $CuS0_4$ to the growth medium, was shown to restore the decreased respiratory capacity of the *ATX1* deleted cells. The analyses of the growth characteristic together with the significantly expressed genes and also integration of interactome and regulome with the transcriptome enabled the identification of the processes, which were affected from the applied perturbations.

3.4.1. The Global Transcriptional Reprogramming in Response to *ATX1* Deletion

The number of the significantly expressed genes in response to gene deletion or change in the copper level revealed that, only 7 % of the transcripts displayed a significant change in response to deletion of *ATX1* gene either independent of copper level or in a

copper level dependent manner as an additive or interaction effect. On the other hand, about 40 % of the genes were significantly expressed between high copper and copper deficient conditions independent of deletion of *ATX1* gene. These results indicated that absence of *ATX1* gene affected only a small group of transcripts whereas, majority of the genes were altered in the same way in response to a change in the copper level in the reference and *ATX1* deleted strains. The similarity between the transcriptional responses of the *ATX1* deleted strain and the reference strain to a change in the copper level might be due to the presence of an Atx1p independent pathway to deliver copper to Ccc2p [18].

Although only a small group of the genes were identified to be significantly expressed in response to deletion of *ATX1* gene, analyses of these genes and also their interacting partners revealed the process that were affected from the absence of *ATX1* gene. Absence of *ATX1* gene led to the down-regulation of the genes associated with ATP synthesis coupled proton (*ATP1*, *ATP14* and *ATP18*) and electron (*EMI5*, *COX8* and *COX5A*) transport processes. The transcripts that are encoding the proteins, which are interacting with the proteins associated with mitochondrial respiratory chain complex IV assembly, were also identified to be significantly expressed in response to the deletion of *ATX1* gene. These findings represented additional evidence for the decreased respiratory capacity of *ATX1* deleted cells. Integration of the protein-protein interaction network with the transcript levels also revealed that transcriptional response to interacting effect of gene deletion and change in the copper was concentrated around the genes, which were significantly enriched with respiration related process. The analyses of the transcripts levels together with the observation of higher ethanol yields in *ATX1* deleted strain under both conditions in comparison to reference strain clearly indicated that this strain preferred fermentation instead of respiration even in the presence of high levels of copper, which is able to restore the decreased respiratory capacity of this strain.

In addition to respiration related genes, the transcripts (*UME6*, *DEP1* and *CSR1*), which were associated with regulation of phospholipid biosynthetic process term (p-val < 0.05), were significantly down-regulated in *ATX1* deleted strain. *UME6* was also a key transcriptional regulator of early meiotic genes and also involved in the regulation of genes associated with arginine catabolism (*CAR1*, *CAR2*) [88]. Integration of the regulatory network with transcript levels also highlighted the fact that transcriptional response to the

deletion of *ATX1* gene was concentrated around transcription factors (Mbp1p, Ace2p and Fkh2p) involved in the regulation of cell cycle progression. In mammalian cells, *ATOX1*, which is the ortholog of *ATX1*, was reported to play a role in copper stimulated cyclin D1 expression and cell proliferation [89]. Although *ATX1* gene has not been previously associated with cell cycle regulation in yeast, identification of the transcription factors associated with cell cycle in response to the deletion of *ATX1* gene might indicate a possible role of this protein in cell proliferation. Moreover, identification of *CLN3*, which is the G1 cyclin involved in cell cycle progression in yeast, to be down-regulated in the *ATX1* deletion mutant in comparison to the reference strain both under high copper and copper deficient conditions also supported this prospect.

The effect of copper on the expression level of the genes (*GLO3*, *YPT1*, *GET2*), which were associated with retrograde vesicle mediated transport from golgi to ER, was observed to be antagonistic in the reference and *ATX1* deleted strains. These genes were up-regulated in response to high copper in the *ATX1* deleted cells, whereas, they were down-regulated in the reference strain. The up-regulation of these genes in the absence of the *ATX1* gene in response to high level of copper might be a cellular response to maintain proper functioning of intracellular transport machinery.

3.4.2. The Global Transcriptional Reprogramming in Response to High Copper Levels

Comparison of the transcription levels under copper deficient and high copper conditions revealed that change in the copper level led to a global transcriptional re-organisation both in the reference and *ATX1* deleted strains. Analyses of the significantly expressed genes revealed that high copper level led to a transcriptional re-organisation mimicking the starvation conditions. Genes associated with ribosome biogenesis were down-regulated, whereas transcripts that play a role in sporulation, autophagy, cellular amino acid metabolic process and cellular response to starvation were up-regulated. Up-regulation of the genes (*ARG1*, *ARG2*, *ARG4* and *ARG5,6*) that encode proteins in the arginine biosynthesis pathway also indicated that high copper levels induced an amino-acid starvation-like transcriptional response. Identification of the key transcription factors also revealed that transcriptional response to a change in the copper levels were concentrated

around 5 transcription factors (Tec1p, Nrg1p, Ume6p, Flo8p and Phd1p) that are involved in pseudohyphal growth, which is an adaptive response to limited nutritional conditions [90].

High copper levels also induced the genes associated iron ion homeostasis. *CCC2* was also observed to be up-regulated under high copper conditions when compared to copper deficient conditions in both strains. Up-regulation of high affinity iron transporter; Fet3p and siderophore iron transporters; Fit1p, Arn1p and Enb1p might indicate that high copper led to iron deficiency both in the reference strain and in the *ATX1* deleted strain. However, analyses of the intracellular and extracellular iron levels revealed that high copper levels did not result in a decrease in the intracellular or extracellular iron levels. The up-regulation of iron transporters under high copper conditions might be as a consequence of enhanced requirement for iron as a cofactor to decompose H_2O_2 in response to increased oxidative stress by high levels of copper [53].

Genes associated with TOR signalling cascade, actin cytoskeleton, endocytosis and cell wall organisation process terms were also up-regulated in response to high copper levels. Up-regulation of these genes could be interpreted as the response of the cells to mediate resistance to high copper concentrations [91]. The up-regulated genes involved in the TOR signalling cascade, specifically the genes encoding the components of TORC2 complex (*TOR2*, *TSC11*, *BIT61*, *SLM2*) coincides with the regulatory role of this complex in actin cytoskeleton organisation and endocytosis. Tor2p, which is required for efficient endocytosis, was proposed to act through Rom2p to stimulate Rho1, which regulates the cell wall synthesizing enzyme 1,3-beta-glucan synthase, Gsc2p [92]. Under high copper conditions, the expression levels of *RHO1*, *ROM2* and *GSC2* were also observed to be up-regulated in both strains.

Expression levels of *BNA1*, *BNA2*, *BNA4*, *BNA5* and *BNA6* were observed to be significantly up-regulated in response to high copper in both strains. This observation provided further support and explanation for the observation of tryptophan requirement for yeast growth under copper overload reported [8].

3.4.3. Does the Transcriptional Response to the Deletion of *ATX1* and *CCC2* Resemble or Differ?

Analyses of significantly expressed genes in response to gene deletion under copper deficient conditions revealed that the genes that play role in respiration related processes were repressed in both strains under copper deficient conditions. Moreover, observation of higher ethanol yield in these strains in comparison to the reference strain also provided additional support for this prospect. The more pronounced repression of the genes, which were associated with ATP synthesis coupled proton transport, in the *CCC2* deleted strain was also in accordance with the observation that the respiratory capacity of the *CCC2* deleted strains were lower than that of the *ATX1* deleted strains (Figure 3.1). Although high copper levels were shown to restore the decreased respiratory capacity of both strains, ethanol yield of the *ATX1* deleted strain was increased under high copper conditions indicating that these cells displayed a fermentative behaviour under high copper conditions. Moreover, the genes, which were associated with aerobic respiration, namely, *MDH1*, which encodes the mitochondrial isozyme of malate dehydrogenase that converts malate to oxaloacetate in the tricarboxylic acid (TCA) cycle, *QCR6*, which encodes the subunit of cytochrome bc(1) complex, and *COQ9*, which is required for respiratory growth, were repressed in the *ATX1* deleted strain in comparison to both reference strain and *CCC2* deleted strain under high copper conditions (Cluster 0). These results indicated that, the *ATX1* and *CCC2* deleted strains behave differently under high copper conditions.

In the absence of *CCC2*, the genes associated with ribosome biogenesis were down-regulated and sporulation related genes were observed to be up-regulated under copper deficient conditions. This fact together with the up-regulation of amino acid catabolic process term might indicate that, absence of the *CCC2* gene generates a starvation-like response under copper deficient conditions. Although a slight increase in the amino-acid catabolic process was observed also in the *ATX1* deleted strain, the absence of this gene seemed to generate a less severe effect. Moreover, comparison of the transcriptional profiles under copper deficient and high copper conditions in the *ATX1* deleted strain and reference strain revealed that high copper levels led to a transcriptional re-organisation mimicking a starvation-like response both in the absence of *ATX1* gene and in the reference strain.

Comparison of the intracellular and extracellular iron and copper levels also revealed that, absence of *ATX1* did not have any differential effect on ion concentrations. However, intracellular iron levels decreased under copper deficient conditions and increased under high copper conditions in comparison to the reference strain in the absence of *CCC2* gene.

These results clearly represented that although the proteins encoded by *ATX1* and *CCC2* genes take part in the consecutive steps in the intracellular transport of copper, the absence of these genes did not yield similar responses. One possible reason for this observation might be the presence of an *ATX1* independent pathway that deliver copper to Ccc2p [18]. Another explanation for the diversity might be the fact that the proteins encoded by *ATX1* and *CCC2* genes might have some other roles rather than copper transport.

3.5. Concluding Remarks

The analysis of the transcriptional response to the deletion of *ATX1* gene under copper deficient and high copper conditions and the further investigation of the transcript levels in conjunction with interactome and regulome revealed the genome-wide effect of the deletion of *ATX1* gene. Comparison of the transcript levels of the *ATX1* deleted strain with that of the *CCC2* deleted strain enabled the identification of the similarities and differences between the genome-wide effects of the absence of these genes.

The down-regulation of the genes associated with ATP synthesis coupled proton and electron transport processes together with the higher ethanol yield obtained in the *ATX1* deleted strain in comparison to the reference strain was in accordance with the decreased respiratory capacity of this strain. Although high copper levels were shown to restore the decreased respiratory capacity, ethanol yields were observed to be higher under high copper conditions in the absence of *ATX1*. This result indicated that the *ATX1* deleted cells preferred fermentation even they were capable of respiring. The integration of transcript levels with interactome and regulome also highlighted the effect of the deletion of *ATX1* gene and the interaction effect of gene deletion and change in the copper level on respiratory mechanism.

The integrated analyses of the transcript levels with regulome showed that the deletion of the *ATX1*gene affected the regulation of the cell cycle. This result together with the down-regulation of *CLN3* in this strain indicated that *ATX1* gene might play a role in the regulation of cyclin expression similar to its mammalian counterpart.

The high copper levels affected the majority of the transcripts in the same way in the reference and *ATX1* deleted strains. High copper levels led to the repression of the genes associated with ribosome biogenesis and induction of genes that play role in the autophagy, amino acid metabolic process and response to starvation processes. This result indicated that high levels of copper induced a starvation-like response both in the reference and *ATX1* deleted strains. Moreover, the key transcriptional regulators identified in response to high copper levels also supported this prospect. High copper levels also induced iron transporters indicating the increased demand for iron in these strains. The up-regulation of processes associated with actin cytoskeleton organisation, cell wall biogenesis and endocytosis together with the TOR signalling cascade under high copper levels might indicate that cellular response to increase resistance to high copper levels was mediated by TORC2 complex.

Comparison of the transcriptional responses and also the growth characteristics of the *ATX1* and *CCC2* deleted cells demonstrated that although the proteins encoded by these genes take part in the consecutive steps in the intercellular transport of copper, the absence of these genes did not yield similar responses. The *CCC2* deleted cells displays a nitrogen starvation-like response under copper deficient conditions, whereas high copper levels leads to a similar response in the *ATX1* deleted cells. The absence of *CCC2* gene resulted in a decrease in the intracellular iron levels on the other hand, any effect of the *ATX1* deletion on iron levels could not be observed. Either the presence of an *ATX1* independent pathway that delivers copper to Ccc2p or the possible role of *ATX1* gene in regulation of cell cycle might be the reason for these diverse responses.

4. A NOVEL STRATEGY FOR SELECTION AND VALIDATION OF REFERENCE GENES IN DYNAMIC MULTIDIMENSIONAL EXPERIMENTAL DESIGN IN YEAST

Understanding the dynamic mechanism behind the transcriptional organization of genes in response to varying environmental conditions requires time-dependent data. The dynamic transcriptional response obtained by real-time RT-qPCR experiments could only be correctly interpreted if suitable reference genes are used in the analysis. The lack of available studies on the identification of candidate reference genes in dynamic gene expression studies necessitates the identification and the verification of a suitable gene set for the analysis of transient gene expression response. In this chapter of the study, the strategy developed for the determination of a candidate reference gene set, which was proposed to be used for the normalization of dynamic expression profiles of the target genes in *S. cerevisiae*, was explained [93]. The methodology followed for the determination and selection of stable expressed genes were described and the validity of this candidate gene set was verified under two different experimental conditions.

4.1. Background

Single time point experiments provide a static measurement of gene expression although transcription is a temporal process. Time series gene expression experiments are useful in elucidating the dynamic mechanism behind a biological process. The high-throughput data obtained from microarray technology enables the identification of differentially expressed genes. Real-time RT-qPCR is the favoured method for conducting a detailed study on a gene set of interest, which is determined from high-throughput studies. It is a widely preferred method for quantitative gene expression analysis due to its high sensitivity, specificity and wider dynamic range. However, data normalization for the elimination of sample-to sample differences is the main obstacle of this method even in non-transient studies [94–97]. The multidimensional and temporal nature of time series data renders its analysis more complicated thus drawing attention to the importance of normalization.

Normalization with internal control genes, generally referred to as the housekeeping or reference genes, is a commonly used method among various normalization techniques for the determination of gene expression using real-time RT-qPCR. A suitable reference gene should be constitutively expressed in the tissues or cells under investigation regardless of the experimental perturbation. A single reference gene was frequently used in many studies in order to normalize mRNA fraction without any validation of stability. However growing evidence indicates the absence of a single universal reference gene, which may be independent of all kind of experimental conditions. Since the normalization of real- time RT-qPCR data using a non-validated single reference gene may engender misleading conclusions, in recent years, the calculation of a normalization factor based on the geometric average of validated multiple reference genes was suggested to discard possible outliers and differences in the abundance of different genes [98].

The general approach in normalization using multiple genes is the selection of reference genes among candidate genes, which have been commonly used for normalization. However a group of genes selected among the commonly used reference genes may not be a suitable reference gene set for each experimental condition. *ACT1*, which is a commonly used reference gene for *Saccharomyces cerevisiae*, was validated to be a suitable reference gene to normalize the expression of genes involved in central carbon metabolism in response to a short-term glucose pulse [99]. On the other hand, the use of *ACT1* and other commonly used reference genes *(PDA1, TDH3, RDN18)* was invalidated as internal control for quantitative analysis of gene expression in the yeast *S. cerevisiae* during long-term growth on glucose [97]. This fact clearly indicates the necessity of the validation of the suitability of reference genes under specific experimental conditions and steers us towards the determination of a candidate reference gene set among novel genes instead of commonly used ones, which is suitable for use under the conditions of interest.

A reasonably successful approach in the compilation of a candidate reference gene set is to select genes from a genome-wide background and to use a large number of microarray datasets for a specific condition and for specific tissues/organisms [95, 96, 101]. Since the determination of a suitable candidate gene set creates a big concern in dynamic expression profiling studies due to the multidimensional and dynamic nature of

the experiments, microarray datasets could be used to identify the transcripts, which display stable expression with respect to time as well as to different genetic and environmental conditions. However, a vigilant human-mediated research is required for the selection of the microarray datasets, which will be used for the identification of the candidate reference gene set.

Following the compilation of the candidate reference gene set, the second key point is the determination of the most stable genes among the candidates under selected conditions. Several software tools were developed for the verification of the suitability of the candidate reference genes such as geNorm [98], NormFinder [101] and Bestkeeper [102]. As designated by the geNorm algorithm, the expression ratio of two suitable reference genes should be constant across all samples. Therefore the inclusion of two correlated genes in the list of candidates may lead to false positive results due to the similarity in their expression profiles. On the other hand, the NormFinder algorithm depends on statistical linear mixed-effects modelling, which accounts for the overall variation in the expression of the candidate reference genes and also for the variation between the sample groups. NormFinder was found to be less sensitive in the case of correlated gene sets. Although the definition of "stability" differs somehow among these algorithms, there are many studies reporting similar results in terms of stability when either one of these software were used [97, 104, 105].

The aim of the present study was to determine a set of candidate reference genes to analyse the dynamic transcriptional response of selected genes to changing environmental conditions by real-time RT-qPCR in *S. cerevisiae*. For this purpose, a set of candidate reference genes, which can be used for the dynamic expression profiling studies in *S. cerevisiae*, was determined for the first time using 31 publicly available and independently generated time-dependent transcriptome data.

This candidate reference gene set was then verified using two separate datasets to identify the most stable reference genes under these two selected environmental conditions and the stability of the reference sets were confirmed by expression analysis of the specific perturbation responsive genes. The stable reference gene sets comprised of both previously reported reference genes and genes that were identified as reference for the first time in

this study. Moreover these newly identified genes were determined to be more stable than the common reference candidates, used either individually or as a multiple gene set, presenting an improvement in the determination of gene expression profiles.

This study revealed that the selection of reference genes among the candidate reference gene set, which was identified in this present study, outperformed the use of reference genes that were frequently reported in the literature. This novel approach allows the reduction of multiple parameters that would be frequently encountered in dynamic gene expression studies down to only one parameter, time, for the determination of a suitable set of reference genes. The high flexibility of this method enables the use of this candidate reference gene set in the determination of reference genes under any experimental condition as long as the supplemented data is of dynamic nature.

4.2. Materials and Methods

4.2.1. Determination of the Reference Gene Candidates

In order to determine the reference candidates displaying stable expression throughout time course experiments, transcriptome data sets were used. Publicly available time series microarray data sets were downloaded from the Array Express database [105]. Thirty one different time-dependent experiments conducted with *S. cerevisiae* using Affymetrix Yeast Genome S98 and Yeast2 arrays (14 and 17 experiments, respectively) were first pre-processed with RMA Express software applying quantile normalization [106]. For the multiple probe sets targeting the same gene, the average expression value would be used if there were no significant difference between the expression levels of the multiple probes and the larger value would be selected if there were a significant difference. The expression levels of the genes that were detected in both platforms were combined and a matrix of dimensions 5424x888 was obtained for the 5424 identified transcripts measured at 888 time points in 31 different experiments.

4.2.1.1. Assessment of Expression Stability by Coefficient of Variation Approach. The candidate reference genes with stable gene expression across time were determined by the coefficient of variation approach [95]. For each gene, the coefficient of variation (CV) of

the expression value, which is defined as the ratio of the standard deviation to the mean was calculated and the CV values of the genes were then ranked.

CV values were determined for the combined data set of 31 different experiments and also individually for each experiment. The CV values were used in the assessment of stable expression profiles and the candidate gene list was determined.

4.2.1.2. Composition of the Combined Datasets. In order to determine the effect of inclusion of an additional dataset in the final cluster of experiments on the stability profile of the genes, combinations of 31 datasets were created. There were 465 possible combinations that contain 2 or 29 datasets and 31 possible combinations that contain 30 datasets. Stability profiles for each of these 496 combinations were identified. However, due to the excessive number of the possible combinations for the subsets containing 3 to 28 datasets, 1000 random combinations were generated for those subsets and the stability analysis was conducted for those random combinations. In the following expression, n_i represents the number of generated subsets containing i datasets and c_i represents the number of all possible subsets containing i datasets, where $1 < i < 31$.

$$n_i = \begin{cases} c_i & if\ c_i < 1000 \\ 1000 & if\ c_i > 1000 \end{cases}$$

4.2.2. Confirmation of the Stability of Reference Genes and Their Validation

The suitability of the candidate reference gene set was confirmed under two different experimental conditions.

4.2.2.1. Strain and Media. Homozygous *ho*Δ/*ho*Δ strain of *S. cerevisiae* from a genetic background of BY4743 (*MAT*a/*MAT*Δ *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/l*eu2*Δ*0 lys2*Δ*0*/+ *met15*Δ*0*/+ *ura3*Δ*0*/*ura3*Δ*0*), purchased from the European *Saccharomyces cerevisiae* Archive for Functional Analysis [27], was kindly provided by Prof. Stephen G. Oliver. The absence of the deleted gene was verified using PCR-based methods [107].

The preculture was incubated in YPD medium (2% [wt/vol] D-glucose, 2% [wt/vol] peptone, 1% [wt/vol] yeast extract) at 30°C and 180 rpm in an orbital shaker. Synthetic defined medium was used in the fermentations [108].

4.2.2.2. Chemostat Culture, Experimental Design and Sample Description. Cells were cultivated in B-Braun Biostat B plus fermenters with 1.5L working volume of ammonium or glucose-limited synthetic defined medium. Ammonium or glucose was injected in an impulse-like manner into their respective limited cultures at steady state. The temperature was controlled at 30^{o}C and the agitation was set to 800 rpm. The pH was controlled at 5.5 with 1M NaOH and HCl. The dilution rate was set to 0.1 hr^{-1}. The air flow into the fermenters was adjusted at 1.5 L/min to keep dissolve oxygen saturation above 80% throughout the experiment. A total of 14 time-dependent RNA samples were collected at steady state, in the first minute with 20 second intervals, with 8 minute intervals for 32 minutes and then hourly for 5 hours following the impulse. The sample volume was 5 ml of culture at an OD range of 1.2-1.4. The samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until further processing.

4.2.2.3. Nucleic Acid Extraction. RNA extraction and quality control were carried out as described in Section 2.2.3.

4.2.2.4. Reverse Transcription. In order to unify the initial concentration of RNA in the analyses, all samples were diluted to the same concentration prior to the real-time RT-qPCR application. Reverse transcription was carried out at 50^{o}C for 30 minutes. The reaction was allowed to proceed with 80 ng/µl in a reaction volume of 12.5 µl. QuantiTect RT mix (Qiagen, USA, Cat no: 204245) was used at a ratio of 0.01 total reaction volume. Assays were conducted in triplicates. The synthesized cDNA template was immediately allowed to proceed with the polymerase chain reaction.

4.2.2.5. qPCR Target Information. The PCR product (amplicon) was determined to be either 100-150 base pairs long or 200-250 base pairs long. The gene symbols, sequence accession numbers, location of the amplicon, amplicon lengths, *in silico* PCR results for specificity is provided in as a supplementary document in the reference article [93].

4.2.2.6. qPCR Oligonucleotides. The primer length was selected to be in the range of 18-24 nucleotides. The GC content of the primers ranged between 50 to 60 per cent. The melting temperatures of the primers were in the range of 55-58^{o}C. Primer3 software was used in the design of the primers except for that for 18S rRNA, which was designed in this study [109]. The complete set of primer pair sequences and RTPrimerDB identification numbers are listed in Table 4.1. The designed primers were manufactured by Alpha DNA (Montreal, Quebec) and desalted. The performance of all primers was experimentally confirmed by conventional PCR to ensure the amplification of a single region with the correct amplicon length.

Table 4.1. Gene primer sequences and efficiencies used in this study.

Primer Name	**Primer Sequence**	**ID**	**Efficiency (%)**
FBA1-F	GACTTGTACACCAAGCCAGA	8325	87
FBA1-R	GATGTCACCAGCGTACAAAC		
PDC1-F	CGCTCGTATCAAGGGTATGT	8326	108
PDC1-R	CTTAGCTTGAGCAGAGATGG		
GCN4-F	GCTAGATGACGCTGTAGTGGAA	8383	95
GCN4-R	CGTCAGTGGTAACTGGAATG		
RPS26A-F	GGTATTACCGGTGGTGAAGAAG	8387	85
RPS26A-R	AGCAATGGAGAAGTTTGGAGAG		
TDH3-F	CAAGGAAACCACCTACGA	8329	86
TDH3-R	CGAAGATGGAAGAGTGAGAG		
ARF1-F	CTAACGATAGATCGCGTATTGG	8389	107
ARF1-R	TCTGGCAAATCTTGCTTGTTAG		
TPI1-F	GTGTCGGTGTCATCTTGTGT	8388	95
TPI1-R	ACGACGTTAGTCCAGTCCTT		
CDC19-F	CAGAGGTGACTTGGGTATTG	8332	103
CDC19-R	GGTTGGTCTTGGGTTGTAAG		
PGK1-F	GGAACACCACCCAAGATAC	8333	82
PGK1-R	GGTGACATCCTTACCCAAC		

Table 4.1. Gene primer sequences and efficiencies used in this study (Continued).

ADH1-F	CAAGTCGTCAAGTCCATCTC	8334	103
ADH1-R	GTAGACAAGCCGACAACCT		
CCW12-F	TTTGGTTTCCACCGCTAC	8335	85
CCW12-R	CAGCAGAGGTGGTGTTCTTT		
ACT1-F	AGAGTTGCCCCAGAAGAACA	8336	93
ACT1-R	GGCTTGGATGGAAACGTAGA		
HAP4-F	CCTCCTCCATTTCATCTCCA	8337	103
HAP4-R	GCAGCAATGGTTTCCACATC		
MEP2-F	CTGGACATGGTGGTCTAGTT	8338	113
MEP2-R	GAGGTGACGGAATGTGGT		

4.2.2.7. qPCR Protocol and qPCR Validation. Qiagen QuantiTect® SYBR® Green one step RT-PCR kit was used for real-time RT-qPCR applications as described by the manufacturer (Qiagen, USA, Cat no: 204245). All kit contents are optimized and validated by the manufacturer. The PCR reactions were performed in a final reaction volume of 12.5 µl containing the final concentration of 2.5 mM of $MgCl_2$ and 0.5 mM of forward and reverse primers. Plates and film sealers were manufactured from Bio-Rad Laboratories (Cat no: MSB1001, MLP9601). The reaction mixtures were prepared manually and the reactions were allowed to proceed in iCycler 5 instrument (Bio-Rad Laboratories). Assays were conducted in triplicates. The annealing temperature optimization and thermocycling parameters are provided in Table S8. RT-PCR sensitivity and reproducibility assays were conducted as described by the manufacturer (Qiagen, USA, Cat no: 204245). The range of the Cq values was determined as 10.9-31.70 for case study I and as 12.00-28.30 for case study II. The presence of no-template control wells were ensured in all reaction plates. Serial dilutions of RNA (5-point 4-fold dilution series, starting with 100 ng/reaction) were used for the measurement of the overall assay efficiency for one step RT-PCR. The log template amount was plotted against the corresponding Cq value and the slope (S) was determined. The efficiency (E) was calculated according to the following formula:

$$E = 10^{(-1/S)} - 1 \tag{4.1}$$

The efficiency values for each of the candidate reference genes and the target genes were provided in Table 5.1. The linear dynamic range of the Cq values was between 14.7 and 35.7. The 95% confidence interval at the lowest limit was determined to be less than 1 for the Cq values that were determined using either forward/reverse primer pair except for *ACT1* and *PDC1* (1.5 and 1.8, respectively). The average of the 95% confidence intervals of the Cq values throughout the range was 0.6.

4.2.2.8. Data Analysis. iCycler^{TM} iQ Optical System Software version 3.0a (Bio-Rad Laboratories) was used with PCR base line subtracted curve fit method for the measurement of quantification cycle (Cq). The geometric average of the Cq values with a standard deviation of less than 0.5 cycles was used in further calculations. Raw Cq values were used to determine the relative gene expression values (Q) using delta-Ct method [110]. Cq values for no-template control wells were excluded from further analysis since the values were greater than 35 or not detected. The data analysis was carried out using Microsoft Office 2007 Excel implemented with the statistical analysis tool.

4.2.2.9. Assessment of Expression Stability by geNorm and NormFinder. The expression stability of candidate reference genes was evaluated using geNorm [98] and NormFinder [100] software programs. The expression values were provided as input for both algorithms. Delta-Ct method was used for the calculation of expression values with the gene having the lowest Cq value being used as the reference gene [98].

4.3. Results

4.3.1. Identification of a Candidate Reference Gene Set

Thirty one different time series datasets from experiments conducted using *S. cerevisiae* strains were collected from the publicly available database of functional genomics experiments; ArrayExpress [105]. The coefficient of variation (CV), which is the ratio of standard deviation to mean, was used to compare the extent of variation among the expression levels of genes and therefore to determine the transcripts, which displayed a stable expression profile throughout different time points. The ranking of the CV values

was defined as the stability profile in this study. Two different approaches were used to determine the candidate reference gene set based on stability profiles.

In the first approach, each of the 31 datasets was analysed separately in order to obtain the individual stability profiles. The top 100 stable genes were identified for each dataset. 4156 transcripts did never rank within the top 100 genes in any of these datasets and interestingly there was no common unique transcript, which appeared among the top 100 stable genes in all cases. *TPI1* and *ACT1* were among the 100 most stable transcripts in 81% of the cases. The 10 transcripts, *TPI1, ACT1, TDH3, FBA1, CCW12, CDC19, ADH1, PGK1, GCN4* and *PDC1*, which ranked within the top 100 in more than 55 % of the datasets, were selected as candidate reference genes. Among these genes *ACT1*, which encodes the single essential gene for actin, and *TDH3*, which encodes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were reported to be commonly used reference genes in yeast [111]. *CCW12*, the cell wall mannoprotein with a role in the maintenance of newly synthesized areas of cell wall; *GCN4*, the transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation, and other genes functioning in the super pathway of glucose fermentation were identified in this candidate reference gene set [107].

As a second approach, the stability profiles of the genes were investigated by combining different datasets in order to identify the genes, which display less fluctuating expression profiles across different time points and under different experimental conditions. The expression values of the genes that are common in all datasets were used to construct the combined dataset. At this point a question of whether there were a prerequisite number of experiments to be included into this analysis arose. In order to observe the effect of the number of datasets on stability profiles of the genes, resampling from the data was carried out via bootstrapping. Random combinations of 31 datasets were generated. The stability profiles for each of these combinations were calculated and the similarities between these stability profiles and the overall stability profile, which was obtained by combining all 31 datasets, were assessed using the Pearson correlation coefficient (PCC). Average of the PCC values were calculated for the combinations having the same number of datasets. The average and the minimum of the PCC values of the

combinations that have the same number of datasets were examined as a function of the number of datasets (Figure 4.1).

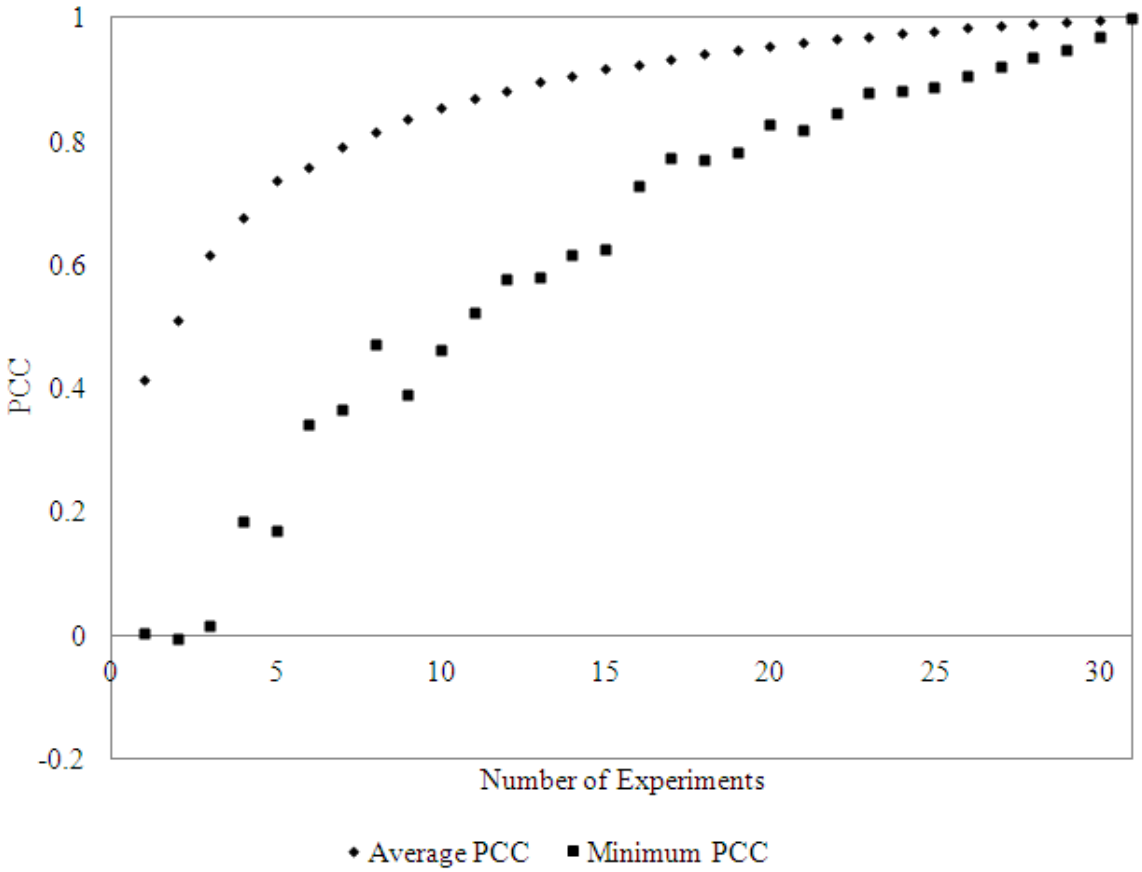


Figure 4.1. Minimum and average PCC values as a function of the number of experiments.

The analysis of these combinations showed that both the average and the minimum PCC increased with the inclusion of increasing numbers of datasets. This observation indicated that, each dataset included in the analysis has a contribution to the stability profile. Therefore the combination of all available data sets was used in the second approach.

The stability profile of this combined dataset, which consists of the expression values of 5423 transcripts across 888 different time points, revealed the genes displaying fewer fluctuations in their expression profiles across all these time points. The top 100 genes displaying the lowest CV value were investigated for the identification of candidate reference genes. The CV values of these genes varied between 0.05 and 0.11. A binned frequency histogram of the available data indicated that a gene pool of 6 genes with the

lowest CV values (corresponding to 1 % of the total population) would be a suitable choice of candidate pool (Figure 4.2).

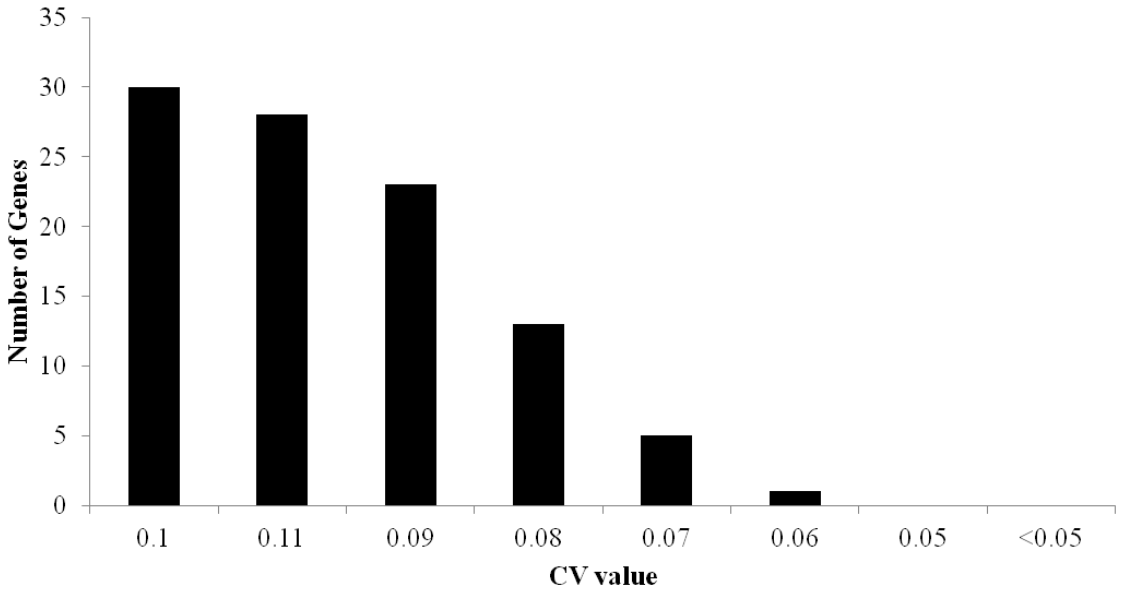


Figure 4.2. A non-cumulative histogram plot of the number of genes assigned to a specific CV value.

Bringing the CV threshold above 0.07 resulted in an observable number of additional genes thus populating the candidate pool with genes having less stable expression profiles, which are likely to be discarded in a stability analysis and rendering the experiments costly. These genes were *TDH3, RPS26A*, *TPI1, CDC19, ARF1* and *ENO1*. Due to the high homology between *ENO1* and *ENO2* (blastn e-value= 4.7 x 10^{-264}), concerns regarding a possible unspecificity in the PCR product led to the elimination of this gene from the candidate set. Therefore the top five genes, *TDH3, RPS26A*, *TPI1, CDC19* and *ARF1* with the smallest coefficient of variation values were selected as the candidate reference genes. Among these genes, *TDH3, CDC19* and *TPI1* were also identified as candidate genes using the first approach. *RPS26A* encoding a protein component of the small ribosomal subunit (40S) has similarity to rat S26 ribosomal protein, which is commonly used as a reference gene in rat tissues [112]. The wheat ortholog of *ARF1*, which encodes an ADP-ribosylation factor, was shown to be one of the most stable reference genes in that particular plant [96].

The candidate reference genes, which were selected using two different approaches, were combined and a total of 12 transcripts were determined as the candidate reference gene set for normalization of dynamic real-time RT-qPCR data.

4.3.2. Identification and Verification of Exclusive Reference Gene Subsets Under Specific Experimental Conditions

In order to test the applicability of this candidate reference gene set on different experimental conditions, this pool of genes were used to identify the most stable condition specific reference genes. For this purpose, perturbations involving nutrient availability in yeast were investigated. Yeast cells grown in glucose-limited (Case study I) or ammonium-limited (Case study II) continuous cultures at steady state were supplemented with the respective limiting nutrient in an impulse-like manner. Dynamic gene expression profiles of the candidate reference genes were investigated in response to recovery from limiting conditions. The expression levels of *HAP4* in Case study I and *MEP2* in Case study II were investigated using a reference gene set, which was determined from the available candidate genes. *HAP4* and *MEP2* were selected such that their expression profiles were responsive to variations in the amount of available glucose and ammonium, respectively. Primers of 12 candidates were designed and their amplification efficiencies were determined. All candidates except for *PGK1,* whose amplification efficiency could not be improved to be higher than 85%, were procured for further analysis. Regions from the 11 candidate reference genes as well as the query genes were PCR amplified and the raw Cq values were transformed into relative quantities using the delta-Ct method [96]. All analyses in these case studies were conducted in compliance with MIQE guidelines for qPCR [113]. The expression stability of these candidates throughout these time series experiments was tested using two different statistical software programs; geNorm and NormFinder.

4.3.2.1. Case Study I: Selection and Validation of the Most Stable Reference Genes During Glucose Perturbation. The presence of any correlated gene pairs among the candidates was previously reported to engender misleading predictions regarding the stability order of the candidate reference genes [98]. In order to identify correlated gene pairs from the candidate list, Pearson correlation coefficients between expression values of each pair of the candidates were calculated. The threshold for the correlation among gene

pairs was decided as ±0.85. A total of three correlated gene pairs were identified. *ARF1* was correlated with two other genes; *TPI1* and *CDC19*. The third correlated gene pair was *FBA1* and *CCW12*. Five different pools of candidate genes were created in order to investigate the effect of the inclusion of correlated gene pairs in the reference gene set. P-0 was the pool including all candidate genes. P-1 and P-3 were created by excluding *ARF1* from the candidate list together with either *CCW12* or *FBA1*, respectively. As an alternative, *ARF1* was kept in the pool and the other two transcripts (*TPI1* and *CDC19*), which showed high correlation with *ARF1*, were excluded from the candidate list in addition to either *CCW12* or *FBA1* (P-2 and P-4). One other alternative pool was constructed by excluding all of these correlated genes from the candidate list (P-5).

In order to identify a suitable set of reference genes, two algorithms; NormFinder and geNorm were used. Both algorithms were used to rank the eleven candidates based on the stability of their expression profiles. Additionally, geNorm algorithm was also used to determine the stability ranking of genes in the pools, in which only the genes with a correlation of < |0.85| among their expression profiles were left (P1-P5) (Figure 4.3).

	NormFinder	geNorm-P-0	geNorm-P-1	geNorm-P-2	geNorm-P-3	geNorm-P-4	geNorm-P-5
TPI1	1	5	2	-	3	-	-
FBA1	2	2	1	1	-	-	-
CDC19	3	1	1	-	2	-	-
ACT1	4	6	4	3	4	4	3
ARF1	5	1	-	1	-	3	-
CCCW12	6	3	-	-	1	2	-
GCN4	7	4	3	2	1	1	5
RPS26A	8	7	7	6	7	7	2
TDH3	9	8	5	4	5	5	1
PDC1	10	9	6	5	6	6	1
ADH1	11	10	8	7	8	8	4

Figure 4.3. Stability analysis of the candidate genes in Case Study I.

The genes were scored using the average value of the stability rankings obtained by NormFinder and geNorm for each pool separately. The total score for each candidate gene was calculated by summing up its individual scores for each case; P-0 to P-5. The genes were then ranked based on their total scores from the minimum score to the maximum score indicating an order from the most stable to the least stable gene. The minimum

number of reference genes to be used was selected as 4 in order to make sure that at least one stable gene could be selected even after the removal of correlated gene pairs in P-4 and P-5. This ranking indicated that the lowest total scores were obtained for *TPI1*, *FBA1*, *CDC19* and *ACT1* (Figure 4.4).

	Score P-0	Score P-1	Score P-2	Score P-3	Score P-4	Score P-5	TOTAL SCORE
TPI1	3.0	1.5	1.0	2.0	1.0	1.0	9.5
FBA1	2.0	1.5	1.5	2.0	2.0	2.0	11.0
CDC19	2.0	2.0	3.0	2.5	3.0	3.0	15.5
ACT1	5.0	4.0	3.5	4.0	4.0	3.5	24.0
ARF1	3.0	5.0	3.0	5.0	4.0	5.0	25.0
GCN4	5.5	5.0	4.5	4.0	4.0	6.0	29.0
CCW12	4.5	6.0	6.0	3.5	4.0	6.0	30.0
TDH3	8.5	7.0	6.5	7.0	7.0	5.0	41.0
RPS26A	7.5	7.5	7.0	7.5	7.5	5.0	42.0
PDC1	9.5	8.0	7.5	8.0	8.0	5.5	46.5
ADH1	10.5	9.5	9.0	9.5	9.5	7.5	55.5

Figure 4.4. Scoring of the candidate genes in Case Study I.

The scores for P-1 and P-2 were the lowest for these four reference genes indicating that these pools provided the optimal configuration. Since two of the most stable genes; *TPI1* and *CDC19*, were removed in P-2 in order to discard the correlations among gene pairs, the scoring for these genes was based solely on the stability ranking obtained from NormFinder. The reference gene selection was carried out based on the stability scoring that was provided for P-1 since both geNorm and NormFinder stability rankings were available for all of these four genes. *TPI1*, *FBA1*, *CDC19* and *ACT1* were also determined as the four most stable genes among all candidates when the NormFinder algorithm was used to perform the stability analysis.

Exclusion of the correlated gene pairs resulted in differences in the stability orders of the candidate genes when geNorm was used. The comparison of the stability rankings of P-0 and P-1 by geNorm with the rankings obtained by NormFinder indicated that elimination of correlations by the removal of *ARF1* and *CCW12* resulted in obtaining similar results using both algorithms. *TPI1* and *ACT1* could not be identified as stable unless correlations that were present among the expression profiles of the genes in the pool were removed.

These results indicated that, correlated gene pairs should be excluded from the candidate list when using geNorm in order to avoid misleading results during the determination of the stable reference genes. Exclusion of the correlated gene pairs facilitates the attainment of comparable results by both algorithms. These two algorithms thus could be used to cross-check conjoint results while selecting the most suitable reference genes.

Table 4.2. Pair-wise variations between the $(n+1)^{th}$ and the n^{th} genes for different pools of candidate sets in Case Study I.

Case Study I	**V2/3**	**V3/4**	**V4/5**	**V5/6**	**V6/7**	**V7/8**	**V8/9**	**V9/10**	**V10/11**
P-0	0.155	0.129	0.101	0.105	0.107	0.127	0.114	0.093	0.096
P-1	0.205	0.154	0.146	0.172	0.127	0.102	0.114		
P-2	0.178	0.194	0.232	0.159	0.116	0.127			
P-3	0.219	0.164	0.148	0.177	0.130	0.103	0.114		
P-4	0.164	0.202	0.240	0.164	0.118	0.127			
P-5	0.279	0.245	0.203	0.172					

geNorm suggested using a varying number of reference genes between 3 and 6 for different pools (with a pairwise variation threshold of 0.15 as suggested by the software developers) for determining the expression profile of *HAP4* (Table 4.2).

A set of at most six reference genes; *TPI1*, *FBA1*, *CDC19*, *ACT1*, *ARF1* and *GCN4* were determined to constitute a suitable pool for the analysis of gene expression in response to an impulse-like glucose perturbation. The change in the expression level of *HAP4* with a known transcriptional response to the amount of glucose in the medium was investigated in order to test the performance of this reference gene pool. *HAP4*, whose gene product is a transcriptional activator and global regulator of respiratory gene expression, was known to be repressed by glucose in order to prevent the activation of respiration [114]. Therefore it could be expected that *HAP4* would show a decreasing expression profile in response to a sudden increase in the amount of available glucose in the medium.

In order to evaluate the importance of determining a suitable reference gene set, which would be used in the normalization of the expression profile of the gene of interest, three different sets of reference genes were used to calculate the relative expression profile

of *HAP4*. Initially *TDH3*; one of the commonly used reference genes was used as the sole gene for normalization. Then, the geometric average of the Ct values for *ARF1-CDC19*; the gene pair, which was identified as the most stable pair in the analysis conducted by geNorm using P-0 was used as the reference gene set for the normalization of *HAP4* expression. In the third case, *TPI1*, *FBA1*, *CDC19* and *ACT1;* the reference genes identified as the most stable genes both by NormFinder and by geNorm using P-1 were employed in the analysis. Similarly the geometric average of the Ct values of these four genes was used for determining the expression profile of *HAP4*. *HAP4* displayed a fluctuating expression profile in response to glucose perturbation when the data was normalized using *TDH3*; a commonly used reference gene. Although normalization using *ARF1* and *CDC19* captured the general trend for *HAP4* expression, the sudden increase within the first minute upon addition of glucose was an unacceptable outcome of this selection of reference genes. In contrast, the relative expression of *HAP4* decreased immediately after the impulse and remained repressed during the time course of the experiment as expected when the four most stable reference genes (*TPI1*, *FBA1*, *CDC19*and *ACT1*) were used for normalization (Figure 4.5a). These results clearly demonstrated that using a commonly preferred reference gene without verification would yield to misleading expression profiles. Moreover the selection of a correlated gene pair would not improve the outcome even though they were identified to be stable by commonly used reference selection software.

The *TPI1-FBA1-CDC19-ACT1* reference gene set was further analysed in order to investigate the effect of having additional genes in the set or the effect of excluding one or more of the members of the set. Since the pairwise variations among the candidate reference genes that were obtained from the geNorm algorithm indicated that a set of six reference genes would be suitable, the contribution of the next fifth and the sixth most stable reference genes in determining the expression profile of *HAP4* were investigated. The results indicated that the inclusion of *ARF1* or *ARF1* and *GCN4* in the reference gene set did not yield any improvements in the determination of the expression profile of *HAP4*. Moreover, the expression trend of *HAP4* was observed to change slightly unfavourably upon addition of *ARF1* in the reference gene set. Further inclusion of *GCN4* in the reference set yielded a similar undesirable outcome (Figure 4.5b). Therefore a pool consisting of at most four reference genes was determined to be sufficient.

The change in the gene expression profile within the first minute upon inclusion of *ARF1* in the reference gene set was similar to the response that was observed when the *ARF1-CDC19* gene pair was used as reference. The common denominator in the *ARF1-CDC19* reference gene set and the *TPI1-FBA1-CDC19-ACT1* reference gene set was *CDC19*, which might indicate that the general trend in *HAP4* expression profile was observed owing to the presence of this gene. The unexpected increase in the gene expression at the 20th second post impulse was observed due to the inclusion of *ARF1* in the reference gene set (Figure 4.5a and Figure 4.5b).

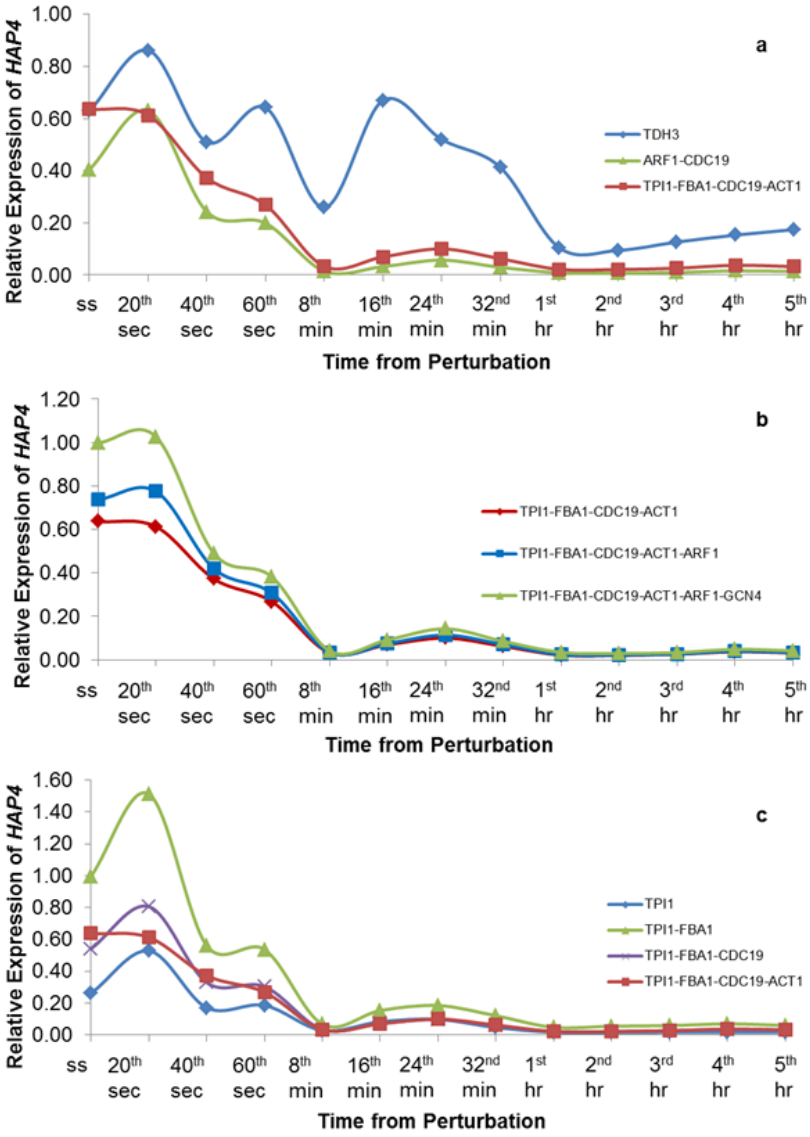


Figure 4.5. Expression profile of *HAP4* in response to the relaxation from glucose limitation calculated using commonly used reference gene (a), using increasing number of reference genes (b) and decreasing number of reference genes (c).

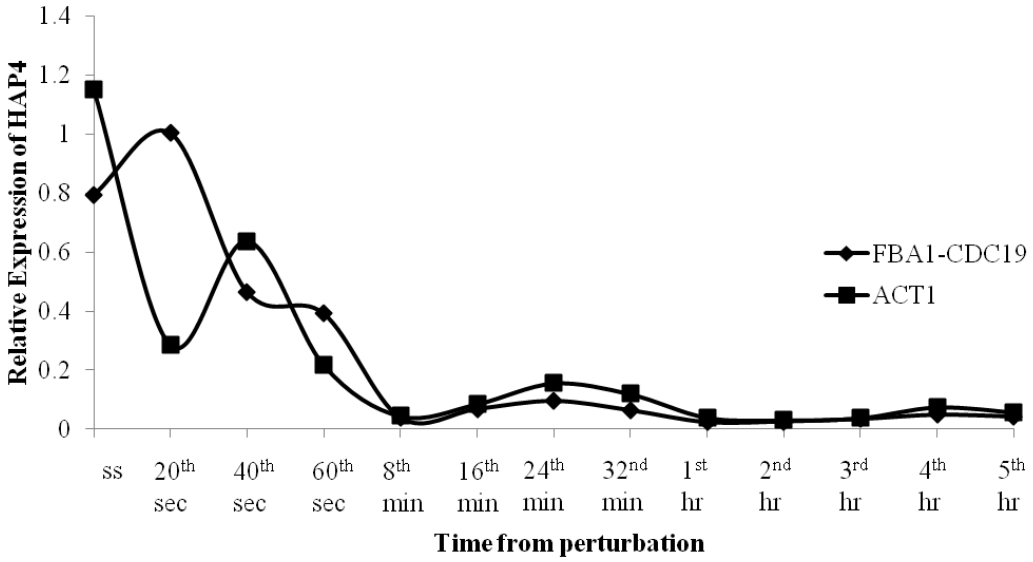


Figure 4.6. Expression profile of *HAP4* using *ACT1* or *FBA1-CDC19* in response to the glucose impulse.

The observation that the inclusion of the more genes in the reference set did not provide any improvement led to the thinking that the reference gene set would be further reduced in size. For this purpose, the number of reference genes included in the set was reduced by one each time starting from the least stable gene in the set. The exclusion of *ACT1* from the reference gene set resulted in an unexpected increase at the 20th second post impulse. A further exclusion of *CDC19* from the set caused an even steeper increase in *HAP4* expression at the 20th second post impulse in addition to unexpected increases in gene expression in the 60th second and another slight increase at the 24th minute. Utilization of *TPI1* as the sole reference gene for the normalization of *HAP4* expression yielded a similar expression profile for this gene to that of using the geometric average of the Ct values for *TPI1*, *FBA1* and *CDC19* (Figure 4.5c). The increase in the expression of *HAP4* at the 20th second could only be prevented via the inclusion of *ACT1* in the reference gene set raised a question of whether this gene could be used as a single reference gene in determining the expression profiles under the stated conditions. Moreover, *ACT1* was frequently reported as a reference gene in yeast gene expression studies via real-time RT-qPCR [99]. However, the use of *ACT1* as the sole reference gene did not yield a decreasing expression profile for *HAP4*. The similarity between the expression profiles when *TPI1* was used alone or in conjunction with *FBA1* and *CDC19* aroused curiosity regarding whether using the geometric average of the Ct values for *FBA1* and *CDC19* alone would result in an adequate expression profile for *HAP4*. However this reference gene pair caused

a steep increase in *HAP4* expression at the 20th second post impulse (Figure 4.6). These results indicated that the reference gene set comprised of *TPI1*, *FBA1*, *CDC19*, *ACT1* was suitable for determining gene expression in response to an impulse-like perturbation in glucose levels.

4.3.2.2. Case Study II: Selection and Validation of the Most Stable Reference Genes During Ammonium Perturbation. In order to identify correlated gene pairs from the candidate list, Pearson correlation coefficients between expression values of each pair of the candidates were calculated. Analysis of the PCCs between the gene pairs among the candidate gene set revealed the presence of one gene pair with a PCC greater than 0.85 in response to relaxation of ammonium limitation. *TDH3* was correlated with *PDC1*. Three different pools of candidate genes were formed in order to investigate the effect of the inclusion of correlated gene pairs in the reference gene set. P-0 was the pool including all candidate genes. P-1 and P-2 were created by excluding *TDH3* and *PDC1* from the candidate list, respectively. One other alternative pool was constructed by excluding both of these correlated genes from the candidate list (P-3).

The eleven candidates were ranked based on the stability of their expression profiles using NormFinder and geNorm as it was the case for the glucose perturbation. Additionally, geNorm algorithm was also used to determine the stability ranking of genes in the pools, in which only the genes with a correlation of < |0.85| among their expression profiles, were left (P-1 to P-3) (Figure 4.7).

The genes were scored such that the average stability rankings were obtained using NormFinder and geNorm for each pool separately. The total score for each candidate gene was calculated by summing up its individual scores for each case; P-0 to P-3. The genes were then ranked based on their total scores from the minimum score to the maximum score indicating an order from the most stable to the least stable gene. Although the minimum number of reference genes to be used could be selected as 3 in order to make sure that at least one stable gene remained even after the removal of the correlated gene pair in P-3, the number of reference genes was selected as 4 for the sake of consistency with Case Study I. Indeed the analysis conducted by geNorm also demonstrated that 4

genes should be used as reference under the stated conditions. This ranking indicated that the lowest total scores were obtained for *FBA1*, *TDH3*, *ACT1* and *CCW12* (Figure 4.8).

	NormFinder	geNorm-P-0	geNorm-P-1	geNorm-P-2	geNorm-P-3
FBA1	1	1	1	1	1
TDH3	2	5	-	5	-
CCW12	3	4	4	4	4
ACT1	4	1	1	1	1
TPI1	5	6	5	6	5
CDC19	6	2	2	2	2
ADH1	7	3	3	3	3
GCN4	8	7	6	7	6
ARF1	9	8	7	8	7
PDC1	10	9	8	-	-
RPS26A	11	10	9	9	8

Figure 4.7. Stability analysis of the candidate genes in Case Study II.

In this case, geNorm did not capture the gene pair with the highest correlation as the most stable pair. However, although the most stable gene was identified as *FBA1* and the 4 least stable genes were identified as *GCN4*, *ARF1*, *PDC1* and *RPS26A* by both geNorm and NormFinder, the stability orders for the remaining of the set were considerably different by either one of the algorithms. Removal of the correlation among the candidate genes did not change the stability rankings determined by geNorm such that both algorithms would yield similar stability profiles. If the three candidate pools (P-1 to P-3) were analysed, *FBA1* and *ACT1* would fall into the stable gene set as it was the case that was determined by NormFinder (Figure 4.7). *FBA1*, *TDH3*, *CCW12* and *ACT1* were determined as the four most stable genes among all candidates as indicated by their scores (Figure 4.8).

A set of four reference genes; *FBA1*, *TDH3*, *CCW12* and *ACT1* were determined to constitute a suitable pool for the analysis of gene expression in response to an impulse-like ammonium perturbation. The change in the expression level of *MEP2*, an ammonium transporter, was investigated in order to test the performance of this reference gene pool. *MEP2*, whose mRNA accumulation was more abundant in cells grown in limiting concentrations of ammonia than in high concentrations [115] was monitored in response to an impulse-like addition of ammonium into its limited culture. Therefore it could be

expected that *MEP2* would display a decreasing expression profile in response to a sudden increase in the amount of available ammonium in the medium.

	Score P-0	Score P-1	Score P-2	Score P-3	TOTAL SCORE
FBA1	1.0	1.0	1.0	1.0	4.0
TDH3	3.5	2.0	3.5	2.0	11.0
ACT1	2.5	2.5	2.5	3.5	11.0
CCW12	3.5	3.5	3.5	2.5	13.0
CDC19	4.0	4.0	4.0	5.0	17.0
TPI1	5.5	5.0	5.5	4.0	20.0
ADH1	5.0	5.0	5.0	5.0	20.0
GCN4	7.5	7.0	7.5	7.0	29.0
ARF1	8.5	8.0	8.5	8.0	33.0
PDC1	9.5	9.0	10.0	10.0	38.5
RPS26A	10.5	10.0	10.0	9.5	40.0

Increasing stability ⟷ Decreasing stability

Figure 4.8. Scoring of the candidate genes in Case Study II.

In order to evaluate the importance of determining a suitable reference gene set, which would be used in the normalization of the expression profile of the gene of interest, three different sets of reference genes were used to calculate the relative expression profile of *MEP2*. Initially *ACT1*; one of the commonly used reference genes was used as the sole gene for normalization. Then, the geometric average of the Ct values for *FBA1-ACT1*; the gene pair, which were identified as the most stable pair in the analysis conducted by geNorm using P-0 was used as the reference gene set for the normalization of *MEP2* expression. In the third case, the geometric average of the Ct values of *FBA1*, *TDH3*, *CCW12* and *ACT1;* the reference genes identified as the most stable genes based on the scores, which were calculated using both NormFinder and geNorm results, was used for determining the expression profile of *MEP2*.

MEP2 displayed a fluctuating expression profile in response to ammonium perturbation when the data was normalized using *ACT1*; a commonly used reference gene or using the most stable gene *FBA1* along with *ACT1*. In contrast, the relative expression of *MEP2* decreased gradually following the impulse and remained repressed during the

time course of the experiment as expected when the four most stable reference genes (*FBA1*, *TDH3*, *CCW12* and *ACT1*) were used for normalization (Figure 4.9a). These results clearly demonstrated that using a commonly preferred reference gene without verification would yield to misleading expression profiles.

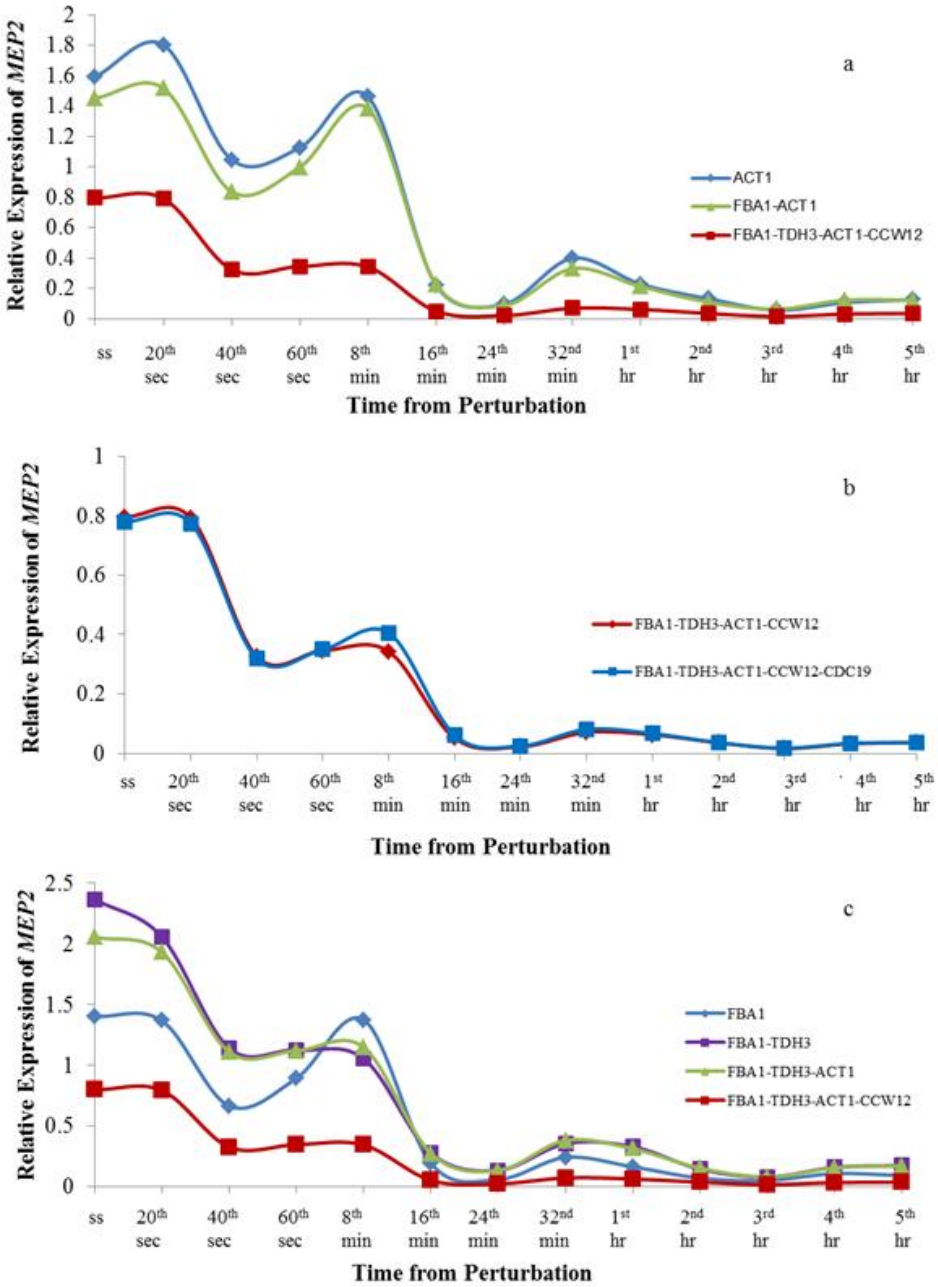


Figure 4.9. Expression profile of *MEP2* as a response to the relaxation from ammonium limitation using commonly used reference gene (a), using increasing number of reference genes (b) and decreasing number of reference genes (c)

The *FBA1-TDH3-CCW12-ACT1* reference gene set was further analysed in order to investigate the effect of having additional genes in the set or the effect of excluding one or more of the members of the set. The contribution of the fifth most stable reference genes in determining the expression profile of *MEP2* was investigated. The results indicated that the inclusion of *CDC19* in the reference gene set did not yield any improvements in the determination of the expression profile of *MEP2* displaying an unexpected increase in gene expression at the 8th minute (Figure 4.9b). Therefore a pool consisting of at most four reference genes, as also suggested by geNorm, was determined to be sufficient.

The observation that the inclusion of the more genes in the reference set did not provide any improvement led to the thinking that the reference gene set would be further reduced in size as it was in Case Study I. For this purpose, the number of reference genes included in the set was reduced by one each time starting from the least stable gene in the set. The exclusion of *CCW12* or *CCW12* and *ACT1* from the reference gene set resulted in an unexpected increase in gene expression during a period from approximately the 32nd minute until the 2nd hour post impulse. A further exclusion of *TDH3* from the set, utilizing *FBA1* as the sole reference gene, caused a fluctuating expression profile for *MEP2* (Figure 4.9c).

4.3.2.3. Determination of the Optimum Number of Reference Genes for Normalization.

The geNorm algorithm provides the contribution of each additional stable gene into the reference gene set in terms of the pair-wise variation between the two sequential normalization factors [98]. The developers suggest using a threshold of 0.15 in the determination of the optimal number of reference genes required for normalization. geNorm suggested using a different number of reference genes varying between 3 and 6 for each pool in the case of glucose perturbation (Table 4.2). This result necessitated a human-mediated evaluation of the expression profiles of the gene of interest obtained by different sets of reference genes. The results presented in this study indicated that the utilization of a set comprised of four reference genes with stable and uncorrelated expression profiles would be sufficient for this case. On the other hand, in the case of ammonium perturbation, the analysis using geNorm revealed that the optimal number of reference genes was 4 for each pool (Table 4.3). Further analysis also demonstrated that

neither increasing nor decreasing the number of reference genes improved the expression profile obtained for *MEP2*.

Table 4.3. Pairwise variations between the $(n+1)^{th}$ and the n^{th} genes for different pools of candidate sets in Case Study II.

Case Study II	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11
P-0	0.111	0.150	0.107	0.098	0.087	0.087	0.081	0.103	0.100
P-1	0.111	0.150	0.107	0.112	0.097	0.086	0.121	0.113	
P-2	0.111	0.150	0.107	0.098	0.087	0.087	0.081	0.113	
P-3	0.111	0.150	0.107	0.112	0.097	0.086	0.131		

Therefore, the number of reference genes suitable for real-time RT-qPCR data normalization would also vary on a case specific manner and a human-mediated evaluation of the obtained results to design real-time RT-qPCR experiments may provide reductions in the number of experiments, thus reducing the unnecessary costs.

4.4. Discussion

Understanding the dynamic mechanism behind a biological process and the identification of the statistically meaningful changes in the expression levels of selected genes involved in the various processes require quantification and comparison of dynamic data. Real-time RT-qPCR is widely used to investigate the relative expression levels of target genes in detail. However, the selection of suitable reference genes imposes problems in the analysis of both transient and non-transient expression profiling studies. The quantification of the dynamic response by real-time RT-qPCR requires the identification of the reference genes, which display constant expression across all time points regardless of the different genetic or environmental perturbations. Although the commonly used reference genes previously reported in literature [97–99] constitute a suitable pool for the analysis of non-transient data, these genes were observed to fall short in fulfilling their roles as reference genes in the analysis of dynamic gene expression. Therefore, a pool of candidate reference genes needs to be identified for dynamic studies. The most suitable set of reference genes would then be determined among the proposed candidates under the studied experimental conditions.

In this study, high-throughput transient gene expression data were used to identify a set of candidate reference genes for real-time RT-qPCR studies. The reference genes were selected from a large pool of dynamic microarray data sets such that they displayed stable expression profiles across time regardless of the type of experimental condition.

The initial stage of this study required the collection of publicly available time series microarray datasets. It was observed that the use of key words such as, “time series” or “time course” was insufficient to extract all the necessary information from the database. This fact signified the importance of human intervention in acquiring information from electronic sources. The content of the datasets needed to be carefully investigated by the researcher to evaluate whether the set was coherent with the context of the study or not. Only then a comprehensive set could be attained.

Two different approaches were used in the identification of a set of candidate reference genes. One approach utilized individual experimental data sets for the determination of stability rankings for each set. The frequency of occurrence of each gene among the most stable 100 genes was determined as a measure of selection criterion. The most frequently encountered genes across all experiments were identified as candidates. In the other approach, however, all available data were merged into a single complete dataset and the overall stability profile based on CV values was used as the second selection criterion. Three genes were identified by both approaches; *TDH3*, *TPI1* and *CDC19*. All three genes were determined as reference in at least either one of the two case studies.

The advantage of this present strategy for the identification of a pool of candidate reference gene sets is the flexibility of the method. It enables the implementation of other approaches in a modular manner. By this means, the candidate gene pool may be extended to meet other specific needs that might be required.

The approach, in which a combination of datasets were used to identify reference gene candidates through the calculation of CV values, showed that the number of the datasets used for the identification of stable genes affected the stability order of the genes. This result indicated the necessity to include as many datasets as possible in the analysis to obtain more reliable results. Thus as many time course experiments conducted using *S.*

cerevisiae as possible were tried to be included in the present study. The strategy is not limited to the currently available datasets but allows the inclusion of additional data sets and this is advantageous in terms of improving the results obtained from this approach.

The stability analysis of the collected time series datasets revealed that the transcripts that take place in the super pathway of glucose fermentation (*FBA1*, *TPI1*, *PGK1*, *CDC19* and *PDC1*) tended to display stable expression profiles in time course studies. Furthermore, these transcripts were verified to display stable expression profiles experimentally thus presenting a good alternative as reference genes in the normalization of real-time RT-qPCR data. The dominance of fermentation–related genes as stable reference candidates in time course studies appears to be an interesting result, which requires further investigation.

In this study, geNorm and NormFinder algorithms were used for the identification of the most stable genes among the candidate list. For this purpose the results obtained from these two software programs were compared and the transcripts, which displayed the most stable expression profiles in both applications, were identified. The stability of the genes was evaluated based on a scoring system that allowed displaying their average stability rankings. However, obtaining similar results using both software programs highly depended on the gene set to be analysed since geNorm algorithm might be very sensitive to the existence of any correlated gene pairs. The results clearly showed that the exclusion of one of the correlated genes altered the stability order and the stability scores of the transcripts would be low only if the correlated genes were excluded from the candidate list. The approach presented in this present study is based on the elimination of the correlated gene pairs according to the results of the real-time RT-qPCR experiments under the selected conditions rather than the *a priori* elimination of genes that were reported to be correlated in the literature. This approach enabled the observation of any possible correlations among genes investigated in the samples of the current case studies and avoided the unnecessary exclusion of the candidates, which could possibly be among the most stable genes.

This study provided additional evidence that, there are no universal reference genes, which could suitably be used under different experimental conditions. Here we propose a

candidate gene set, which can be used for the normalization of dynamic expression profiles of the target genes in *S. cerevisiae*. In order to confirm the suitability of this candidate gene set, we investigated the transcriptional profiles of *HAP4* and *MEP2* genes under two different experimental conditions using both the newly identified reference genes and the commonly used ones. These analyses clearly demonstrated that the newly identified reference genes outperformed the conventional candidates in dynamic expression profiling analysis.

The responses of *HAP4* and *MEP2* to an impulse-like addition of glucose and ammonium, respectively, were reported in several studies in the literature [115, 116]. Investigation of the expression profiles of genes with well-documented responses in an experimental condition was shown to aid the evaluation of the optimum number of reference genes selected from the candidate reference gene set. Therefore it would be suggested to conduct evaluations regarding the candidate reference gene set using such control genes with known expression profiles prior to conducting the analyses on the genes of interest.

It should be noted that for each specific experimental condition that would be investigated, a different set of reference genes would selected from the candidate gene pool. The nature of the perturbation or the experimental setup would result in the identification of different reference genes for each specific experimental condition throughout the dynamic range of the experiment. In fact in Case Study I, in which the amount of glucose used as the sole carbon source was varied, *ACT1* was identified among the most stable reference genes although it alone was not sufficient for normalization. On the other hand, *ACT1* expression was shown to be unstable in another experimental setup for investigating diauxic shift, in which the cells starving for glucose switched to utilizing other carbon sources such as ethanol [97]. Yet another study utilized *ACT1* as reference for monitoring the expression levels of glycolytic genes in response to a switch from growing on ethanol to growing on glucose [99].

Several methodologies were used previously in the selection of RT-qPCR reference genes. One of the most commonly utilized strategies is the selection of one or more of the reference genes that are frequently cited in the literature [97, 99, 104, 117–121]. In rare

instances, several different approaches were also utilized. In a study for validating reference genes for quantitative expression analysis by real-time RT-qPCR in *S. cerevisiae*, Teste *et al*. selected suitable microarray datasets, in which the culture conditions reported for these datasets were closest to their experimental setup. The potential reference genes were selected among the transcripts displaying stable expression profiles in these microarray datasets. Additionally traditionally used reference genes were also included in the study [97]. Another study focused on systematically collecting microarray data for selecting reference genes under a specific set of conditions [94]. In yet another study, the candidate reference genes were determined via stability of their expression levels across different tissues using their EST profiles [96]. Existence of a pool of commonly used reference genes has proven very useful in time invariant RT-qPCR analysis. However, these reference genes were often found to display far from stable expression profiles in dynamic studies mandating researchers to seek alternative ways to identify novel reference gene candidates [97]. Unfortunately currently utilized approaches usually fail in the identification of a universal set of candidate reference genes to be used in dynamic studies regardless of experimental conditions. Our approach may help serve this purpose by gathering dynamic microarray datasets having as diverse experimental conditions as possible for the identification of a universal pool of candidate reference genes, from which users may select subsets that would be suitable for their particular needs.

The present approach allows its users a vast space to manoeuvre using the proposed candidate reference genes. Although the specific cases presented in this study focused on transient changes in the amount of available glucose or ammonium in the fermentation medium in yeast, suitable reference genes would be selected among the candidate pool for completely different experimental conditions regardless of whether such an experimental setup was previously analysed or not. The candidate pool itself was created from a collection of dynamic datasets with a diverse set of experimental conditions. Among these, environmental variations including osmotic stress, heat shock, cell cycle, DNA damage, nutrient availability, chemical treatments, desiccation stress, nitrosative stress and genetic mutations including deletion and overexpression of genes were included. The genes in the candidate pool were identified such that regardless of the diversity of the experimental conditions under which the microarray data were generated, the expression profile of each candidate was stable. This strength of the method of selection increases the possibility of

identifying reference gene among the pool of candidates, which would have stable expression profiles across time in a specific experimental condition that was not previously analysed.

It can be concluded that the pool of candidate reference genes determined in this study; *TPI1, ACT1, TDH3, FBA1, CCW12, CDC19, ADH1, PGK1, GCN4, PDC1, RPS26A* and *ARF1*, may be used to identify the set of suitable reference genes in the analysis of dynamic transcriptional data by real-time RT-qPCR in *S. cerevisiae* under different experimental conditions. However, it is also undeniable that, as the number of the publicly available time course microarray datasets increases, this candidate gene set may be improved. The flexibility of the methods used in this approach enable the inclusion of additional datasets thus constant update of the candidate pool. Additional methods could also be implemented in a modular manner to enhance the results obtained from this study. This study showed the significance of researchers' intervention at various stages of reference gene selection both during the inclusion of new dynamic datasets to be used for the determination of the candidate set and during the exclusion of the correlated gene pairs from the candidate pool while identifying the most stable genes.

4.5. Concluding Remarks

Understanding the dynamic mechanism behind the transcriptional organization of genes in response to varying environmental conditions requires time-dependent data. The dynamic transcriptional response obtained by real-time RT-qPCR experiments could only be correctly interpreted if suitable reference genes are used in the analysis. The lack of available studies on the identification of candidate reference genes in dynamic gene expression studies necessitates the identification and the verification of a suitable gene set for the analysis of transient gene expression response.

In this study, a candidate reference gene set for RT-qPCR analysis of dynamic transcriptional changes in *Saccharomyces cerevisiae* was determined using 31 different publicly available time series transcriptome datasets. Ten of the twelve candidates (*TPI1*, *FBA1*, *CCW12*, *CDC19*, *ADH1*, *PGK1*, *GCN4*, *PDC1*, *RPS26A* and *ARF1*) we identified were not previously reported as potential reference genes. Our method also identified the

commonly used reference genes *ACT1* and *TDH3*. The most stable reference genes from this pool were determined as *TPI1*, *FBA1*, *CDC19* and *ACT1* in response to a perturbation in the amount of available glucose and as *FBA1*, *TDH3*, *CCW12* and *ACT1* in response to a perturbation in the amount of available ammonium. The use of these newly proposed gene sets outperformed the use of common reference genes in the determination of dynamic transcriptional response of the target genes, *HAP4* and *MEP2*, in response to relaxation from glucose and ammonium limitations, respectively.

A candidate reference gene set to be used in dynamic real-time RT-qPCR expression profiling in yeast was proposed for the first time in the present study. Suitable pools of stable reference genes to be used under different experimental conditions could be selected from this candidate set in order to successfully determine the expression profiles for the genes of interest.

5. ANALYSES OF DYNAMIC EXPRESSION PROFILES OF IRON TRANSPORTERS IN RESPONSE TO A PERTURBATION IN COPPER LEVEL

The analyses of the transcriptional levels of the genes encoding iron transporter proteins in the reference and *CCC2* deleted strains under copper deficient and high copper conditions revealed that the effect of high copper levels on the expression levels of these genes in reference and *CCC2* deleted strains was contrary. This part of the thesis was concerned with the transient response of iron transporters to a sudden change in the extracellular copper levels in the reference and CCC2 deleted strains. Expression levels of high affinity (*FET3*), low affinity (*FET4*) and siderophore-iron transporters (*ARN1, ARN2, ENB1, FIT1, FIT2, FIT3*) as well as that of high affinity copper transporter (*CTR1*) were investigated by real-time RT-qPCR. The transient change in the intracellular and extracellular copper and iron concentrations were also analysed in conjunction with the transcript levels.

5.1. Background

Iron is an essential cofactor in a variety of reactions in all cell types. It is present in metabolic reactions, cytochromes, iron-sulfur proteins and ribonucleotide reductases [121]. Similar to copper ion, excessive amount of iron leads to toxic effect by generating hydroxyl radicals via Fenton reactions. Thus, maintenance of iron homeostasis is essential to prevent iron-dependent damage and also to maintain mitochondrial functionality [122].

Iron homeostasis is tightly regulated in *Saccharomyces cerevisiae*. There are two different iron uptake systems in yeast. The first system, which is the reductive iron transport mechanism, includes the high affinity (Fet3p/Ftr1p) and low affinity (Fet4p and Smf1p) transporters. These transporters import Fe^{+2}, which is reduced by membrane bound reductases (Fre1p and Fre2p). Fet4p was also shown to transport other metals such as copper [123]and zinc [124]. Fet3p oxidizes Fe^{+2} and transfers Fe^{+3} to Ftr1p to transport

into the cell. Fet3p requires copper for this oxidise activity [52] thus high affinity iron transport mechanism requires copper for proper functioning.

The second iron transport system is the non-reductive mechanism, which is capable of transporting ferric ions. In this mechanism, iron transport is not dependent on the activity of ferric reductases but iron bound by siderophores are transported by siderophore transporters (Arn1-4, Fit1-3). Although *S. cerevisiae* do not produce any siderophores by itself, it can use siderophores excreted by other micro-organisms under low iron condition [125].

The high and low affinity iron transport and also the siderophore transport mechanisms are regulated by iron responsive transcription factor, Aft1p. Previous studies reported that when cells were transferred from iron replete conditions to iron deplete conditions, reductive iron transport mechanism was induced much higher than non-reductive mechanism, but when cells were cultured in iron-deficient medium, the induction in siderophore transport mechanism was much higher [125]. These findings indicated that non-reductive iron transporters are induced under more severe conditions. The iron transporters were also regulated by the copper availability. Non-reductive iron transporters were induced under copper deprived conditions although cellular iron levels were not decreased [23]. In another study, high-affinity reductive iron transporter, Fet3p was reported to be induced by copper although no difference was observed in cellular iron pools [6]. Since increased copper levels did not result in a decrease in the cellular iron levels, the most plausible explanation for the induction of iron transporter seems to be the increased requirement for iron as a cofactor to decompose H_2O_2 generated by high copper levels.

The analyses of the transcriptional response to the deletion of *CCC2* gene under high copper and copper deficient conditions revealed that, siderophore iron transporters were induced under copper deprived conditions and repressed under high copper conditions in the *CCC2* deleted strain when compared to the reference strain. High copper levels led to up-regulation of these non-reductive iron transporters in the reference strain but same level of copper led to down-regulation of these genes in the *CCC2* deleted strain in comparison to copper deficient conditions. This observation brought the question whether the transient

responses of these genes to a sudden change in the copper level were similar in these two strains or not. In this part of the study, the transient responses of reductive and non-reductive iron transporters to a sudden increase in the extracellular copper levels were investigated both in the reference and the *CCC2* deleted strain. Analyses of the intracellular and extracellular iron and copper concentrations together with the transcription profiles revealed the similarities and differences in the transcriptional responses of iron transporters to changing copper and iron levels between these two strains.

5.2. Materials and Methods

5.2.1. Strain, Growth Conditions and Sampling

The experiments were performed using the strains presented in Section 2.2.1. The preculture conditions were also given in Section 2.2.1. Cells were cultivated in B-Braun Biostat B plus fermenters with 1.5L working volume of synthetic defined medium prepared by excluding $CuSO_4$ in order to create copper deficient condition. $CuSO_4$ was injected in an impulse-like manner into copper deficient cultures at steady state. The fermentation conditions were given in Section 4.2.2.2. A total of 6 time-dependent RNA and metabolite samples were collected at steady state, 1 minute, 5 minutes, 30 minutes, 1 hour and 2 hour after than the impulse injection. 1 ml of RNA samples and 2 ml of metabolite samples were collected at each sampling point. The samples were immediately frozen in liquid nitrogen and stored at -80^{o}C until further processing.

5.2.2. Nucleic Acid Extraction and Real-Time RT-qPCR Analysis

The RNA extraction and quality checks were carried out as described in Section 2.2.3.

The primer design, reverse transcription, qPCR analyses, data analyses and stability analyses were performed as described in Sections 4.2.2.4, 4.2.2.5, 4.2.2.6, 4.2.2.7, 4.2.2.8 and 4.2.2.9 in compliance with MIQE guidelines for qPCR [113]. The primer sequences and the primer efficiencies are provided in Table 5.1.

Table 5.1. Gene primer sequences and efficiencies used in this study.

Primer Name	Primer Sequence	Efficiency (%)
CTR1-F	CCCTGATCCAATTGCTGT	84
CTR1-R	CATCCCTCTTCTTGTGTAGG	
FET4-F	GATTGCTAACACGCCAAC	82
FET4-R	CGGTAACCTCCACTCTTCT	
FET3-F	CCCCTACGATTACGATGAG	86
FET3-R	TCAAGTTCTGTGGGATGG	
ARN1-F	GAAGTCAGTCCCATTCAGG	80
ARN1-R	GACGGCAGATTGTACAGAGA	
ARN2-F	GGACTTGTCGGTGTTATGCT	84
ARN2-R	CTGCCTCCACTACACTACCT	
FIT3-F	TGTCTGGACTGGTGAAGG	78
FIT3-R	GAGGATGTAGCAGAGGAAGA	
FIT1-F	GGCAAGCTTACACAGAGAG	84
FIT1-R	ATACCAGCGGTAGTGGTTTG	
FIT2-F	CAAAGGTTGTCACCGAAG	72
FIT2-R	GGAGGATGAGGAGGATGTAG	
ENB1-F	TCGGTATAGCTGGTGGTATG	95
ENB1-R	CTACTGCGTCTCCGATTT	

5.2.3. Intracellular and Extracellular Ion Determination and Sample Preparation

2 ml of sample was collected at each time point for the determination of extracellular and intracellular ion concentrations. The samples were centrifuged at 400g for 3 minutes at 4 ^{o}C. The supernatants that contain the extracellular metabolites were directly used.

The cell pellets were washed with 1mM KCN and centrifuges at 400g for 3 min. After digestion in 2 ml of nitric acid at 100°C for 2 h, the samples were centrifuged at 10000g for 2 min and diluted with 1 ml of water.

The intracellular and extracellular iron, copper and zinc concentrations were kindly determined by Redokslab Analytical Systems Inc., *Istanbul, Turkey*, using iCap Q ICP-MS (Thermo Scientific Inc., USA).

5.3. Results

Regions from the 11 candidate reference genes (*ACT1, ADH1, ARF1, CDC19, CCW12, FBA1, GCN4, PDC1, RPS26A, TDH3, TPI1*) as well as the query genes (*CTR1, FET3, FET4, ARN1, ARN2, ENB1, FIT1, FIT2, FIT3*) were PCR amplified and the raw Cq values were transformed into relative quantities using the delta-Ct method [110]. NormFinder [101] and geNorm [98] algorithms were used to rank the eleven candidates based on the stability of their expression profiles.

5.3.1. Selection and Validation of Most Stable Reference Genes in the Reference Strain

In order to identify correlated gene pairs from the candidate list, Pearson correlation coefficients between expression values of each pair of the candidates were calculated. In the reference strain, *ARF1*, *CDC19*, *FBA1* and *RPS26A* were observed to be highly correlated with each other (|Pearson correlation| > 0.85). In order to eliminate any misleading results due to the presence of this highly correlated group, these genes were excluded from the further analyses in reference strain and the suitable reference genes were selected from the remaining 7 candidate genes in this strain.

When the stabilities of the remaining candidate reference genes were investigated in reference strain, *TDH3-ACT1* gene pairs were identified as the most stable genes by both algorithms (Figure 5.1). Although same genes were identified as most stable by both algorithms, any possible effect of the presence of correlated gene pairs among the remaining candidate set, namely *CCW12-TDH3*, *CCW12-ADH1* and *TDH3-PDC1*, on the stability profile obtained by geNorm algorithm was investigated. P-1 and P-2 were created by excluding *CCW12* from the candidate list together with either *TDH3* or *ADH1*, respectively. As an alternative, *CCW12* was kept in the pool and the other two transcripts

(*TDH3* and *ADH1*), which showed high correlation with *CCW12*, were excluded from the candidate list.

	NormFinder	geNorm-P-0	geNorm-P-1	geNorm-P-2	geNorm-P-3
TDH3	1	1	-	1	1
ACT1	2	1	1	1	-
GCN4	3	4	2	3	2
ADH1	4	3	1	-	1
TPI1	5	6	3	4	3
CCW12	6	5	-	-	4
PDC1	7	7	4	5	-

Figure 5.1. Stability analysis of the candidate genes in the reference strain.

The genes were scored using the average value of the stability rankings obtained by NormFinder and geNorm for each pool separately. The total score for each candidate gene was calculated by summing up its individual scores for each case; P-0 to P-3. The genes were then ranked based on their total scores from the minimum score to the maximum score indicating an order from the most stable to the least stable gene. This ranking indicated that the lowest total scores were obtained for *TDH3*, *ACT1*, *GCN4* and *ADH1* (Figure 5.2). These results showed that, the presence of the correlated genes did not alter the stability profiles obtained by geNorm algorithm for this specific case.

	SCORE P-0	SCORE P-1	SCORE P-2	SCORE P-3	TOTAL SCORE
TDH3	1	1	1	1	4
ACT1	1.5	1.5	1.5	2	6.5
GCN4	3.5	2.5	3	2.5	11.5
ADH1	3.5	2.5	4	2.5	12.5
TPI1	5.5	4	4.5	4	18
CCW12	5.5	6	6	5	22.5
PDC1	7	5.5	6	7	25.5

Figure 5.2. Scoring of the stability orders of candidate reference genes in the reference strain.

In order to determine the optimal number of reference genes required for normalization, the contribution of each additional stable gene, which is provided by geNorm algorithm, into the reference gene set in terms of the pair-wise variation between

the two sequential normalization factors were investigated. It was observed that contribution of each additional gene was below the threshold of 0.15, which was suggested by developers of geNorm. This fact indicated that, whether using 3 or 4 reference gene would not affect the obtained expression profiles. In order to test the performance of the reference set and also observe the effect of changing number of reference gene on expression profile, the expression profile of *CTR1*, a high affinity copper transporter, was investigated (Figure 5.3).

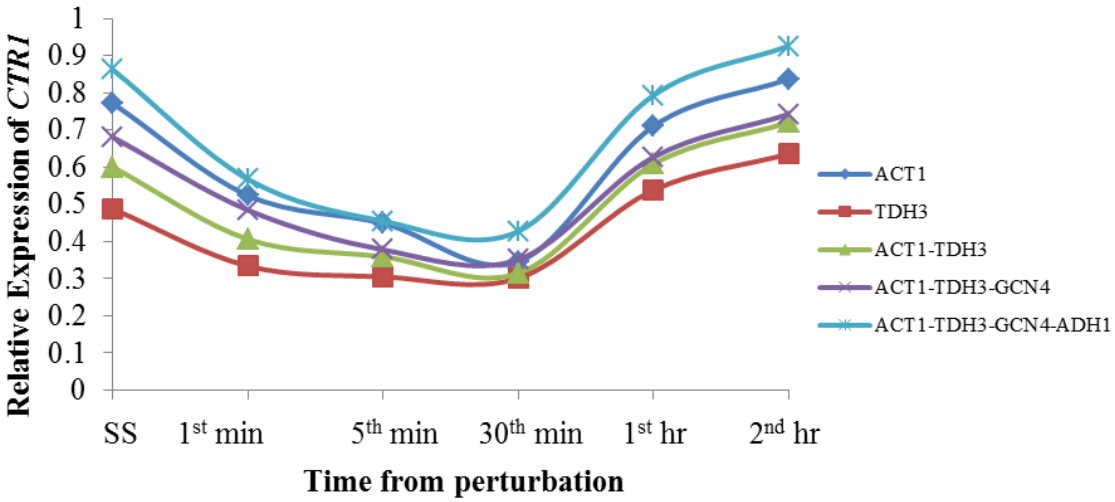


Figure 5.3. Expression profile of *CTR1* in response to a perturbation in copper level in the reference strain.

Five different sets of reference genes were used to calculate the relative expression profile of *CTR1* in response to copper impulse. The expression profile of *CTR1* was calculated using, the most stable genes, *ACT1* and *TDH3*, separately and also using the geometric average of these two reference genes. Then the number of the reference genes was increased up to 4, by including the next stable gene in each step.

The expression profiles of *CTR1* revealed that using 1 or 4 reference genes did not affected the obtained profiles. Three most stable reference genes (*TDH3*, *ACT1* and *GCN4*) were determined as the reference gene set and this set was used to calculate the relative expression profiles of iron transporters in reference strain.

5.3.2. Selection and Validation of Most Stable Reference Genes in the *CCC2* Deleted Strain

When the Pearson correlation coefficients between expression values of each pair of the candidates were calculated in the *CCC2* deleted strain, two pairs of genes (*CCW12-ACT1* and *TDH3-CDC19*) were identified to be correlated with each other. Four different pools of candidate genes were created in order to investigate the effect of the inclusion of correlated gene pairs in the reference gene set. P-1 and P-3 were created by excluding *TDH3* from the candidate list together with either *CCW12* or *ACT1*, respectively. P-2 and P-4 were created by excluding *CDC19* from the candidate list together with either *CCW12* or *ACT1*, respectively. *ACT1-ADH1* and *CDC19-TDH3* gene pairs were identified as the most stable genes by NormFinder and geNorm algorithms, respectively. However, *ACT1* and *ADH1* gene pair was also identified to be the most stable genes by geNorm when the stability analysis was performed using candidate gene set pools (P1 to P4),. This result indicated that elimination of the correlated gene pairs resulted in obtaining similar results using both algorithms.

Gene name	NormFinder	geNorm-P-0	geNorm-P-1	geNorm-P-2	geNorm-P-3	geNorm-P-4
ACT1	1	3	1	1	-	-
ADH1	2	2	1	1	2	1
FBA1	3	4	3	2	3	2
CDC19	4	1	2	-	4	-
TDH3	5	1	-	3	-	1
GCN4	6	8	5	5	1	4
TPI1	7	5	6	6	5	5
PDC1	8	7	4	4	1	3
CCW12	9	6	-	-	6	6
ARF1	10	9	7	7	7	7
RPS26A	11	10	8	8	8	8

Figure 5.4. Stability analysis of the candidate genes in the *CCC2* deleted strain.

The genes were scored using the average value of the stability rankings obtained by NormFinder and geNorm for each pool separately. The total score for each candidate gene was calculated by summing up its individual scores for each case; P-0 to P-4. The genes were then ranked based on their total scores from the minimum score to the maximum score indicating an order from the most stable to the least stable gene. This ranking

indicated that the lowest total scores were obtained for *ACT1*, *ADH1*, *FBA1* and *CDC19* (Figure 5.5).

Gene name	SCORE P-0	SCORE P-1	SCORE P-2	SCORE P-3	SCORE P-4	TOTAL SCORE
ACT1	2	1	1	1	1	6
ADH1	2	1.5	1.5	2	1.5	8.5
FBA1	3.5	3	2.5	3	2.5	14.5
CDC19	2.5	3	4	4	4	17.5
TDH3	3	5	4	5	3	20
GCN4	7	5.5	5.5	3.5	5	26.5
TPI1	6	6.5	6.5	6	6	31
PDC1	7.5	6	6	4.5	5.5	29.5
CCW12	7.5	9	9	7.5	7.5	40.5
ARF1	9.5	8.5	8.5	8.5	8.5	43.5
RPS26A	10.5	9.5	9.5	9.5	9.5	48.5

Figure 5.5. Scoring of the stability orders of candidate reference genes in *CCC2* deleted strain.

In order to determine the optimal number of reference genes required for normalization, the contribution of each additional stable gene, which is provided by geNorm algorithm, into the reference gene set in terms of the pairwise variation between the two sequential normalization factors were investigated. Similar to the previous case, the contribution of each additional gene was below the threshold of 0.15. In order to test the performance of the reference set and also observe the effect of changing number of reference gene on expression profile, the expression profile of *CTR1* was investigated.

Similar to the analyses conducted for reference strain, five different sets of reference genes were used to calculate the relative expression profile of *CTR1* in response to copper impulse in CCC2 deleted strain. The expression profile of *CTR1* was calculated using, the most stable genes, *ACT1* and *ADH1*, separately and also using the geometric average of these two reference genes. Then the number of the reference genes was increased up to 4, by including the next stable gene in each step. The expression profiles of *CTR1* revealed that using 1 or 4 reference genes did not affected the obtained profiles. Three most stable reference genes (*ACT1*, *ADH1* and *FBA1*) were determined as the reference gene set and this set was used to calculate the relative expression profiles of iron transporters in *CCC2* deleted strain.

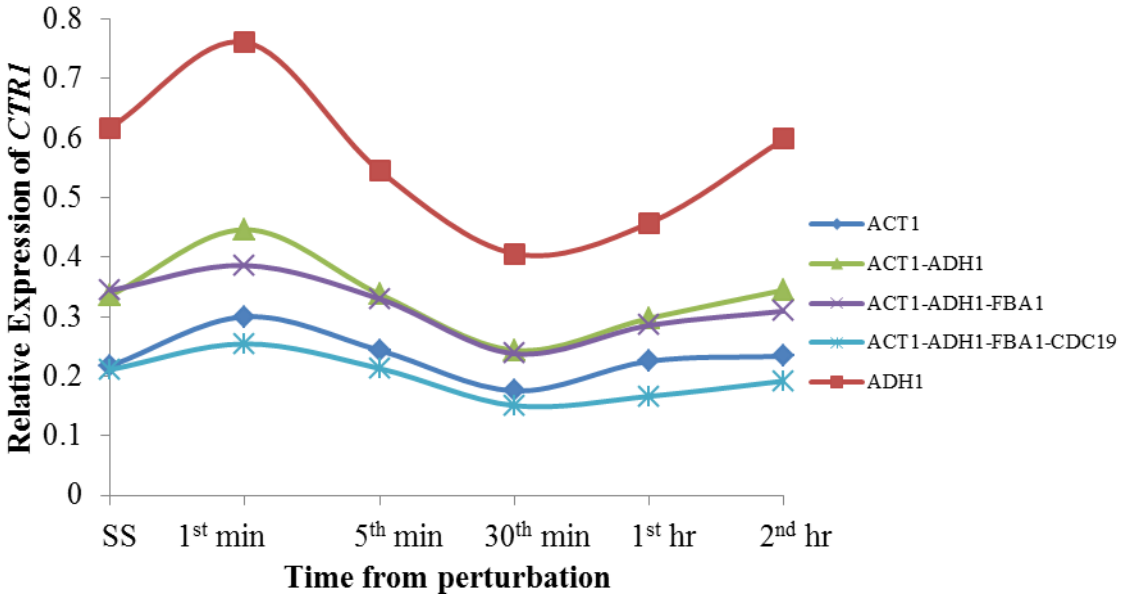


Figure 5.6. Expression profile of *CTR1* in response to a perturbation in copper level in the *CCC2* deleted strain.

5.3.3. Analysis of Expression Profiles of Iron Transport Genes in Response to Copper Impulse

The transcriptional profiles of *FET3*; high affinity iron transporter, *FET4*; low affinity iron transporter, *ARN1*, *ARN2*, *ENB1*; siderophore iron transporters, and *FIT1*, *FIT2*, *FIT3*; mannoprotein involved in the retention of siderophore-iron in the cell wall, were investigated in response to a sudden increase in the copper level in the absence of *CCC2* gene and as well as in the reference strain.

High affinity iron transporter, *FET3* was observed to be induced in response to copper impulse within the first minute and remained at higher values in comparison to steady state levels in both strains (Figure 5.7a). On the other hand, the low affinity iron transporter, *FET4*, which also imports several other transition metal ions, including zinc and copper, showed a changing expression profile in response to copper impulse (Figure 5.7b). In the reference strain, the expression profile of *FET4* showed a slight increase within the first minute following the perturbation and then it decreased below its steady state levels within the five minutes following the impulse. The expression level of *FET4* recovered to its steady state levels 2 hours later than the pulse. On the other hand, in the

CCC2 deleted strain, the expression level of *FET4* did not display any differential change until 2 hours later than the impulse, at which it was repressed by 1.5 fold.

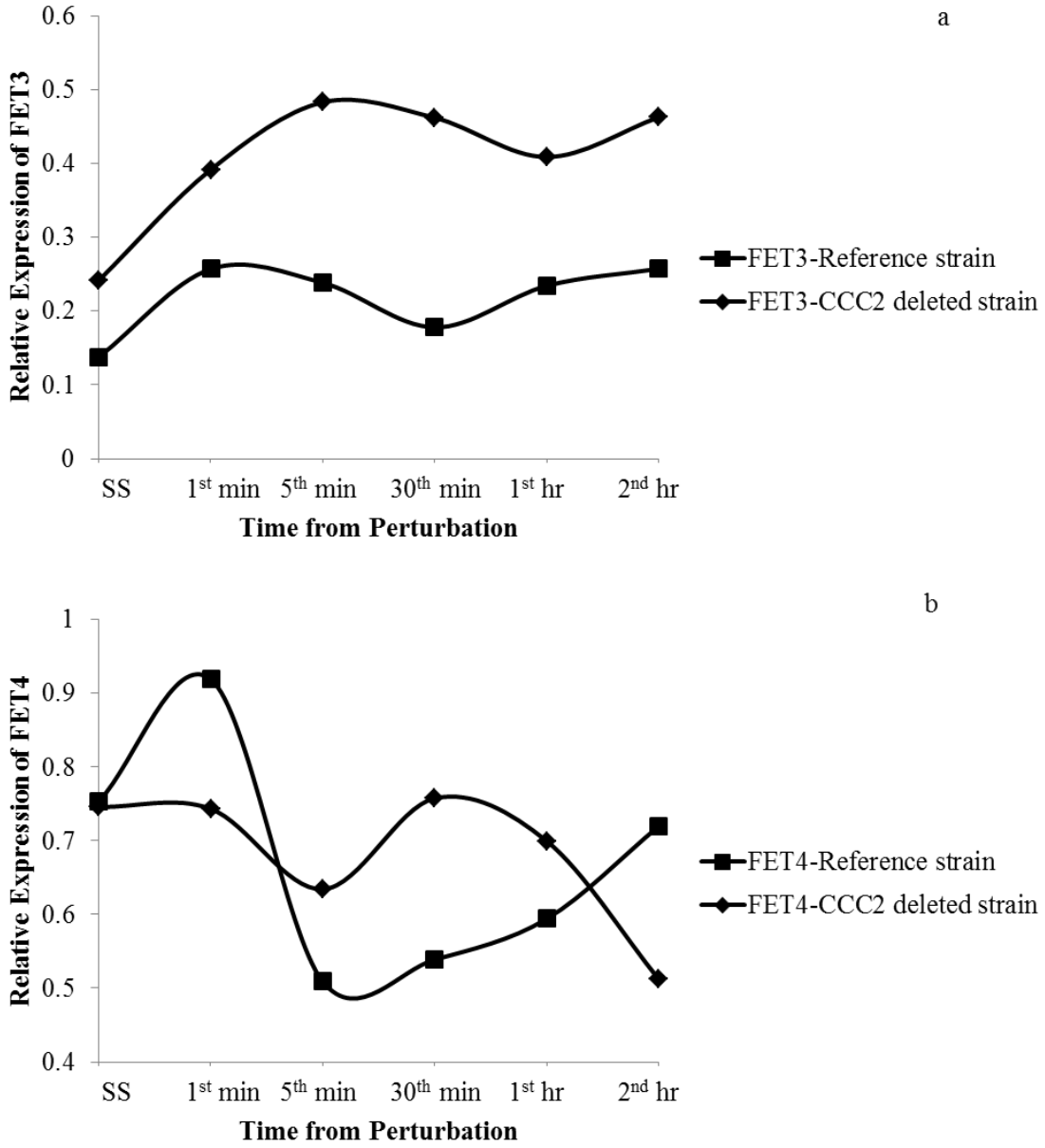


Figure 5.7. Transcriptional profiles of *FET3* (a) and *FET4* (b) in response to copper impulse in the reference and *CCC2* deleted strains.

When the transcriptional profiles of siderophore iron transporters were investigated, it was observed that expression level of *ARN1* gene showed a gradual increase following the perturbation in both strains (Figure 5.8). Two other siderophore transporters; *ARN2* and *ENB1* were down-regulated in response to a sudden increase in the copper level in the reference strain (Figure 5.9, Figure 5.10). Although the expression levels of both genes

started to recover after an initial repression, they remained at lower values than their respective steady state values during the course of experiment. On the other hand, in the *CCC2* deleted cells, *ARN2* and *ENB1* were observed to be induced by copper impulse.

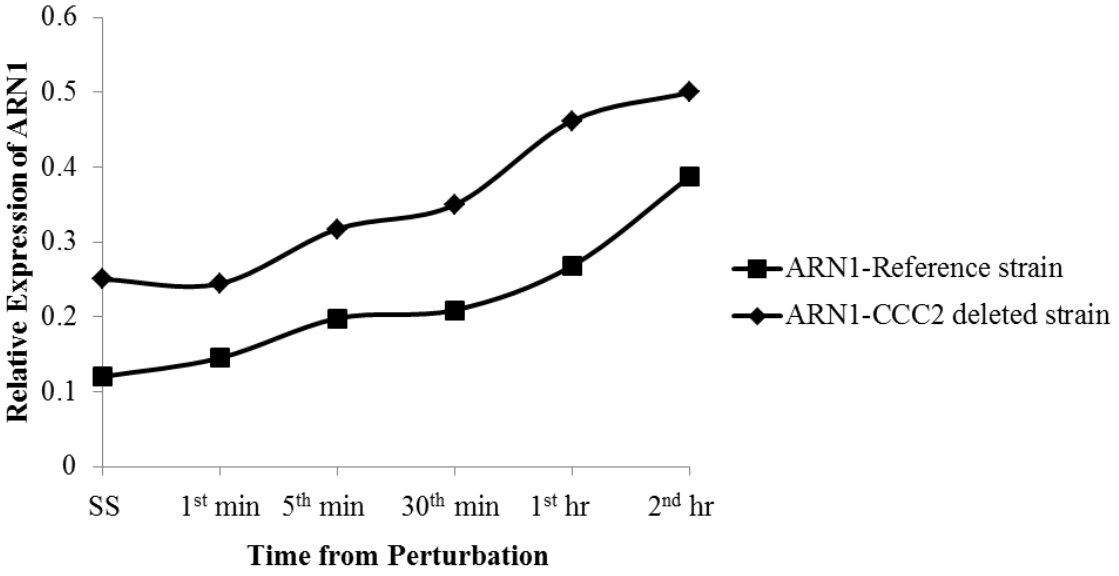


Figure 5.8. Transcriptional profiles of *ARN1* in response to copper impulse in the reference and *CCC2* deleted strains.

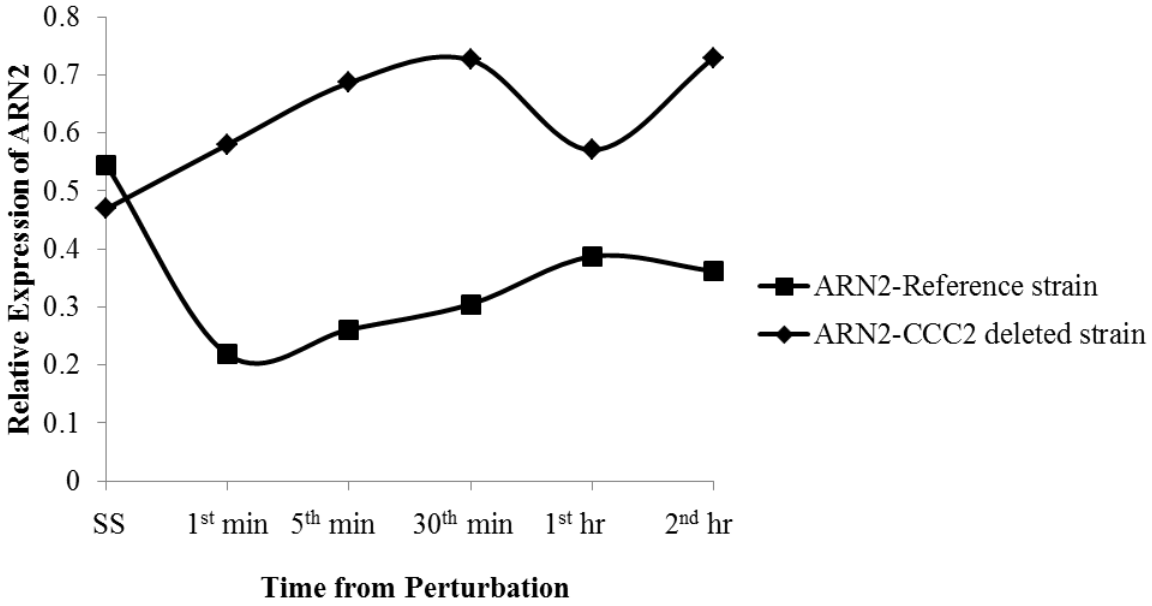


Figure 5.9. Transcriptional profiles of *ARN2* in response to copper impulse in the reference and *CCC2* deleted strains.

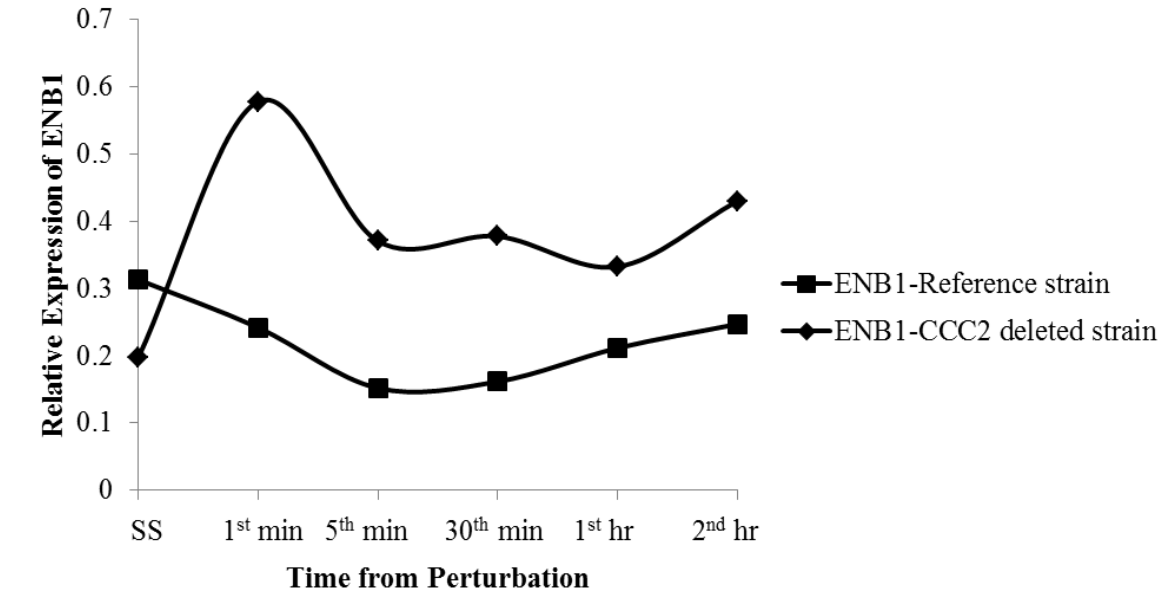


Figure 5.10. Transcriptional profiles of *ENB1* in response to copper impulse in the reference and *CCC2* deleted strains.

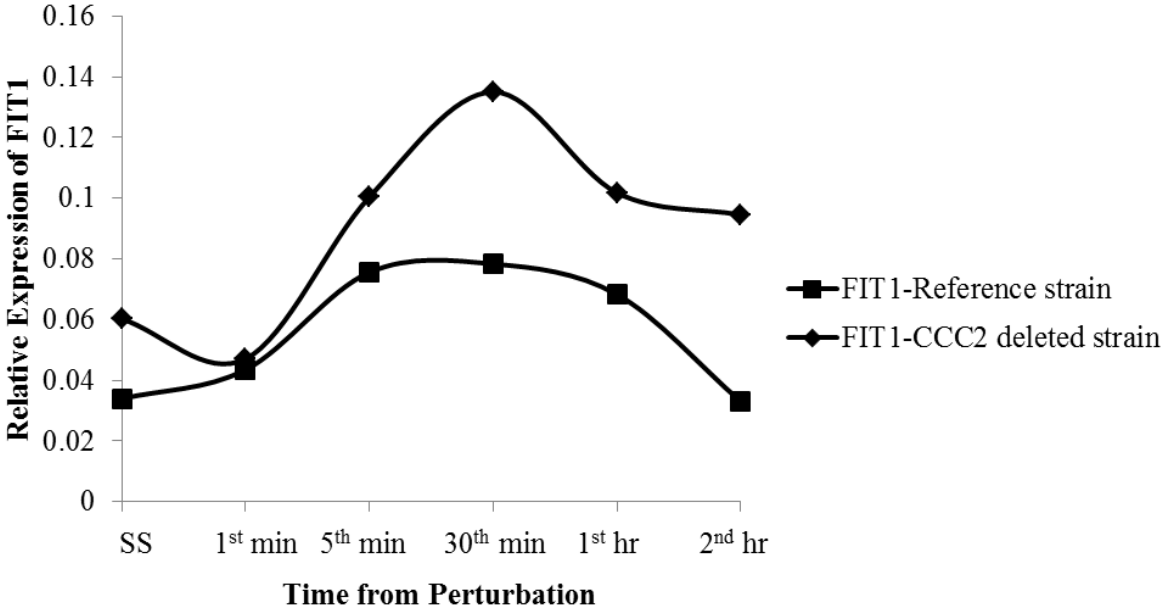


Figure 5.11. Transcriptional profiles of *FIT1* in response to copper impulse in the reference and *CCC2* deleted strains.

The transcript levels of *FIT1* and *FIT3* did not display any differential change within the first minute following the impulse but then these genes were induced and showed higher expression profiles until two hours later than the impulse in the reference strain (Figure 5.11 and Figure 5.13). In the absence of *CCC2* gene, these genes were also observed to be up-regulated 5 minutes later than the impulse however, even after 2 hours

later than the perturbation; the expression levels of these genes remained to be high in comparison to steady state condition in the *CCC2* deleted strain. On the other hand, expression level of *FIT2* showed a 1.8 fold repression within the first minute following the perturbation and then remained approximately constant during the course of experiment in the reference strain (Figure 5.12). In the absence of *CCC2* gene, the expression level of *FIT2* did not changed in response to copper pulse.

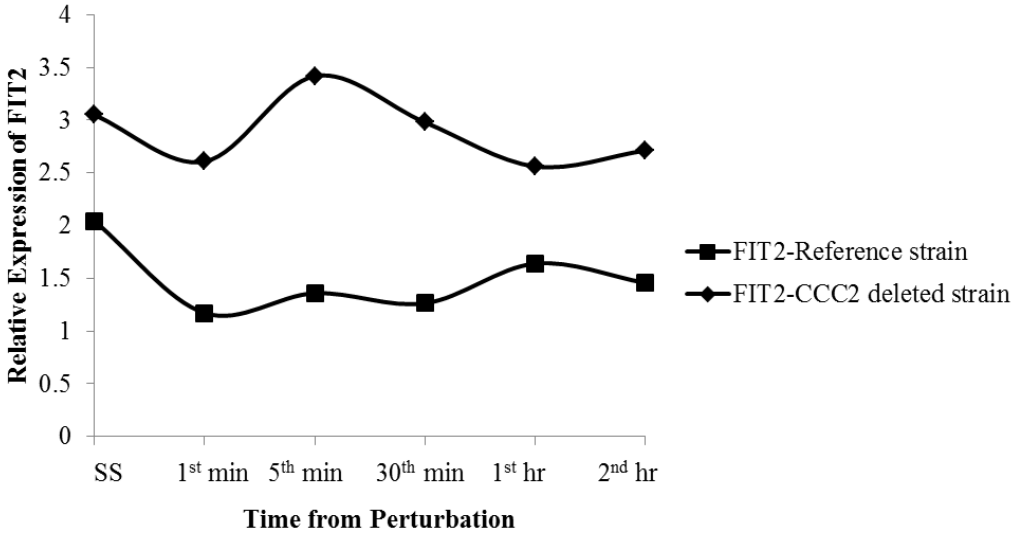


Figure 5.12. Transcriptional profiles of *FIT2* in response to copper impulse in the reference and *CCC2* deleted strains.

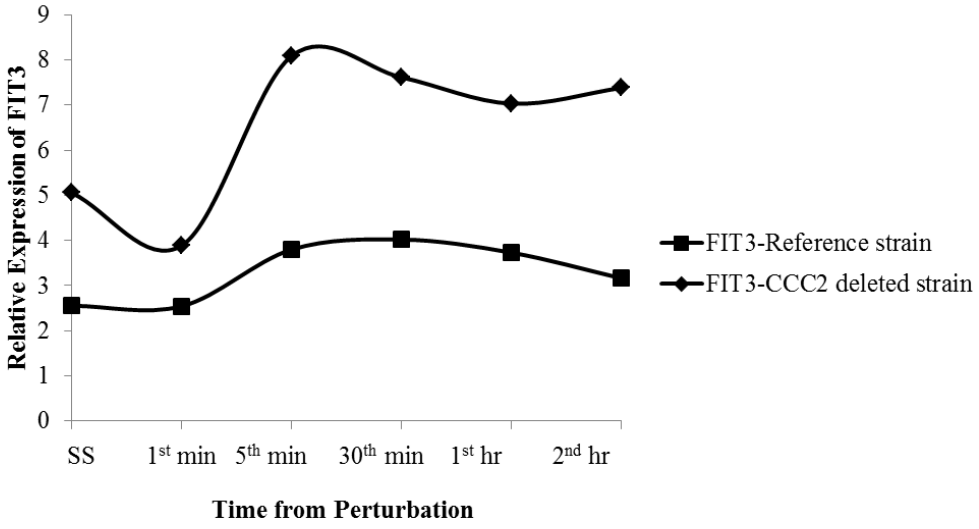


Figure 5.13. Transcriptional profiles of *FIT3* in response to copper impulse in the reference and *CCC2* deleted strains.

5.3.4. Analysis of Expression Levels Under Copper Deficient Conditions

The steady state expression levels of iron transporters and also that of *CTR1* were investigated in the *CCC2* deleted strain in comparison to reference strain. Under copper deficient conditions, *ENB1* and *CTR1* were observed to be repressed in the *CCC2* deleted strain by 1.7 and 2 fold, respectively. On the other hand, the high affinity iron transporter, *FET3* and siderophore iron transporter proteins, *ARN1*, *FIT1*, *FIT2* and *FIT3* were induced by at least 1.5 fold in the *CCC2* deleted strain (Figure 5.14).

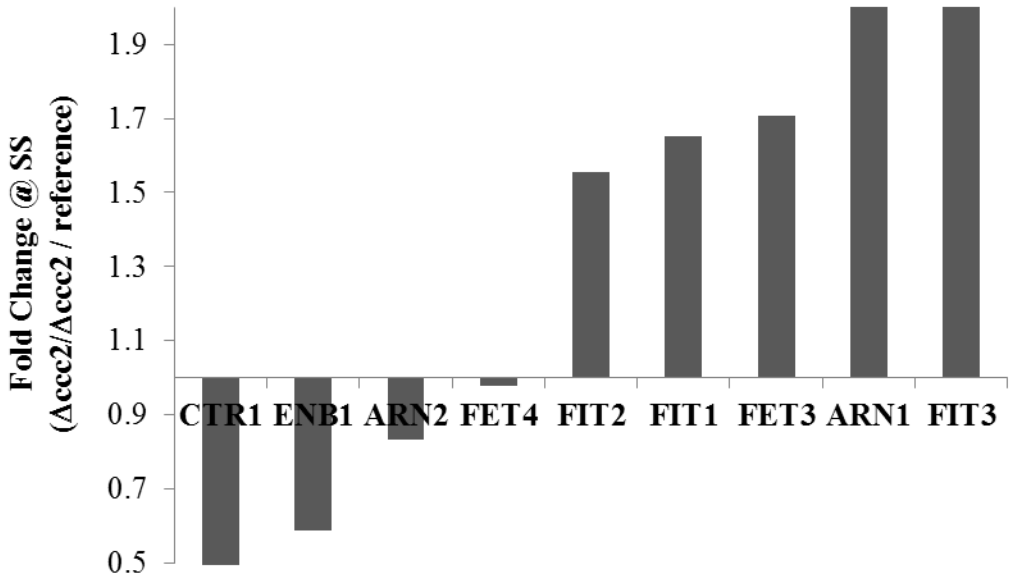


Figure 5.14. Fold changes in the expression levels of iron transporters between *CCC2* deleted strain and reference strain under copper deficient conditions.

5.3.5. Analyses of the Extracellular and Intracellular Iron and Copper Levels in Response to Copper Impulse

Extracellular and intracellular copper and iron levels were quantified in response to copper impulse in both strains using ICP-MS (Figure 5.15). The extracellular copper (Figure 5.15a) levels decreased at a continuous rate following the impulse as expected in both cultures. The extracellular iron levels were observed to be not affected from the change in the extracellular copper levels in both cultures (Figure 5.15c).

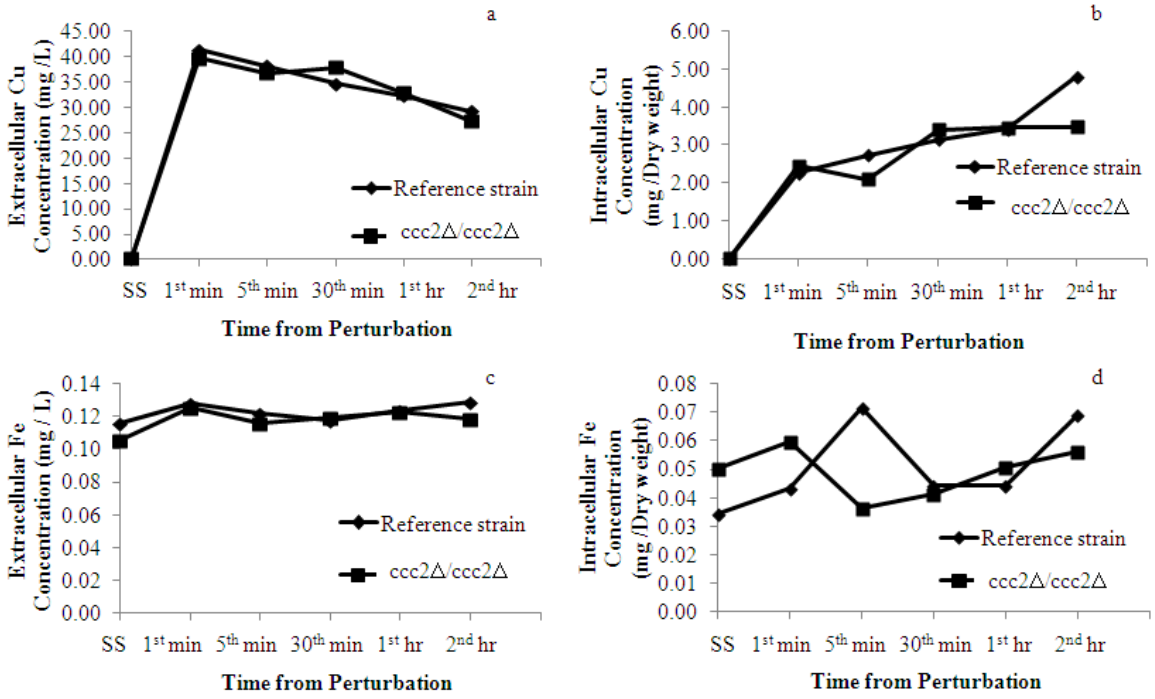


Figure 5.15. Extracellular (a, c) and intracellular (b, d) copper and iron levels.

When the intracellular copper levels of the reference and *CCC2* deleted strains (Figure 5.15b) were compared, it was observed that intracellular copper levels showed a sharp increase within the first minute following the impulse and then continued to increase slightly during the course of experiment in both strains. When the intracellular iron levels (Figure 5.15d) were investigated, it was observed that iron levels showed a fluctuating behaviour in response to copper impulse. In reference strain, intracellular iron levels showed an increasing behaviour during the first five minute following the pulse but then decreased to its steady state values. Two hours later than the impulse, iron levels were observed to increase again. In *CCC2* deleted strain, iron level showed a slight increase in response to copper impulse within the first minute after the perturbation but it decreased below its steady state values within the first five minutes. In the later hours, iron levels showed an increasing profile and reached to its steady state values 1 hour later than the copper impulse.

5.3.6. Comparison of the Intracellular Iron Levels of Batch and Chemostat Cultures

Intracellular iron levels identified using the samples collected at the steady state of the copper deficient chemostat culture were compared with the previously identified

intracellular iron levels using the samples collected at the mid-exponential phase of a copper deficient batch cultures for the reference and *CCC2* deleted strains. This comparison revealed that intracellular iron level of the reference strain grown in copper deficient chemostat culture showed 3 fold reduction in comparison to cells grown in copper deficient batch cultures, whereas in the case of CCC2 deleted strain, approximately same intracellular iron levels were identified in batch and chemostat cultures (Figure 5.15).

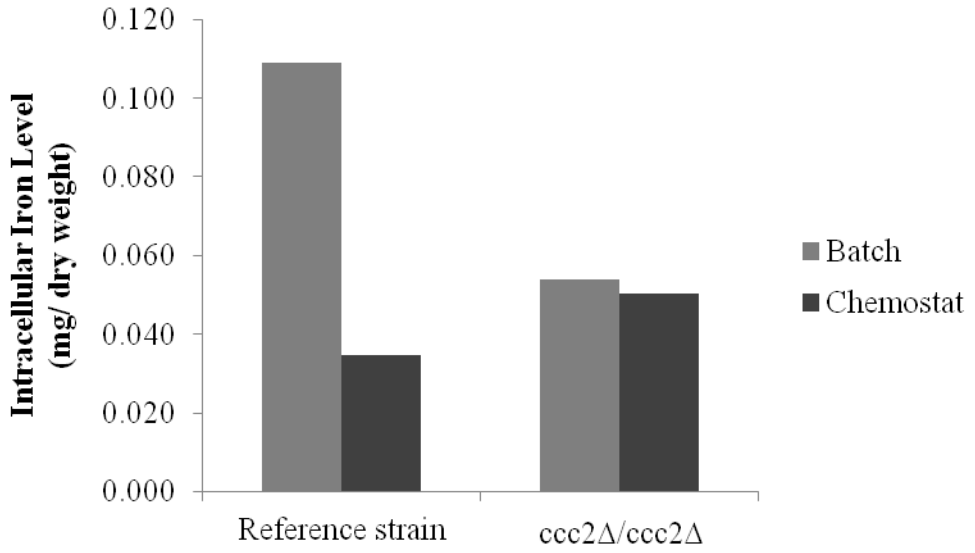


Figure 5.16. Comparison of the intracellular iron levels of batch and chemostat cultures.

5.4. Discussion

The transcriptome analyses of the cells growing under copper deficient or high copper conditions revealed that siderophore iron transporters were up-regulated under high copper conditions in comparison to copper deficient conditions in the reference strain. On the other hand, in the absence of *CCC2* gene, an opposite response was observed and iron transporters were down-regulated under high copper conditions in comparison to copper deficient conditions. The previous reports regarding the dynamic transcriptional response of iron transporters in response to copper deprivation and discrepancy between the expression levels of iron transporter in the reference and *CCC2* deleted strain, brings the question whether, the dynamic transcriptional response of these genes to high levels of copper were similar in these two strain or not. For this purpose, the dynamic transcriptional response of the siderophore, high and low affinity iron transporters to a sudden increase in the extracellular copper levels were analysed by real-time RT-qPCR. The changes in the

intracellular and extracellular iron and copper levels were also analysed in response to copper impulse.

Correct interpretation of the dynamic transcriptional profiles obtained by real-time RT-qPCR is only possible when suitable reference genes are used. For this purpose the candidate reference gene set, which was proposed to be used in dynamic real-time RT-qPCR expression profiling studies in yeast [93], were used to determine the most suitable pool of reference genes. *ACT1* was observed to be stably expressed in response to a change in the copper level both in the reference and *CCC2* deleted strains. Additionally, *TDH3-GCN4* and *ADH1-FBA1* gene pairs were also among the stably expressed genes in the reference and the *CCC2* deleted strains, respectively.

In order to test the performance of these reference genes, the expression profile of *CTR1*, which was known to be repressed at high copper conditions [5, 124] was investigated. Expression profile of *CTR1* in the reference strain was calculated either by using the most stable reference genes, *TDH3* and *ACT1* alone or by using the geometric average of the Ct values of up to four most stable genes. Although the expression profile of *CTR1* was not affected from the number of the reference genes used for normalization, 3 most stable genes, which is the minimum recommended number [98] were selected as reference gene set. In reference strain, *CTR1* expression was observed to be repressed within the first minute of the copper impulse and showed a decreasing expression profile for 30 minutes following the impulse. Expression level of *CTR1* was observed to be recovered to its steady state values 1 hour later than the injection although the extracellular copper levels were much higher in comparison to steady state levels (Figure 5.3). This might indicate the presence of a possible regulatory feedback mechanism that controls the expression level of *CTR1* gene to maintain the copper homeostasis.

When the transcriptional response of *CTR1* to copper impulse was investigated in the *CCC2* deleted strain, it was observed that, in contrast to reference strain, *CTR1* showed a slight increase within the first minute of the copper impulse (Figure 5.6). However, after this slight induction, the expression of *CTR1* was repressed and reached its lowest value 30 minutes later than the copper impulse. Similar to the reference strain, expression level of *CTR1* was recovered to its steady state levels 1 hour later than the perturbation. The initial

induction in the *CTR1* expression in the *CCC2* deleted strain might be an indication of a more severe copper deficiency in comparison to the reference strain and requirement for copper import in this strain.

Requirement of copper for high affinity iron transport links copper and iron metabolism in *S. cerevisiae*. Although copper deficiency was previously reported to be not affecting cellular iron levels, decreased cellular copper resulted in the up-regulation of iron transport mechanism at transcriptional level [23]. The possible reason for this transcriptional response was proposed to be the presence of a much smaller regulatory iron pool , which enables cells to respond to a disturbance in the iron level before its depletion [23]. The quantification of the cellular iron levels using the samples collected from the copper deficient batch cultures in the framework of this study also supported this prospect. On the other hand present study revealed that long term exposure to copper deficient conditions resulted in a differential reduction in the intracellular iron levels in the reference strain, whereas in the *CCC2* deleted strain, long term exposure did not lead to a further reduction in the cellular iron levels.

When the transcriptional responses of iron transporters to a sudden increase in the extracellular copper level were investigated, it was observed that expression level of *FET3* was induced and remained at higher values in comparison to copper deficient conditions in both strain. This induction could be interpreted as a response to restore the iron deficiency, which arose from the copper scarcity. In the reference strain, the low affinity iron transporter, *FET4*, was observed to be repressed after an initial slight induction following the copper impulse. On the other hand, in the absence of *CCC2* gene, *FET4* was unresponsive to a change in the copper level within the two hours of the injection. Since *FET4* was reported to import several other transition metal ions, including zinc and copper, the regulation of the transcription of the *FET4* might be not directly related to change in the availability of only one ion.

Analyses of the siderophore iron transporters revealed that, expression level of *ARN1*, *FIT2* and *FIT3* were increased in response to copper impulse in both strains. The expression levels of other siderophore transporters; *ARN2*, *ENB1* and *FIT1* were observed to be repressed in the reference strain. On the other hand, in the *CCC2* deleted strain, the

expression of *ARN2* and *ENB1* was induced and *FIT1* expression remained constant in response to a sudden change in the copper level. The induction of siderophore transporters could also be explained by the cellular response to increase iron levels by the elimination of copper limitation. However, there were discrepancy between transcriptional responses of *ARN2*, *ENB1* and *FIT1* to copper impulse in the reference and the *CCC2* deleted strains. Moreover, when the steady state levels of these transcripts in the reference and the *CCC2* deleted strains were compared, it was observed that the expression of *ENB1* and *ARN2* were repressed in the *CCC2* deleted strain in comparison to reference strain, whereas the other siderophore transporters, *FIT1*, *FIT2*, *FIT3* and *ARN1*, were up-regulated in the *CCC2* deleted strain. These results might indicate that, *ENB1* and *ARN2* were regulated by a different mechanism from that of other siderophore transporters and absence of *CCC2* gene affected the regulation of these transcripts. Moreover, the deletion mutants of siderophore iron transporters other than that of *ENB1* showed decreased copper resistance, whereas the *ENB1* deletion mutant showed an increased copper resistance [23]. This observation has also indicated that this transcript might have another role rather than being a siderophore iron transporters. Further studies are required to elucidate the role of *ENB1* possibly in copper homeostasis. Another possible explanation of the down-regulation of these genes in the reference strain but not in the *CCC2* deleted strain might be the fact that the increased iron level in the reference strain in response to copper impulse might establish a negative feedback loop to represses the expression of these genes.

The comparison of the expression levels of *ARN2* and *ENB1* between the reference and *CCC2* deleted strains in batch and continuous cultures also showed discrepancies. When the expression levels of *ARN2* and *ENB1* were analysed using the samples collected at the mid-exponential phase, these genes were observed to be up-regulated in the *CCC2* deleted strain in comparison to the reference strain under copper deficient conditions. However, when the expression levels of these genes were analysed in the samples collected from copper deficient chemostat cultures, they were identified to be down-regulated in the *CCC2* deleted strain. The up-regulation of *ARN2* and *ENB1* in the reference strain when compared to the *CCC2* deleted strain in copper deficient chemostat culture might be due to the iron deficiency observed in the reference strain under this condition. On the other hand, the up-regulation of other siderophore transporters in the *CCC2* deleted strain in comparison to the reference strain both in batch and chemostat cultures might indicate that

the induction of those transporters was due to the defective high affinity iron transport, which was observed in the absence of *CCC2* gene.

When the transient change in the intracellular iron levels in response to a sudden change in the copper levels were compared in the reference and CCC2 deleted strains, it was observed that cellular iron levels was increased by 2 fold in the reference strain within the first 5 minutes following the impulse. On the other hand, in the absence of *CCC2* gene, a similar increase could not be observed in response to copper impulse. These results indicated that, although cultivation of the *CCC2* deleted cells under high copper conditions could restore the iron deficiency in this strain, a sudden increase in the extracellular copper levels was not able to restore this deficiency in the short term. On the other hand, the iron deficiency in the reference strain, which stem from the long term exposure to copper deficiency, could be restored by the elimination of copper limitation.

5.5. Concluding Remarks

In this study, the dynamic transcriptional re-organisation of the high affinity, low affinity and siderophore iron transporters and also the transient change in the cellular iron and copper levels in response to a sudden relaxation of copper deficiency have been identified.

For the analysis of the transcript levels using real-time RT-qPCR, the most suitable pool of reference genes were selected among the previously identified candidate reference gene set. In the reference strain, *ACT1*, *TDH3* and *GCN4* genes were stably expressed, whereas in the *CCC2* deleted strain, *ACT1*, *ADH1* and *FBA1* displayed the most stable expression profiles. The relative expression profile of high affinity copper transporter, *CTR1*, which was expected to be down-regulated under high copper conditions, was analysed in order to test the suitability of these reference gene sets to analyse the dynamic transcriptional profiles of the genes of interest in response to a sudden increase in the copper level. This analysis revealed that the expression of *CTR1* was immediately repressed in the reference strain, whereas in the *CCC2* deleted cells, the expression of this gene was repressed after an initial slight induction indicating the more severe copper deficiency in this strain in comparison to the reference strain.

The analyses of the cellular iron levels of the cells cultivated in copper deficient chemostat cultures revealed that long term exposure to copper deficient resulted in iron deficiency also in the reference strain. On the other hand, long term exposure of the CCC2 deleted cells to copper deficiency did not led to a further iron deficiency in comparison to short term exposure. The induction of the high affinity iron transporter, *FET3* and siderophore iron transporters, *ARN1*, *FIT1* and *FIT3* in response to copper impulse in both strains revealed that elimination of copper limitation resulted in a transcriptional response to restore iron deficiency. Quantification of the cellular iron levels showed that sudden increase in the copper levels led to an increase in the cellular iron levels in the reference strain, however, in the *CCC2* deleted strain, a similar increase could not be observed in the short term although the iron transporters were up-regulated. Further studies are required to identify whether copper impulse resulted in an increase in the cellular iron levels in the later hours or not.

The transcriptional responses of *ARN2* and *ENB1* to high copper levels were observed to be antagonistic in two strains. In the *CCC2* deleted strain, these two siderophore iron transporters were induced in response to copper impulse, however in the reference strain the expression of these two genes were repressed by high levels of copper. These results might indicate that the transcription of these genes were regulated by a different mechanism from that of other siderophore transporters or the feedback signal generated by the increased iron levels led to repression of these genes in the reference strain. The comparison of the steady state expression levels of these genes in the reference and *CCC2* deleted strains also depicted that the expression levels of *ENB1* and *ARN2* showed a different behaviour from that of the other siderophore transporters. *ENB1* and *ARN2* were down-regulated in the *CCC2* deleted strain in comparison to the reference strain under copper deficient conditions, whereas the other siderophore transporters were induced under same conditions. This observation was not in accordance with the previous results obtained in Chapter 2 however this difference could stem from the decreased iron levels in the reference strain in response to long term exposure to copper deficiency. The up-regulation of *ENB1* and *ARN2* in the reference strain under copper deficient conditions together with their down-regulation in response to copper impulse, which resulted in an increase in the cellular iron levels, might indicate that the transcriptional regulation of

these genes were iron level dependent and even small changes in the iron levels led to a transcriptional re-organisation in the expression level of these genes.

6. CONCLUSION

Conservation of the biochemical and molecular functions between organisms facilitates the use of model organisms to understand the molecular mechanisms behind the Mendelian disorders. The aim of this thesis was to provide a systems level understanding of the copper related disorders such as Wilson and Menkes diseases by making use of the highly conserved copper homeostasis pathway between human and yeast.

In the first part of this study, the genome-wide effect of the absence of the yeast ortholog of Wilson and Menkes disease causing gene, *CCC2*, was investigated at transcriptional and metabolic level under changing copper conditions. These analyses revealed that the transcriptional response to the deletion of *CCC2* gene was dependent on the extracellular copper level. Under copper deficient condition, deletion of *CCC2* gene resulted in the deficiencies in iron, NAD^+, amino acid, pyridoxine metabolisms. The iron deficiency observed under copper deficient conditions caused the up-regulation of siderophore iron transporters in comparison to the reference strain. This defect was restored under high copper condition. However, high copper level led to an iron deficient like response in the reference strain indicating the increased demand for the cellular iron. NAD^+ biosynthesis from tryptophan was induced in response to the deletion of *CCC2* gene under copper deficient condition. On the other hand, high copper levels made a similar effect on the NAD^+ metabolism in the reference strain. These observations indicated that the copper overload response of the healthy cells resembles the transcriptional response to the absence of *CCC2* gene under copper deficient conditions. This might indicate that deletion mutant of *CCC2* gene can be used as a model for Wilson disease, in which copper accumulation was observed. On the other hand, arginine metabolism was observed to be affected from the deletion of *CCC2* gene both under copper deficient and high copper conditions. Integration of the transcriptome and regulome also highlighted a concentrated transcriptional change around the regulatory pathways related with amino acid availability in response to the interacting effect of gene deletion and change in the copper level. Moreover, metabolomic analyses of amino acid levels complemented these observations and revealed that intracellular arginine and alanine levels were lower in the *CCC2* deleted

strain when compared to the reference strain. Additionally the intracellular arginine, alanine, glutamine and tyrosine levels were significantly lower under copper deficient and high copper conditions in comparison to the condition containing adequate level of copper in both strains. Pyridoxine biosynthetic pathway was also among the processes, which was observed to be disturbed by the deletion of *CCC2* gene under copper deficient condition. The improvement in the respiratory capacity of the *CCC2* deleted cells with the supplementation of pyridoxine might indicate that absence of *CCC2* gene leads to pyridoxine deficiency, which might manifest iron and respiratory deficiencies. The absence of *CCC2* gene, and also the high copper level, affected the glucose transport mechanism. High affinity glucose transporters were repressed in response to the deletion of *CCC2* gene under copper deficient conditions in comparison to the reference strain, indicating that the absence of *CCC2* resulted in a transcriptional response mimicking the high glucose response under this condition. On the other hand, high level of copper resulted in the induction of high affinity transporters and repression of low affinity glucose transporter indicating that a glucose starvation-like response was induced by high copper level in both strains.

In the second part, the effect of the absence of the *ATX1*, which is the copper transporting chaperone to Ccc2p and the ortholog of *ATOX1* in human, was investigated at transcriptional level. The growth characteristics and the transcript levels were also analysed in comparison to the *CCC2* deleted strain under high copper and copper deficient conditions in order to elucidate the similarities and differences between these two strains. This comparative analysis revealed that although the proteins encoded by *ATX1* and *CCC2* genes take part in the consecutive steps in the intercellular transport of copper, the absence of these genes did not yield similar responses. Under high copper condition, the *ATX1* deleted cells displayed a fermentative behaviour, whereas *CCC2* deleted cells showed an increased respiratory behaviour. High copper levels induced a starvation-like response both in the reference and *ATX1* deleted strains; however, a similar response was observed in the *CCC2* deleted strain under copper deficient conditions in comparison to these strains. The integrated analyses of the transcript levels with regulome revealed the effect of the deletion of the *ATX1*gene on the regulation of the cell cycle. This result indicated that *ATX1* gene might play a role in the regulation of cyclin expression similar to its mammalian counterpart. Either the possible role of *ATX1* gene in the regulation of cell cycle or the

presence of an *ATX1* independent pathway to transport copper to Ccc2p might be the reason for the difference between the transcriptional re-organisation in response to the deletion of *ATX1* and *CCC2*.

The following two chapters were concerned with the transient gene expression analyses using real-time RT-qPCR. In the third chapter, the novel methodology that was developed for the determination of candidate reference genes, which can be used in the times series gene expression analyses using real-time RT-qPCR, was presented. The validity of the candidate pool was verified under two different experimental conditions. Then, in the last part of the study, this candidate reference gene set was used to select the suitable pool of reference genes, which will be used for the analyses of the transient response of the high, low and siderophore iron transporters to a sudden change in the extracellular copper level in the reference and *CCC2* deleted cells. The transient change in the iron transporters were analysed in conjunction with the changes in the cellular iron and copper levels. These analyses revealed that the expression of high affinity iron transporter, *FET3* and that of siderophore iron transporters, *ARN1*, *FIT1* and *FIT3*, were induced in response to high copper levels in both strains. This transcriptional re-organisation might be interpreted as a transcriptional response to restore cellular iron levels. A sudden increase in the extracellular copper levels led to an increase in the intracellular iron levels of the reference strain however, a similar increase could not be observed in the *CCC2* deleted strain during the course of the experiment. The down-regulation of *ARN2* and *ENB1* in response to copper impulse in the reference strain might be explained with the increase in the iron levels in the reference strain. On the other hand, in case of the *CCC2* deleted strain, the intracellular iron levels, which remained to be low in response to copper impulse, might be the reason for the increasing expression profiles of these genes. Moreover, *ENB1* and *ARN2* were down-regulated in the *CCC2* deleted strain in comparison to the reference strain under copper deficient conditions, whereas the other siderophore transporters were induced under same conditions. These results also provided further evidence for the regulation of the transcriptional levels of *ENB1* and *ARN2* with iron availability. On the other hand, the absence of *CCC2* gene, which led to a defective high affinity iron transport, seemed to be the regulating factor of the expression of the other siderophore transporters.

In conclusion, the absence of the *CCC2* gene, which is the yeast ortholog of Wilson and Menkes disease causing genes, led to deficiencies in iron, NAD^+, amino acid and pyridoxine metabolisms under copper deficient condition. Deletion of *CCC2* gene also resulted in a transcriptional response mimicking high glucose response under copper deficient conditions. When the *CCC2* deleted cells were grown under high copper condition, the deficiencies in the iron, NAD^+, pyridoxine and glucose transport mechanism were observed to be restored. However, high copper level led to iron deficiency and also the disturbance of NAD^+ homeostasis in the presence of the *CCC2* gene. However, a similar response was not observed in the *CCC2* deleted strain under high copper conditions. This might indicate that, Ccc2p might play a role in the cellular responses observed in the iron and NAD^+ metabolisms in response to high copper level. High copper level resulted in nitrogen and glucose starvation-like responses in the reference, *CCC2* and *ATX1* deleted strains. Observation of these changes also in the deletion mutants indicated that the transcriptional responses observed in these processes were not affected from the absence of these genes. Additionally, comparison of the cellular iron levels observed in the case of batch and continuous cultivations revealed that although short term exposure did not yield iron deficiency in the reference strain, long term exposure resulted in iron deficiency in this strain. This observation presented the difference between the batch and continuous cultivations.

Further investigation of the genome-wide transcriptome levels using the steady state samples collected from copper deficient chemostat culture may highlight the processes that were affected from the deletion of *CCC2* gene when cells were exposed to copper deficient conditions for longer period of time. Moreover, the analyses of the transient change in the transcriptome may provide further inside into the transcriptional re-organisation of these strains in response to a sudden change in the copper level. Additionally, further analyses of the transcript levels under iron deficient and high iron conditions may further emphasize the deficiencies due to the absence of the *CCC2* gene.

APPENDIX A: ENRICHED GO TERMS IN PART II

Enriched GO biological process terms among the significantly expressed genes in response to the deletion of *CCC2* gene, change in the copper level and interaction effect of gene deletion and change in the copper level were given in Table A.1, Table A.2 and Table A.3.

Table A.1. Enriched go-terms among the significantly expressed genes in response to change in the copper level in the CCC2 deleted and reference strain.

<table>
<tr><td colspan="2"></td><td colspan="2">Enriched GO-Biological Process Terms</td></tr>
<tr><td colspan="2">EFFECT OF COPPER LEVEL</td><td>CCC2Δ/ CCC2Δ</td><td>Reference strain</td></tr>
<tr><td rowspan="2">UP</td><td>High Cu vs Low Cu</td><td></td><td></td></tr>
<tr><td>High Cu vs Cu Def</td><td>Actin cortical patch assembly (p-val≤ 9.35e-04)</td><td></td></tr>
<tr><td rowspan="5">DOWN</td><td>High Cu vs Low Cu</td><td></td><td>Ribosome biogenesis (p-val≤ 4.95e-07)
Maturation of SSU-rRNA process (p-val≤ 4.54e-03)
Copper ion import (p-val≤ 3.22 e-03)</td></tr>
<tr><td rowspan="3">High Cu vs Cu Def</td><td>Copper ion import (p-val≤ 3.75e-07)</td><td rowspan="3">Ribosome biogenesis (p-val≤ 8.82e-03)</td></tr>
<tr><td>Oxidation-reduction process (p-val≤ 2.21e-03)</td></tr>
<tr><td>Iron ion homeostasis (p-val≤ 1.68e-02)</td></tr>
<tr><td>Low Cu vs Cu Def</td><td>Copper ion import (p-val≤ 1.41e-06)</td><td></td></tr>
</table>

Table A.2. Enriched go-terms among the significantly expressed genes between changing copper levels in response to interaction effect of the change in the copper level and the deletion of *CCC2*.

		Enriched GO-Biological Process Terms	
INTERACTION EFFECT		**CCC2Δ/ CCC2Δ**	**Reference strain**
UP	**High Cu vs Low Cu**	Arginine metabolic process (p-val≤ 2.89e-2) Carboxylic acid metabolic process (p-val≤ 5.43e-3) ATP synthesis coupled electron transport (p-val≤ 1.41e-2)	Iron ion homeostasis (p-val≤ 3.04e-2) Siderophore transport (p-val≤ 2.19e-2)
	High Cu vs Cu Def		Iron ion homeostasis (p-val≤ 5.98e-3) Siderophore transport (p-val≤ 1.11e-2) "*de novo*" NAD biosynthetic process from tryptophan (p-val≤ 3.55e-2) "*de novo*" UMP biosynthetic process (p-val≤ 4.27e-2) Response to copper ion (p-val≤ 3.55e-2) Cellular cell wall organization or biogenesis (p-val≤ 3.55e-2) Nucleotide biosynthetic process (p-val≤ 1.27e-2) Regulation of sporulation (p-val≤ 4.27e-2)
DOWN	**High Cu vs Cu Def**	Iron ion homeostasis (p-val≤ 4.63e-3) Siderophore transport (p-val≤ 1.52e-6)	
	Low Cu vs Cu Def	Arginine metabolic process (p-val≤ 2.20e-03) Ornithine (p-val≤ 8.34e-3) and urea(p-val≤ 5.24e-3) metabolic process Drug membrane transport (p-val≤ 8.34e-3) Cellular keton metabolic process(p-val≤ 9.47e-3) "*de novo*" UMP biosynthetic process (p-val≤ 7.83e-3)	

Table A.3. Enriched go-terms among the significantly expressed genes between two strains in response to interaction effect.

		Enriched GO-Biological Process Terms	
INTERACTION EFFECT		**Copper Deficient Conditions**	**High Copper Conditions**
UP	**CCC2Δ/ CCC2Δ vs Reference strain**	Iron ion homeostasis (p-val≤ 3.89e-4) Siderophore transport (p-val≤ 1.30e-6) Arginine metabolic process (p-val≤ 3.94e-2) Cellular keton metabolic process (p-val≤ 1.99e-2) Drug membrane transport (p-val≤ 1.68e-2) Urea (p-val≤ 1.68e-2) and ornithine (p-val≤ 2.68e-2) metabolic process "*de novo*" NAD biosynthetic process from tryptophan (p-val≤ 1.91e-2) "de novo" UMP biosynthetic process (p-val≤ 3.94e-2) Allantoin (p-val≤ 2.55e-2) and pyridoxal phosphate (p-val≤ 3.06e-2) metabolic process	
DOWN		Glucose transport (p-val≤ 3.65e-2) Polyphosphate metabolic process (p-val≤ 3.65e-2) Ribosome biogenesis (p-val≤ 3.65e-2)	Iron ion homeostasis (p-val≤ 8.58e-3) Siderophore transport (p-val≤ 1.5e-3) "*de novo*" NAD biosynthetic process from tryptophan (p-val≤ 1.04e-3) Purine ribonucleoside triphosphate biosynthetic process (p-val≤ 2.41e-2) Oxidation reduction process (p-val≤ 3.51e-2)

APPENDIX B: ENRICHED GO TERMS IN PART III

Enriched GO biological process terms among the significantly expressed genes in response to the deletion of *ATX1* gene, change in the copper level, additive and interaction effect of gene deletion and change in the copper level were given in the following tables.

Table B.1. Enriched go-terms among the significantly expressed genes in response to deletion of *ATX1* gene.

Enriched GO-Biological Process Terms	**Corr. p-value**
regulation of phospholipid biosynthetic process	0.0292

Table B.2. Enriched go-terms among the significantly up-regulated genes in response to high copper levels.

Enriched GO-Biological Process Terms	**Corr. p-value**
actin cytoskeleton organization	4.79E-06
cell wall organization	2.43E-05
endocytosis	9.63E-05
response to starvation	7.78E-04
autophagy	1.14E-03
"de novo" NAD biosynthetic process from tryptophan	1.06E-02
cellular amino acid metabolic process	1.74E-02
sporulation	3.84E-02
iron ion homeostasis	4.07E-02
TOR signalling cascade	4.39E-02

Table B.3. Enriched go-terms among the significantly down-regulated genes in response to high copper levels.

Enriched GO-Biological Process Terms	**Corr. p-value**
ribosome biogenesis	9.26E-49

Table B.4. Enriched go-terms among the significantly up-regulated genes in response to additive effect of change in the copper level and gene deletion.

Enriched GO-Biological Process Terms	**Corr. p-value**
carnitine metabolic process	2.83E-03

Table B.5. Enriched go-terms among the significantly down-regulated genes under copper deficient conditions and also in the *ATX1* deleted strain.

Enriched GO-Biological Process Terms	**Corr. p-value**
mitochondrial ATP synthesis coupled electron transport	7.45E-04

Table B.6. Enriched go-terms among the significantly up-regulated genes under copper deficient conditions and also in the *ATX1* deleted strain.

Enriched GO-Biological Process Terms	**Corr. p-value**
Post-replication repair	5.49E-03

Table B.7. Enriched go-terms among the significantly up-regulated by high copper levels in the *ATX1* deleted strain and down-regulated in the reference strain.

Enriched GO-Biological Process Terms	**Corr. p-value**
Golgi vesicle budding	1.94E-02
retrograde vesicle-mediated transport, Golgi to ER	3.62E-02

Table B.8. Enriched go-terms among the significantly up-regulated under high copper conditions and down-regulated under copper deficient conditions in the *ATX1* deletion mutant.

Enriched GO-Biological Process Terms	**Corr. p-value**
vesicle targeting	4.20E-02

Table B.9. Enriched go-terms among the significantly down-regulated under high copper conditions and up-regulated under copper deficient conditions in the *ATX1* deletion mutant.

Enriched GO-Biological Process Terms	**Corr. p-value**
telomere maintenance	2.43E-02
'de novo' UMP biosynthetic process	2.16E-02

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