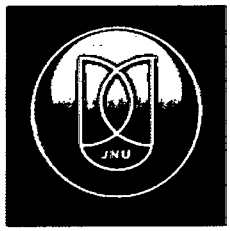
Studies on genes involved in acetic acid tolerance of yeast *Saccharomyces cerevisiae*



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YASHPAL

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2011

Dedicated

to my

Grandmother

इमटैक
IMTECH

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CERTIFICATE

This is to certify that thesis entitled **"Studies on genes involved in acetic acid tolerance of yeast *Saccharomyces cerevisiae*"** comprises the work done by **Mr. Yashpal** under my guidance at the Institute of Microbial Technology, Chandigarh. The work is original and has not been submitted in part or full for any other degree or diploma of any other University/Institute.

Date: 30.8.2011

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Acknowledgement

It has been long but satisfactory journey at the end, as I am submitting my Ph.D. thesis after 6 years. This whole period came out as learning experience both scientific as well as about life. I have learned to work independently and most importantly taking challenges and winning over them. Here I made some good friends and most importantly got my life partner, so it was even more satisfactory at personal level. This thesis will be incomplete without acknowledging all those who supported and guided me throughout this period.

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Yashpal

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Abbreviations:

Δ	Deletion
µCi	Microcurie
~	Approximately
↑	Overexpression of gene
°C	Degree Celsius
aa	Amino acid
ATP	Adenosine tri-phosphate
BLAST	Basic local alignment search tool
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CEN	Centromeric
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetate
g	Gram
g/l	Gram per liter
G418	Geneticin
GPD	Glyceraldehyde-3-phosphate dehydronase
HA	Hemagglutanin A
His	Histidine
hr	Hour
Kb	kilo base
kD	kilo Dalton
Leu	Leucine
LiAcTE	Lithium acetate Tris-EDTA
Lys	Lysine

M	Molar
MCS	Multiple cloning sites
Met	Methionine
µg	Micro gram
µg/ml	Micro gram per milliliter
Min	Minutes
ml	Milli liter
µM	Micro molar
mM	Milli molar
ng	Nano gram
ng/µl	Nano gram per microliter
nm	Nano meter
O.D.	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH_i	intracellular pH
Pmol	Pico mole
PNK	Poly nucleotide kinase
RNA	Ribonucleic acid
RNAse	RibonucleaseA
ROS	Reactive oxygen species
rpm	Revolutions per minutes
RT	Room temperature
SC	Synthetic complete
SD	Synthetic dextrose

SDS	Sodium dodecyl sulphate
Ser	Serine
SGD	Saccharomyces genome database
TAE	Tris-acetate EDTA buffer
TE	TrisCl and EDTA
TEF	Translational elongation factor EF-1
Tris	Tris (hydroxymethyl) amino methane
Ura	Uracil
WT	wild type
YFP	Yellow fluorescence protein

Introduction

Depletion of nonrenewable sources of energy resources has lead to worldwide efforts for developing alternative technologies for generating energy from renewable and economically viable resources. An attractive source of renewable energy is conversion of lignocellulosic biomass in bioethanol, but the biomass has to be first pretreated to release sugars for fermentation. Mild acid hydrolysis and enzymatic treatments are commonly employed pretreatment methods. Upon acid hydrolysis various inhibitory compounds are also released which inhibit the growth as well as fermentation ability of yeast strains. Acetic acid is one of such inhibitor generated during acid hydrolysis of lignocellulosic substrates. It is also produced as a byproduct of ethanolic fermentation and is known to function synergistically with ethanol to inhibit fermentation and growth of yeast. Therefore we wished to study the mechanism of acetic acid tolerance in yeast by identifying genes involved in adaptation yeast to acetic acid. The identified genes might be useful for improving the acetic acid tolerance of yeast strains; such strains are expected to perform better in ethanolic fermentation of lignocellulosic hydrolysates.

Stress response of yeast to acetic acid and other weak acids has been previously described. At low pH, weak acids predominantly exist in undissociated form and can readily diffuse into the yeast cell. Once inside they dissociate at the near neutral pH of cytosol, releasing protons and acid anions, reducing the cytosolic pH. At acidic pH, while metabolic activities of yeast cells are inhibited, however ATP consumption is increased to pump out protons. Acid anions being impermeable to plasma membrane accumulate inside the cytosol, resulting in increased turgor pressure and generation of free radicals, leading to oxidative stress. These inhibitory effects of acetic acid and other weak acids reduce the viability of yeast cells as well as their ability to ferment carbon substrates into ethanol. Nevertheless, yeast cells can tolerate significant concentration of acetic acid in growth media, by modulating its cellular machinery to minimize the accumulation of acids in the cell. These involve activation of plasma membrane ATPases to efflux protons, various membrane transporters to efflux acid anions, alteration in membrane permeability and various metabolic changes to compensate for weak acid stress.

We screened yeast transformants of a multicopy yeast genomic DNA library to identify genes that upon overexpression increase acetic acid tolerance. The advantage of this approach is that identified genes can straightaway be tested for improving the acetic acid tolerance and possibly ethanol productivity of industrial yeast strains. Many acetic acid tolerant clones

obtained carried *HAA1*, a gene encoding a transcription factor known to be involved in acetic acid tolerance. We also identified *YLR297w,* an uncharacterized gene, which upon overexpression provides resistance to acetic acid as well as lipophilic acids such as octanoic acid and benzoic acid. *YLR297w* mediated acetic acid resistance was seen only in late phase of growth and not in exponentially growing cells. Low pH and presence of acetic acid in pregrowth media were found to be important for development of acetic acid tolerance conferred by *YLR297w* overexpression (Chapter-3).

HAA1 gene found in few acetic acid resistant clones were truncated encoding Haa1p missing the C-terminal region. These were even better than wildtype Haa1p in conferring acetic acid tolerance. To further understand the role of Haa1p domains, various deletions of *HAA1* gene lacking either N-terminal or C-terminal region were constructed, expressed in *haa1Δ* strain, and the level of acetic acid tolerance conferred by them was studied. Ability of *HAA1* deletions to induce the expression of selected Haa1p target genes was also monitored. Like full-length Haa1p, C-terminal deletions can fully complement *haa1Δ* strain in acetic acid sensitivity and induction of target genes tested, but the N-terminal deletion can only partially complement. The regulatory regions present in the upstream regions of Haa1p target genes *YGP1* and *TPO2* were identified by DNA binding and promoter mapping experiments. We identified that *YGP1* has two regulatory elements present in upstream region, one responding to acetic acid shock and another active in untreated cells; on the other hand *TPO2* has a single regulatory element in upstream region mainly responding to acetic acid (Chapter-4).

Expression of full-length Haa1p under the constitutive and strong GPD promoter was found to be toxic; however such toxicity was not seen with Haa1p deletion H400, although it was providing acetic acid tolerance better than full-length Haa1p. Such toxicity was possibly resulting from unwanted activity of Haa1p, thus it is likely that Haa1p activity is very tightly regulated. Proteins interacting with Haa1p may have possible role in its regulation and affect its activity. Therefore, proteins having possible interaction with Haa1p were searched from interaction datasets available in online databases, such as Biogrid. Acetic acid sensitivity of strains deleted in selected genes encoding Haa1p interacting proteins was checked. Effect of *HAA1* overexpression in these strains was also checked on acetic acid tolerance and growth. Based on our results and known functions of Haa1p interacting proteins, attempts were made to identify their role in regulation of Haa1p function. We found that Msn5p is important for controlling the nuclear localization of Haa1p whereas Ngg1p functions as repressor of Haa1p

activity upon acetic acid shock. Furthermore, preliminary role of the Pho85p-Pho80p CDK-cyclin complex in targeting Haa1p to Msn5p and Ngg1p mediated regulation was also identified (Chapter-5).

Chapter 1
Review of literature

1.1 Weak acid stress in yeast:

The budding yeast *Saccharomyces cerevisiae* is subjected to multiple and changing stress conditions during its growth. These conditions include hyperosmotic shock, nutrient limitation, temperature variation, accumulation of various inhibitors in media, reduction in pH etc. Weak organic acids such as acetic acid, lactic acid, propionic acid, sorbic acid and benzoic acid are inhibitory to growth of *Saccharomyces cerevisiae*. Since various natural habitats of yeast and fungi contain high concentration of weak acids as a result of bacterial fermentation as well as secreted by certain yeasts such as *Brettanomyces* and *Dekkera* (Pretorius, 2000). *S. cerevisiae* has evolved various mechanisms to grow at substantial concentrations of such acids in growth media. Yeast cells are also subjected to acid stress caused by weak acids produced as byproducts of fermentation (Lafonlafourcade et al., 1983) as well as when subjected to fermentation of acid hydrolyzed lignocellulosic materials (Liu, 2006). So studying the process of acid stress as well as mechanism of adaptation to weak acids in yeast, *Saccharomyces cerevisiae*, is of importance. A good understanding of such mechanisms of weak acid tolerance will help to improve our strategies to address the problem of food spoilage as well as efficient utilization of non-fermentable sugars for production of bioethanol.

1.2 Ethanolic fermentation and weak acid stress:

Ethanolic fermentation is accompanied by drastic reduction of pH and weak organic acids like acetic acid, levulinic acid and formic acid are produced as by-products. Weak acids can also be produced in fermentation media by lactic and acetic acid bacteria, which happen to be common contaminants of yeast ethanolic fermentation broths. These byproducts of fermentation, besides ethanol, reduce ethanol yield as well as growth of fermenting yeast (Lafonlafourcade et al., 1984; Viegas et al., 1989). While ethanol produced during fermentation does not accumulate inside the cell (Loureiro and Ferreira, 1983), weak acids such as acetic acid accumulate inside the yeast cells at low pH, which in turn results into inhibition of fermentation. In the presence of ethanol, the inhibitory effect of acetic acid is more drastic and can be observed at slightly higher pH, causing synergistic inhibition of fermentation (Pampulha and Loureirodias, 1989). This synergistic inhibitory effect is thought to be a result of inhibition of glycolytic enzymes, such as enalose (Pampulha and Loureirodias, 1990). Moreover acetic acid, at concentrations occurring in wine fermentation, induces cell death (Pinto et al., 1989).

As interest in alternative energy sources is rising, the concept of biomass conversion to ethanol has become attractive. In recent years, there has been a rapid increase in ethanol production that has been derived from sucrose (in Brazil) and corn starch (in the USA). However, utilization of common crops for ethanol production is hugely debatable as it results into increase in food prices and demand for arable land. Lignocellulosic biomass as substrate for ethanolic fermentation is an attractive option, as it is generated from agricultural or forest wastes thus have least associated cost. However, one major barrier in implementing this process is inhibitory compounds generated during biomass pre-treatment using dilute acid hydrolysis to release monomeric sugars, interfere with microbial growth and subsequent fermentation (Liu, 2006). Acid pretreatment of lignocellulose results in hydrolysis of hemicelullose, followed by enzymatic or chemical hydrolysis of the remaining cellulose (Sun and Cheng, 2002). The release of monomeric sugars during the pretreatment is accompanied by the generation of inhibitors of fermenting yeasts, which strongly affects the fermentation performance (Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000a, b).

The lignocelluloses mainly compose of cellulose, hemicelluloses and lignin, though composition varies from one plant source to another. Pretreatment of lignocellulosic biomass generate a broad range of compounds. D-glucose is mainly obtained from hydrolysis of cellulose. D-galactose, D-mannose, D-rhamnose (hexoses) as well as D-xylose and L-arabinose (pentoses) and uronic acids are released from the hemicellulosic fraction. Hydrolysis of lignin and further degradation of monomeric sugars generate three major categories of compounds that inhibit fermentation. These are furan derivatives, weak acids and phenolics (Almeida et al., 2007). Further details of their type, source and inhibitory effects are as summarized in table 1.1 and figure 1.1 and 1.2.

1.2.1 Furan Derivatives:

The furan compounds 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde are formed by dehydration of hexoses and pentoses, respectively (Ulbricht et al., 1984; Williams and Dunlop, 1948). HMF and furfural decrease the volumetric ethanol yield and productivity, as well as inhibit the growth or give rise to a longer lag phase. These effects depend on the furan concentration and on the yeast strain used, moreover synergistic effects of HMF and furfural have been demonstrated (Taherzadeh et al., 2000). *In vitro* studies have shown that HMF and furfural directly inhibited alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH), aldehyde dehydrogenase (ALDH) and glycolytic enzymes hexokinase and GPDH. Moreover

Inhibitor Class	Inhibitor name	Source	Effect
Furan Derivative	5-hydroxymethyl-2-furfural	Dehydration of hexoses	Inhibition of glycolytic enzymes and ADH
	2-furfuraldehyde	Dehydration of pentooses	Reduced NADPH and ATP level, etc.
Weak Acids	Acetic acid, formic and Levulinic acid	Deacetylation of hemicelluloses and HMF breakdown	Uncoupling , intracellular anion accumulation and ATP depletion
Phenolics	Acetosyringone, Hydroxybenzoic acid and vanillin	Lignin breakdown and Carbohydrate degradation	Disrupt membrane integrity and Inhibit Electron transport chain

Table 1.1: Type, source and inhibitory effect of inhibitors generated from hydrolysis of lignocellulosic biomass .

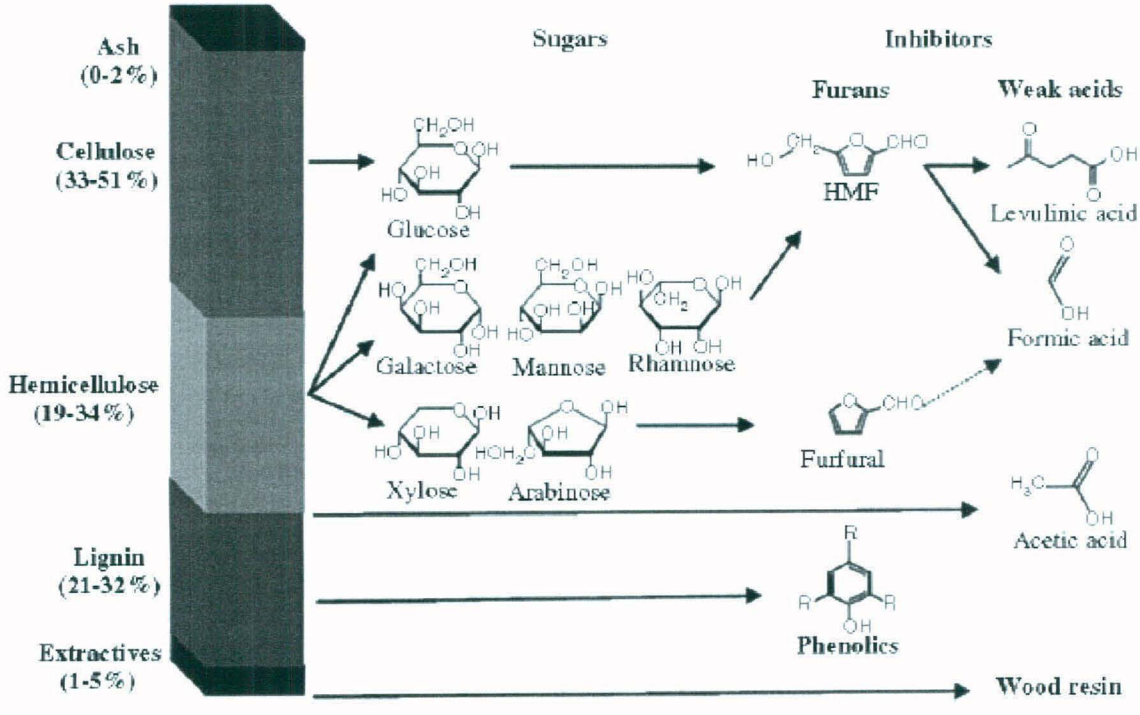


Figure 1.1: Average composition of lignocellusic biomass and main derived hydrolysis products (Almeida et al., 2007).

furans reduces intracellular ATP and NAD(P)H levels by affecting glycolytic and TCA fluxes (Horvath et al., 2003).

1.2.2 Weak acids:

Acetic acid, formic acid and levulinic acid are most common weak acids present in lignocellulosic hydrolyates. Acetic acid is formed by de-acetylation of hemicelluloses, while formic and levulinic acids are products of HMF breakdown. Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures (Ulbricht et al., 1984; Williams and Dunlop, 1948). Weak acids inhibit yeast fermentation by reducing biomass formation and ethanol yields (Larsson et al., 1999). The inhibitory effect of weak acids has been ascribed to uncoupling and intracellular anion accumulation. According to this theory, at pH lower than pKa value of weak acid, it exists in undissociated form which is permeable to yeast plasma membrane. Once inside the cell these weak acids dissociate into protons and anions, which results into acidification of cytosol and accumulation of anions (Russell, 1992). Moreover ethanolic fermentation using xylose as carbon source is more strongly inhibited by acetic acid, as it enhances xylitol formation (Bellissimi et al., 2009).

1.2.3 Phenolics:

A wide range of phenolics are generated due to breakdown of lignin and subsequent acid hydrolysis of carbohydrate, depending on biomass composition and its chemical complexity (Perez et al., 2002). Inhibitory mechanism of phenolics involves disruption of membrane integrity, which further leads to loss of membrane barrier, destruction of electrochemical gradient across the membrane and loss of enzyme matrices (Heipieper and Debont, 1994; Heipieper et al., 1994).

1.3 Weak acids as food preservatives:

Yeasts are able to grow in foods with a low pH (5.0 or lower) in the presence of sugars, organic acids and other easily metabolized carbon sources. During their growth, yeasts metabolize some food components and produce metabolic end products. These products change the physical, chemical, and sensible properties of a food which in turn causes food spoilage (Kurtzman, 2006). For centuries variety of substances such as natural herbs, spices and weak acids, have been used as preservatives to protect food from spoiling. Weak organic acids like benzoic, acetic, sorbic and propionic acids are widely used food preservatives in

Weak acid	Typical food use	Concentration ppm
Formic acid	Semi preserved fish product Pickles, mustard Fruit juice concentrates	300-1000 100 4000
Acetic acid	Pickles, Chutneys, Sauces Salad creams and dressings Vinegars	A few thousand up to % levels in vinegars
Propionic acid	Bread Flour confectionary Jams, tomato puree Non –emulsified sauces	2000-5000 1000-3000 1000 1000
Sorbic acid	Non alcoholic drinks Alcoholic drinks Semi-preserved fish product Processed fruits and vegetables Fruits and dairy based desserts Sugar based confectionary Bakery products Mayonnaise and sauces Salads Fat based spreads Mustard	100-1000 200 500-2000 500-2000 500-1000 500-2000 1000-2000 1000-2000 1000 100-2000 250-1000
Benzoic acid	Non-alcoholic drinks Alcoholic drinks Semi-preserved fish product Fruit products Vegetables, pickles, preserves Sugar and flour based confectionary Mayonnaise and sauces Salads mustard Fat based spreads	100-500 200 100-4000 500-2000 250-2000 1000 250-2500 1000 100-1000
Lactic acid	Fermented meat and dairy products Carbonated drinks Salad dressings Pickled vegetables Sauces	A few thousand up to % levels
Citric acid	Non-alcoholic drinks Jams and jellies Bakery products Cheese Canned vegetables Sauces Packet dry soup and cake mixes	A few thousand up to % levels

Table 1.2: Major food grade weak acids and conditions of use (Stratford and Eklund, 2003).

Weak Acids	Structure	log P	pKa	Inhibitory mechanisms
Formic acid	HCOOH		3.77	Highly toxic, but less studied
Acetic acid	CH_3COOH	-0.24	4.76	Induce apoptosis, inhibit metabolic pathways, generate oxidative stress and ATP depletion
Propionic acid	CH_3CH_2COOH	0.32	4.87	Inhibit metabolic pathways, generate oxidative stress and ATP depletion
Butyric acid	$CH_3CH_2CH_2COOH$	0.83	4.87	Inhibitory action not well studied, lies between propionic and octanoic acid
Octanoic acid	$CH_3(CH_2)_6COOH$	3.05	4.88	Disrupt plasma membrane structure, inhibit oxidative phosphorylation and generate oxidative stress
Sorbic acid	$CH_3(CH)_4COOH$	1.63	4.76	Same as above
Benzoic acid	C_6H_5COOH	1.71	4.2	Same as above

Table 1.3: Chemical structure, dissociation constant (pKa), lipophilic tendency (log P) and inhibitory action of some weak acids.

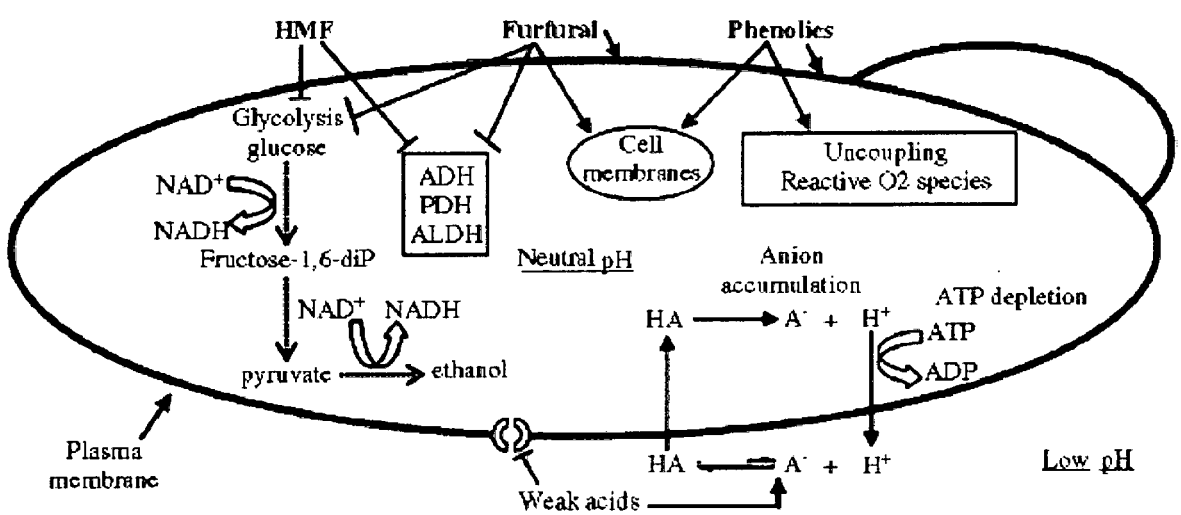


Figure 1.2: Schematic view of known inhibition mechanism of furans, weak acids, and Phenolics in yeast *Saccharomyces cerevisiae* (Almeida et al, 2007).

large scale food and beverage preservation (Stratford and Anslow, 1996). Weak acids are also present naturally in a number of foods, notably fruits and fruit juices, such as citrus food contains citric acid. Use of weak acids as preservatives is controlled by various regulatory agencies worldwide and restricted to certain limits based on legislation, taste and cost (Table-1.2). For instance sorbic acid and benzoic acids are accepted as having GRAS (Generally recognized as safe) status in USA and their use in soft drinks is limited by only taste considerations, whereas in European Union they are regarded as preservatives and permitted at conc. not exceeding 300 and 150 ppm respectively. Similarly, in European Union, acetic acid is equally accepted as acidulant, preservative at higher concentration or as flavor compound whereas formic acid is only accepted as preservative. These acids inhibit microbial growth at different concentration depending on their polarity and carbon chain length. Furthermore, the presence of weak acids can increase the efficacy of physical preservative treatments, such as heat or ultra high pressure. The heat resistance of microbes is greatly reduced in acidic conditions, thus shortening the duration of food processing (Stratford and Eklund, 2003).

1.4 Mechanism of inhibition of yeast growth by weak organic acids:

In general organic acids including acetic acid are weak acids and do not dissociate completely in water, where the strong mineral acids do. Dissociation and re-association of proton and anion in such weak acids depends on the pH of the solution. The pH at which there exists equilibrium of dissociation and re-association between proton and conjugate base of weak acid ($HA \rightleftharpoons A^- + H^+$) is called pKa value. The organic acids have pKa values below 5, such as formic acid (pK_a 3.75 at 20°C), acetic acid (pK_a 4.75), sorbic acid (pK_a 4.76) and benzoic acid (pK_a 4.19) (Table-1.3). The antimicrobial effects of weak organic acids at low pH are result of intracellular acidification and anion accumulation (Russell, 1991; Salmond et al., 1984); though mechanism of action may not be identical for different weak acids (Figure-1.3). The comparative studies of antimicrobial action of acetic acid and sorbic acid have shown that acetic acid mainly acts through acidification of cytosol and anion accumulation, whereas sorbic acid mainly acts by disruption of membrane integrity and oxidative stress caused by associated effects on respiratory chain function (Bracey et al., 1998; Piper, 1999). Such a different mechanism of action defines quite high concentration of acetic acid (80-150mM) is required to completely inhibit the growth of *S. cerevisiae* at pH 4.5. In contrast, highly lipophilic sorbic acid with almost identical pK_a value, 1-3mM concentration was

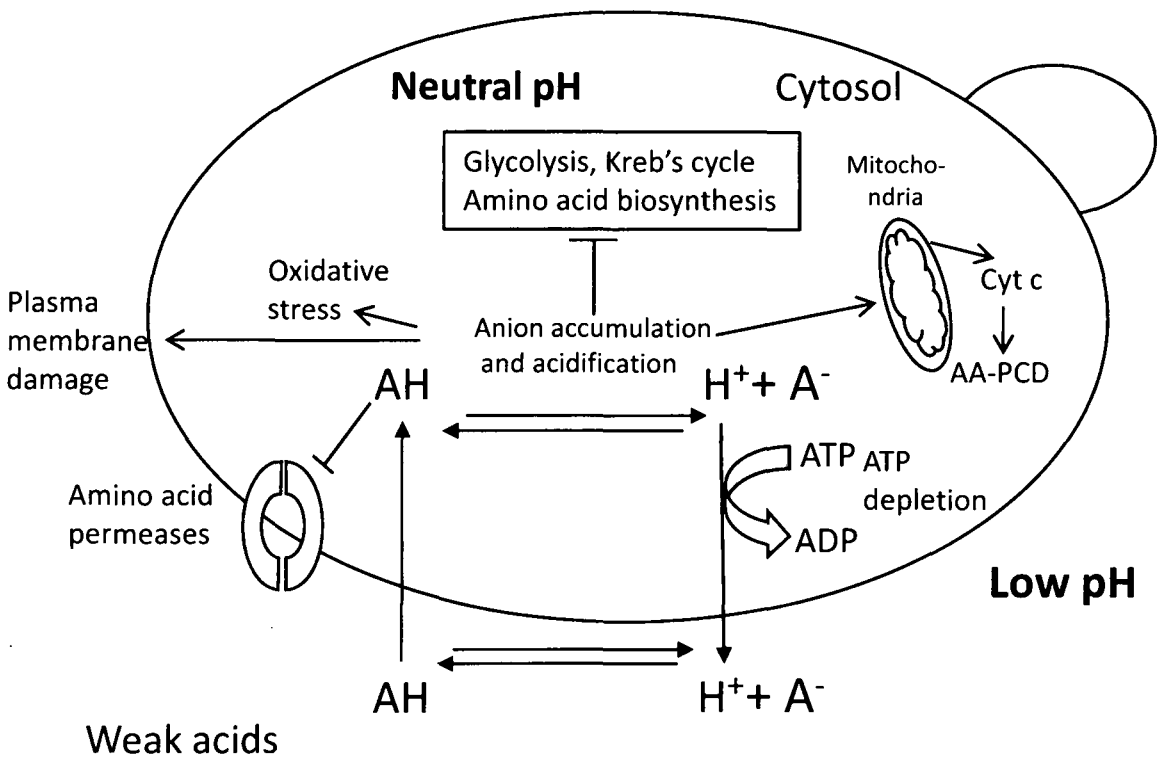


Figure 1.3: Schematic model for weak acid induced stress responses in yeast *Saccharomyces cerevisiae.*

enough to cause similar effect. Thus, as the weak acids become more lipophilic, its antimicrobial ability is enhanced due to destructive effects on yeast plasma membrane in comparison to less lipophilic monocarboxylic acids (Holyoak et al., 1999; Piper et al., 1998; Stratford and Anslow, 1998).

1.4.1 Uncoupling and intracellular anion accumulation:

At low pH weak acids exist predominantly in undissociated form, a form in which they are potent growth inhibitors. The undissociated acid, being uncharged, readily diffuses across the cell membrane only to further dissociate in the higher pH environment of the cytosol. Such dissociation of weak acid generates protons and the acid anion in cytosol, resulting in acidification of cytosol (Krebs et al., 1983). Reduction in *S. cerevisiae* intracellular pH (pH_i) has been demonstrated following the addition of acetate (Arneborg et al., 2000) and benzoate (Krebs et al., 1983), though reduction in pH_i is not always a feature of organic acid stress. The *S. cerevisiae* maintains a relatively constant intracellular pH by pH homeostasis machinery of cell. The decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. Consequently less ATP is available for cellular metabolism and cells encounter shortage of energy pool for growth and multiplication (Palmqvist et al., 1999; Russell, 1991; Verduyn, 1991). Continuous inflow of undissociated acid into the cell by diffusion across plasma membrane results into gradual acidification of cytosol (Bracey et al., 1998). The acidification of cytosol inhibits the activity of cellular machinery, as optimal pH required for enzymatic activity is disturbed. The presence of acetic acid in yeast cell negatively regulates the activity of glycolytic enzymes. The activity of enolase is most severely affected along with inhibition of hexokinase and phosphofructokinase, while glucose transport is not affected (Pampulha and Loureiro, 1989).

Dissociation of weak acid also releases anions into the cytosol; anions being negatively charged are relatively membrane impermeant and accumulate intracellularly to very high level. This high anion accumulation may generate an abnormally high turgor pressure, resulting in free radical production and severe oxidative stress. The anion accumulation also causes protein aggregation, lipid peroxidation, inhibition of membrane trafficking and perturbation of plasma and vacuolar membrane (Piper et al., 2001; Teixeira et al., 2007). This in turn further affect the yeast cells ability to grow and ferment carbon substrate into ethanol, such inhibition of fermentation is synergistically affected by ethanol. Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the

medium, probably as a consequence of strong inhibition of Tat2p amino acid permease (Bauer et al., 2003). Moreover in presence of weak acids at low pH, acidification of external environment may affect the cell wall structure and alter the conformation of proteins on the outer side of plasma membrane (Stratford and Booth, 2003).

1.4.2 Acetic acid mediates apoptosis in yeast:

The role of acetic acid has been also implicated in yeast apoptosis and chronological life span. Acetic acid induced apoptosis in yeast results in mitochondrial swelling, reduction in cristae number and formation of myelinic bodies. Additionally, mitochondrial ROS accumulation, a transient hyperpolarization followed by depolarization, and a decrease in the activity of COX affecting mitochondrial respiration is seen. Mitochondria become permeabilized, allowing the release of lethal factors like cytochrome *c* (Ludovico et al., 2002). However, acetic acid induced programmed cell death (AA-PCD) may occur without cytochrome *c* release, as AA-PCD is significantly observed in *yca1Δ* and presence of Yca1p is essential for cytochrome *c* release (Guaragnella et al., 2010). Acetic acid produced by fermentative growth in stationary phase induces oxidative stress, a factor previously implicated in chronological aging of yeast and other organisms (Burtner et al., 2009). The accumulation of acetic acid in stationary phase cultures inhibits growth arrest of cells in G1 and is preferentially toxic to cells that fail to undergo a G1 arrest. In budding yeast and other fungi acetic acid mediated intracellular acidification activates the same highly conserved Ras2 and cAMP-dependent signaling pathways that respond to glucose. Therefore in stationary phase, when glucose is completely consumed, nutrient depleted cells are subjected to acetic acid-induced growth signals that promote entry into S phase. The absence of nutrients or the regulatory mechanisms required for the synthesis of dNTPs and efficient DNA replication result in replication stress and induction of apoptotic manifestation (Burhans and Weinberger, 2009).

1.5 Mechanism of weak acid tolerance in yeast:

Despite all these toxic and inhibitory conditions encountered by yeast cells in presence of acetic acid, they are able to survive and adapt to significant concentration of acetic acid and other weak acids. Adaptation to low pH and high weak acid concentration or inherent acquisition of tolerance to acid would require yeast cells to modify their cellular machinery to minimize the accumulation of acids within the cell. As earlier described, different weak acids

have different inhibitory action depending on lipophilicity. Thus, yeast cells uses different resistance mechanism to adapt against stress caused by different weak acids (Figure-1.4).

1.5.1 Preadaptation and weak acid tolerance:

Yeast cells exposed to acetic acid show a decrease in cell viability in a concentration and time dependent manner. On exposure to 80 mM acetic acid in YPD at pH 3.0, cell viability decreases to 60% after 60 minutes, 30% after 90 min and virtually all cells are unviable after 200 min (Giannattasio et al., 2005). But it is well-known that pretreatment with one kind of stressing agent may induce cross-resistance against another type of stress agent in yeast (Evans et al., 1998). Therefore, exponentially growing yeast cells preincubated in YPD medium at pH 3.0 for 30 min remained fully viable after 200 min exposure to 80 mM acetic acid. Resistance to acetic acid observed in such pretreated cells was attributed to high intracellular level of both catalase and super oxide dismutase activities (Giannattasio et al., 2005). Moreover, when exponentially growing yeast cells encounter inhibitory concentrations of weak acid, growth is arrested and cells enter a period of growth latency. During this lag phase cell viability is reduced and growth resumes after extended period of time. However, when these adapted cells are reinoculated into a fresh-growth medium at identical pH and supplemented with the same weak acid concentration, no delay in cell growth is observed (Carmelo et al., 1998; Teixeira and Sa-Correia, 2002). Therefore, yeast cells previously adapted to weak acids or other stress have better tolerance to weak acid stress.

1.5.2 Plasma and vacuolar membrane ATPases reduce intracellular acidification:

Diffusion of weak acids into the yeast cell causes acidification of cytosol by increased H^+ influx. To avoid dissipation of plasma membrane potential and maintain the intracellular pH homeostasis to allow physiological activities of cell, yeast cells activate plasma membrane H^+ ATPase (Pma1p) to efflux protons in ATP dependent manner (Mira et al., 2010c). Plasma membrane ATPases also play important role in activity of H^+ antiporters efluxing acid anions from cytosol, thus reducing the stress caused by anion accumulation (Tenreiro et al., 2002). H^+ ATPases present in the vacuolar membrane (V-ATPase) are also been proposed to play similar role. By seizing protons into the vacuolar lumen, V-ATPase activity helps in maintaining pH homeostasis and membrane potential across vacuolar membrane (Carmelo et al., 1997; Marcantoni et al., 2007). For maximal tolerance to weak acids, role of V-ATPases is also implicated in endocytosis, targeting of newly synthesized lysosomal enzymes,

intracellular trafficking, Pma1p sorting to the plasma membrane and compartmentalization of metabolites (Caba et al., 2005; Mira et al., 2009; Mollapour et al., 2004; Teixeira et al., 2005).

1.5.3 Membrane transporters efflux weak acid anion:

Influx of weak acids into the yeast cells also results into anion accumulation. Removal of counter anions is therefore essential and several specific transporters have been implicated in efflux of such anions from yeast cytosol. Pdr12p, a plasma membrane transporter of the ATP binding cassette superfamily is involved in the active efflux of propionate, sorbate, or benzoate anions (Holyoak et al., 1999; Piper et al., 1998). On the other hand, Pdr12p was found to play no detectable role in the active expulsion of counterions of more lipophilic acids such as octanoic acid, decanoic acid, artesunic acid and 2,4-Dichlorophenoxyacetic acid (2, 4-D) (Alenquer et al., 2006; Piper et al., 1998; Teixeira and Sa-Correia, 2002), or of the more hydrophilic acetate anion (Piper et al., 1998). So Pdr12p is an ABC transporter that provides resistance to those carboxylate compounds that can, to a reasonable extent, partition into both lipid and aqueous phases. Pdr12p exports anions by binding to acid anion incorporated within inner leaflet of plasma membrane, and then acts as a flippase to transport them to periplasmic side of membrane. Once on periplasmic side, the anions are readily protonated in acidic environment and released into external media (Mollapour et al., 2008). A close homolog of Pdr12p, Pdr5p is involved in expulsion of anions of highly lipophilic weak acids such as 2, 4-D, artesunic acid and artemisinic acid (Alenquer et al., 2006; Teixeira and Sa-Correia, 2002). A number of genes encoding drug:H^+ antiporters belonging to the major facilitator superfamily (MFS) have also been found to contribute to yeast tolerance to different weak acids: *AQR1* and *AZR1* genes are required for maximal tolerance to acetic and propionic acids (Tenreiro et al., 2002; Tenreiro et al., 2000). *TPO1* gene was found to be a major determinant of resistance to 2,4-D, artesunic and mycophenolic acids (Alenquer et al., 2006); whereas *TPO1* close homologues *TPO2* and *TPO3*, were found to provide protection against acetic, propionic, and benzoic acids (Fernandes et al., 2005). The role of MFS–MDR transporters in reducing the intracellular concentration of different weak acid counterions might also result from the transport of endogenous metabolite(s) that may affect the partition of the weak acid between the exterior and the intracellular environment, through an alteration of intracellular pH and/or plasma membrane potential (Sa-Correia et al., 2009).

1.5.4 Alteration in cell wall and plasma membrane structure:

Cell wall is major barrier that protects cell from rapidly changing external environment. Cell wall avoids the bursting of cells due to outer osmotic changes and maintains an intracellular water activity that is appropriate for biochemical reactions. Cell wall has a dynamic organisation, which changes for different cellular and physiological requirements and on induction of stress. Yeast cell wall is layered structure, with inner layer composed of glucan polymers and chitin and outer layer of highly glycosylated mannoproteins (Levin, 2005). Cell wall assembly is regulated by cell surface sensors coupled with cascades of signalling molecules which activates a set of effector molecules in response to external conditions (Heinisch et al., 1999). On encountering inhibiting concentration of weak acids, yeast cells activate cell wall integrity pathway and so modulate the cell wall architecture to reduce its porosity and entry of undissociated acid into the cell. Exposure to lipophilic weak acids like benzoic acid and 2,4-D results in increased expression of glycosylphosphatidylinositol cell wall protein (GPI-CWP) Spi1p resulting into cell wall remodelling (Abbott et al., 2008; Schuller et al., 2004). The Spi1p mediated cell wall remodelling reduces the porosity of cell wall and reduced plasma membrane damage (Kapteyn et al., 2001; Simoes et al., 2006). At low pH, when glucose repressed *S. cerevisiae* cells are challenged with high concentration of acetic acid, acid floods into the cells through Fps1p aquaglyceroporin an open glycerol channel of plasma membrane. This transiently activates Hog1p MAP kinase, which in turn phosphorylates the Fps1p at two 12 amino acid regions in the cytosolic surface of membrane that are implicated in turgor mediated channel closure. This phophorylation is signal for Fps1p to become ubiquitinated and then endocytosed to the vacuole (Mollapour and Piper, 2007). Exposure to lipophilic acid may also result in altered lipid composition; an increase in the ratio of saturated membrane fatty acids over unsaturated one was reported (Viegas et al., 2005).

1.5.5 Weak acids induced metabolic changes:

Low concentration of acetate and propionate can be utilized by yeast via acetyl-CoA and propionyl CoA pathways of central carbon metabolism. Moreover, low concentration of acetic acid is known to enhance the yield of ethanol during fermentation (Pampulha and Loureirodias, 1990). However in the presence of fermentable sugars like glucose, *S. cerevisiae* cannot use acetate as carbon source and yeast cells as such cannot metabolize sorbate or benzoate (Mollapour et al., 2008). Transcriptomic and proteomic analyses have

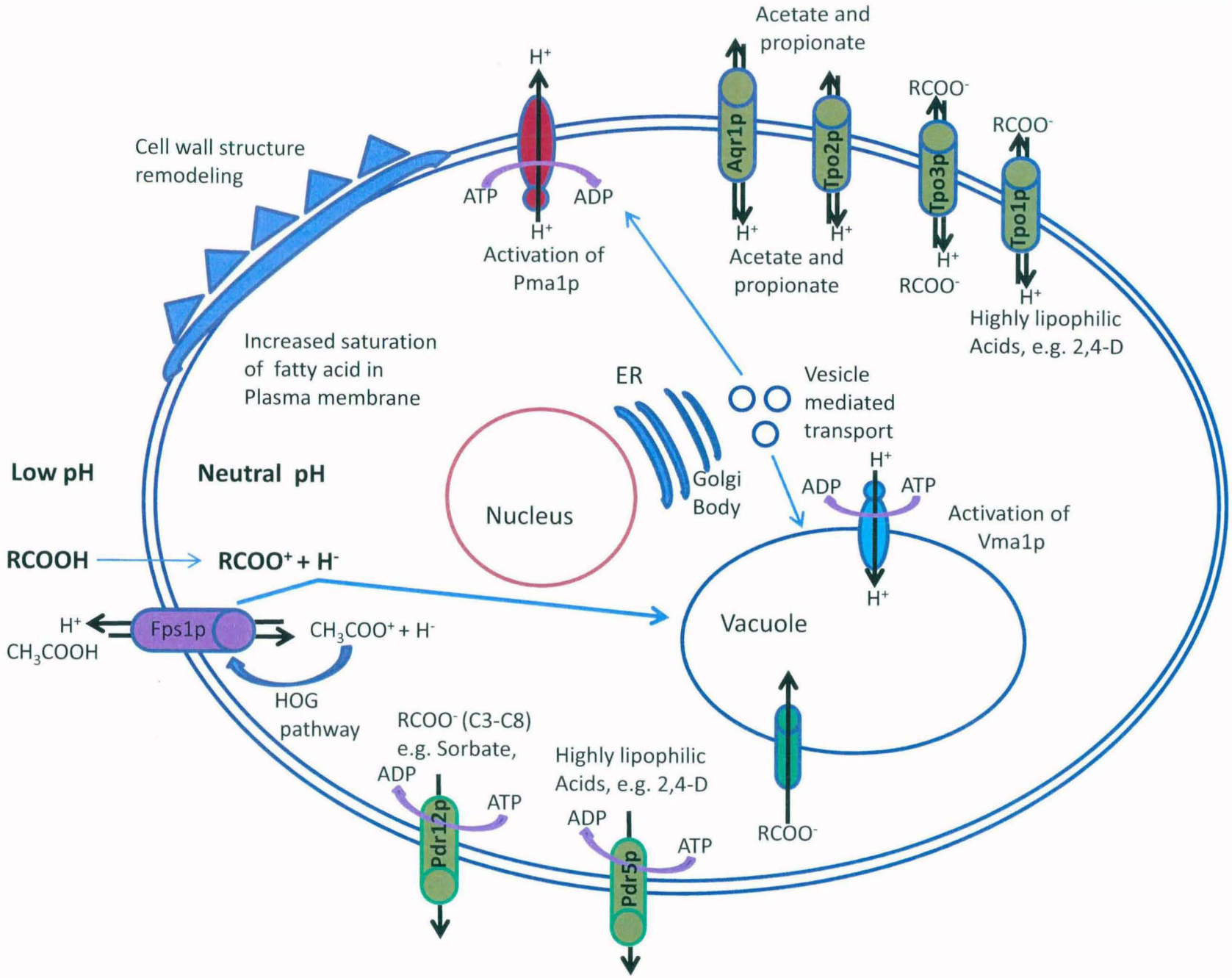


Figure 1.4: Mechanistic model for the adaptive yeast response to weak acid induced stress (Modified from Mira et al., 2010c).

shown that exposure to acetic acid results in activation of the TOR (Target of Rapamycin) pathway, which in turn regulates the yeast response to nutrient starvation. Presence of weak acids results in upregulation of genes encoding proteins involved in amino acid uptake and biosynthesis. Weak acid stressed yeast cells also show increased expression of genes encoding enzymes involved in glycolysis and krebs cycle (Almeida et al., 2009; Mira et al., 2009; Mira et al., 2010b; Mira et al., 2010c). Such responses are proposed to compensate for increased energy consumption (Holyoak et al., 1996) and inhibition of glycolytic enzymes (Pampulha and Loureirodias, 1990).

1.6 Regulation of weak acid tolerance:

The weak acid response in yeast cells result in activation of large number of genes, which make yeast cells resistant to one or few weak acids. Studies show that different weak acids induce different set of genes; however, they may be involved in similar biological processes, such as protein folding, lipid metabolism, cell wall function and multidrug resistance (MDR). In case of MDR protein encoding genes, expression of *TPO3,* a polyamine transporter is induced by all weak acids studied so far, whereas more lipophilic sorbic acid and others specifically induce expression of *PDR5, YOR1, PDR12* and *SNQ1,* and more hydrophilic acetic and propionic acid activate *TPO2* (Abbott et al., 2007; Mira et al., 2010c). Therefore, adaptation to different weak acids requires expression of mostly different set of genes and possibly activated by different regulatory pathways. So far four regulatory pathways, dependent on the transcription factors Haa1p, Rim101p, Msn2p/Msn4p and War1p have been identified in mediating yeast response to weak acid stress (Mira et al., 2010a).

1.6.1 The Msn2p/Msn4p regulon:

Msn2p and Msn4p are two homologous transcription factors involved in yeast response to environmental stress including adaptation to weak acid stress (Gasch et al., 2000). Msn2p/Msn4p complex mediates the induction of genes with STRE elements during acid stress response (MartinezPastor et al., 1996). Most of the genes upregulated by Msn2p and Msn4p in response to weak acids stress encode proteins of the environmental stress response such as molecular chaperones (Hsp26p, Sse2p), enzymes of the carbohydrate metabolism (Hxk1p, Gpd1p), and of the antioxidant defence system (Ctt1p, Gpx1p) (Schuller et al., 2004; Simoes et al., 2006). The induction of Msn2p/Msn4-regulon in response to multiple weak acids provides the cells necessary flexibility to respond to a wide range of challenges,

although the physiological response brought by this activation does not always result in an increased resistance to the weak acid (Mira et al., 2010c).

1.6.2 The Haa1p regulon:

The Haa1p was initially identified as homologous to copper (Cu^{+}) requiring transcription factor Ace1p (Keller et al., 2001). Haa1p is involved in yeast adaptation to hydrophilic weak acids such as acetic acid and propionic acid, and its contribution decreases with increase in lipophilicity of weak acid. Haa1p regulates the expression of genes encoding MDR transporters and other plasma membrane and cell wall associated proteins, such as *TPO2, TPO3, YGP1, PHM8,* and *YLR297w.* However activation of only Tpo2p, Tpo3p and Ygp1p was found essential for resistance to acetic acid, whereas other Haa1p regulated genes play marginal or no role in adaptation to acetic acid (Fernandes et al., 2005). Based on microarray studies, Haa1p is recently shown to be involved in the expression of *SAP30* (Rpd3L histone deacetylase) and *HRK1* (a protein kinase involved in phosphorylation of plasma membrane transporters) genes; deletion of these genes severely decreases the ability of yeast cells to adapt to acetic acid (Mira et al., 2010a).

1.6.3 The War1p regulon:

The War1p is a transcriptional regulator involved in expression of ATP binding cassette (ABC) transporter Pdr12p, which effluxes weak acids such as sorbic acid and benzoic acid. The War1p belongs to the family of Zn_2Cys_6 zinc finger regulators; it recognizes a cis acting weak acid response element (WARE) in the *PDR12* promoter (Kren et al., 2003). Deletion of *WAR1* leads to hyper sensitivity to moderately lipophilic weak acids such as propionic acid, sorbic acid and benzoic acid, but not to hydrophilic acetic acid (Schuller et al., 2004). War1p activity is controlled by direct binding of weak acid counterions, increasing the affinity of this transcription factor for the promoter region of its target genes. In particular, the ability of sorbate anion to induce a conformational change in War1p structure has been demonstrated to activate *PDR12* expression (Gregori et al., 2008).

1.6.4 The Rim101p pathway:

Rim101p transcription factor has been implicated in adaptation of yeast cells to alkaline pH and in cell wall remodelling (Castrejon et al., 2006; Lamb and Mitchell, 2003). Recently it was shown to be required for resistance to weak acid stress at pH 4.0 and its contribution

decreases with weak acid lipophilicity. Rim101p does not protect against low pH as such, when a strong acid is used as the acidulant. Based on the comparison of the transcriptomes of wild-type and *rim101Δ* cells after weak acid shock, Rim101p was found to induce the expression of known Rim101p regulon. On acid shock Rim101p also induces the expression of new target genes, in particular *KNH1*, involved in cellwall β-1, 6-glucan synthesis and the uncharacterized gene *YIL029c*, both of which are required for maximal propionic acid resistance in yeast. Clustering of the genes that provide resistance to propionic acid show the enrichment of genes involved in cell wall function, protein catabolism through the multivesicular body pathway, homeostasis of internal pH and vacuolar function (Mira et al., 2009).

1.6.5 The other weak acid induced regulons:

Besides transcription factors that are solely involved in adaptation to weak acids, there are certain other transcription factors having important role in adaptation to weak acid stress. Pdr1p and Pdr3p transcription factors controlling multidrug resistance in yeast (Gulshan and Moye-Rowley, 2007), are involved in adaptation to lipophilic acids such as 2,4-D and artesunic acid (Ro et al., 2008), by upregulating the expression of Pdr5p and Tpo1p (Alenquer et al., 2006). Based on clustering of genes induced upon weak acid stress with respect to their documented transcriptional regulators using YEASTRACT database (Teixeira et al., 2006), the Yap1p, Sfp1p and Sok2p are also found to be involved in general weak acid transcriptional response. Yap1p is known to play a key role in oxidative stress in yeast; Sfp1p regulates the expression of ribosomal genes in response to nutrient starvation and Sok2p is involved in general stress response in yeast cells. Thus activation of these transcription activators may not be a direct effect of weak acid stress, but a consequence of weak acid induced stress (Mira et al., 2010a).

1.6.6 Genes involved in acetic acid adaptation in yeast:

Genes transcriptionally activated upon acetic acid stress belongs to diverse functional classes like stress response, signal transduction, regulation of carbohydrate metabolism and drug transport, whereas repressed genes belongs to amino acid catabolism and ion transport (Mira et al., 2010b). The *S. cerevisiae's* transcriptional response to acetic acid stress mainly involves expression of Haa1p regulated genes. Since role of Haa1p in acetic acid tolerance is

discussed in separate section, here other aspects of cellular response to acetic acid stress are discussed.

A genome wide screening of EUROSCARF haploid collection for susceptibility phenotype to acetic acid (70-110 mM acetic acid and pH 4.5 in minimal media) by Mira et al., 2010, has identified 650 determinants of tolerance to acetic acid. Clustering of these acetic acid-resistance genes based on their biological function indicated the enrichment of genes involved in transcription, internal pH homeostasis, carbohydrate metabolism, cell wall assembly, biogenesis of mitochondria, ribosome and vacuole, and in the sensing, signaling and uptake of various nutrients, in particular iron, potassium, glucose and amino acids. Ion and proton transport play important role in maintenance of pH homeostasis in weak acid stress, thus genes of this functional class such as *PMP1, VMA8,* and *VPH2* are critical. Other genes in this class enriched were, those involved in uptake of potassium (*TRK1*), ammonium (*MEP3*), phosphate (*PHO88*) and iron (*FET3* and *FRE3*). Deletion of genes encoding protein involved in glycolysis (*HXK2, PFK1* etc), Krebs cycle (*FUM1, PYC1* etc), pentose phosphate pathway (*ZWF1* and *RPE1*), components of respiratory chain (*ATP1, ATP4* and *COX9* etc) and proteins of mitochondrial ribosomes sensitized yeast cells to acetic acid. Deletion of genes involved in cell wall biosynthesis and remodeling (*FKS1, KRE1, CHS1, GAS1* etc) also makes yeast cell sensitive to acetic acid. A number of genes related to sensing, signaling and uptake of amino acids were also identified as determinants of acetic acid tolerance (*GTR1, SLM1, STP1, CYS3, MET4* etc.), which is consistent with the observation that acetic acid caused general amino acid limitation. This screen also uncovered 28 transcription factors required for resistance to acetic acid including *HAA1, RIM101* and *MSN2* whose role in response to acetic acid stress is known (Mira et al., 2010b).

A proteomic approach to study role of acetic acid in apoptosis of yeast by Almeida et al, 2009 has revealed number of proteins whose expression is altered in the presence of acetic acid. Upon acetic acid induced apoptosis 53 spots were affected in 2D electrophoresis, with 41 spots showing decrease and 12 spots with increased intensity and subsequent MS analyses of these spots correspond to 28 proteins. These results indicate involvement of several cellular processes in acetic acid induced apoptotic condition. Upon acetic acid treatment chaperones of Hsp70 family (Ssa1p and Ssa2p etc.) decreased in intensity whereas intensity of Hsp90 family chaperone (Hsc82p) increased. Three proteins involved in nucleotide biosynthesis were affected, Ade6p involved in purine nucleotide biosynthesis increased in spot intensity

whereas intensity of spots corresponding to Rnr2p and Rnr4p proteins that supply DNA precursor for replication and repair decreased. These results indicate that upon acetic acid induced apoptosis, nucleotide biosynthesis is downregulated. Among proteins involved in carbohydrate metabolism, Pfk2p, Fba1p and Pdc3p decreased in spot intensity, further validating the inhibitory effect of acetic acid on carbohydrate metabolism. Acetic acid stress also results in amino acid starvation in yeast, as proteins involved in amino acid biosynthesis (Leu1p, Ilv3p and Thr4p), lysyl-tRNA synthetase Ksr1p and ubiquitin activating enzyme Uba1p showed decreased expression upon acetic acid treatment. However, intensity for a fragment of beta subunit of phenyl-alanyl-tRNA synthatase (Frs1p) increased. These results show that acetic acid treatment results in amino acid starvation reduced processivity of tRNAs, fragmentation of proteins and decreased degradation of amino acid permeases. The transcriptional repressor Wtm1p involved in regulation of meiosis and silencing showed increased expression; whereas expression of proteins involved in translation initiation (Tif1p/Tif2p) and elongation (Eft1/2p, Tef1/2p and Yef1p) is decreased (Almeida et al., 2009).

These studies indicate that acetic acid affects diverse cellular processes in yeast. On the one hand acetic acid inhibits many life governing processes, such as carbohydrate metabolism, amino acid biosynthesis and transport and nucleotide biosynthesis. On the other hand it activates many life saving processes, such as cell wall remodeling, ion transport and pH homeostasis, inhibition of growth and increased recycling of cellular resources.

1.6.7 Genes involved in adaptation to other weak acids in yeast:

Schuller et al., (2004) have used a two pronged approach to identify the genes involved in resistance to sorbic acid, by simultaneously screening for sensitivity of yeast deletion mutants to weak acids and by global transcriptome analysis upon exposure to sorbic acid in yeast. Global transcriptome analysis showed differential expression of 100 genes upon induction with sorbic acid, which were further classified based on transcription factors involved in their regulation. War1p regulates expression of at least four genes including *PDR12* which is most important determinant of resistance to sorbic acid. Msn2/4p regulates expression of 35 genes (*SPI1, HSP26, CTT1, HXT7, PDR15* etc.) and the expression of 13 genes is coregulated by War1p and Msn2/4p (*GPD1, GLK1, TPS1, LSM4* etc.), 21 other genes are regulated by neither War1p nor Msn2/4p (*HSP30, YGP1, TPO2* and *TPO3* etc.) (Schuller et al.., 2004).

Expression of *YGP1, TPO2* and *TPO3,* which were initially categorized as War1p and Msn2/4p independent were further shown to be regulated by Haa1p transcriptional activator (Fernandes et al., 2005). Moreover mutants identified for sorbic acid sensitivity belong to diverse cellular functions, including energy metabolism (*PFK1, ADH1* etc), mitochondrial function, ergosterol biosynthesis (*ERG2, 3, 6* and *28*), aromatic amino acid biosynthesis (*TRP1, 2, 5* and *ARO2*) and transcription and translation (*SWI3, SSN6, RPB9* etc) (Schuller et al., 2004).

In a study by Mira et al (2009), the deletion of 254 genes made yeast cells sensitive to propionic acid. These genes were further clustered into following functional classes: intracellular trafficking (*VPS16, PEP5* etc), protein catabolism through the multivesicular body system (*VPS28, STP22, SNF8* etc), generation of ATP and energy, ergosterol biosynthesis, posttranslational modification and Rim101 proteolytic processing (*RIM8, RIM13* etc) and pH homeostasis (*VMA1, TFT2, VPH2* etc). Deletion of genes encoding Hog1 kinase (HOG signaling pathway) and Slt2 kinase (Cell wall integrity pathway) also leads to propionic acid sensitivity. Furthermore, gene expression analysis has shown that Rim101p plays essential role in resistance to propionic acid. Upon activation with propionic acid Rim101p regulates expression of 22 new genes which were previously not reported to be Rim101p dependent. The Rim101p dependent genes encode proteins involved in cell wall metabolism, iron homeostasis, transcription factors and few proteins of unknown function (Mira et al., 2009). Immediate adaptation to propionic acid also requires expression of Haa1p regulated genes (Fernandes et al., 2005).

1.7 Haa1p, a principle regulator of yeast adaptation to acetic acid:

1.7.1 Haa1p is yeast transcription factor:

Haa1p was discovered as transcription factor homologous to gene encoding the copper activated transcription factor Ace1p (Keller et al., 2001). It was further implicated in rapid adaptation of yeast cells to hydrophilic weak acids, such as acetic acid and propionic acid (Fernandes et al., 2005). Ace1p is a transcription factor with an N-terminal DNA biding domain (DBD) and C-terminal transactivation domain. The DNA binding domain of Ace1p consists of two sub domains, a domain with 40 residues stabilized by one Zn (II) ion and a 70 residues copper regulatory domain (CuRD) that binds four Cu (I) ions through eight cysteinyl thiolates forming a polycopper cluster (Graden et al., 1996). DNA binding by Ace1p requires

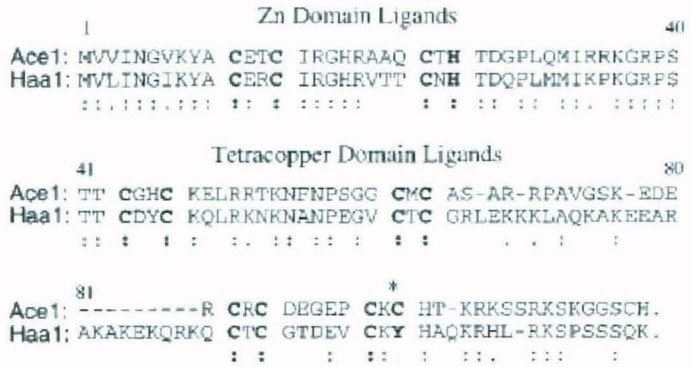
both the Zn and copper regulatory domains for major groove and minor groove DNA interactions (Dobi et al., 1995). The Haa1p transcription factor has a region homologous to both the N-terminal Zn module and copper regulatory domain of Ace1p, but it lacks any similarity to Ace1p beyond DBD (Figure-1.5 (a)). Haa1p-GFP fusion is shown to localize to nucleus (Figure-1.5 (b)), when expressed from under GAL1 promoter upon galactose induction. Haa1p target genes were shown to code for plasma membrane proteins, MDR proteins and cell wall associated proteins, mostly uncharacterized till then. Expression of Haa1p target genes was found to be independent of the copper status of cells and absence of copper metalloregulation is not associated with transactivation domain of Haa1p. Nevertheless striking similarity between the CuRD of Ace1p and Haa1p suggests that, possibly polycopper thiolate cluster may stabilize the DNA binding conformer of Haa1p as it do for Ace1p DBD (Keller et al., 2001).

1.7.2 Role in adaptation to weak acids:

Fernandes et al. (2005) have shown that Haa1p is involved in yeast response to weak acid preservatives, especially in adaptation to hydrophilic weak acids. Haa1p mediated resistance to weak acids decreases steeply with the increase of the liposolubility of the weak acids, being maximal for acetic acid and negligible for octanoic acid. Exposure to weak acids is known to result in decreased viability and extended lag phase of yeast cells. Deletion of *HAA1* further decreases the viability and increases lag phase duration. Furthermore it was shown that yeast cells upon exposure to propionic acid or octanoic acid upregulate 9 out of 10 Haa1p regulated genes described by Keller et al (2001). Among these genes, the rapid activation of *TPO2, YGP1, YIR035c, YLR297w, YPR157w, YER130c* and *YRO2* is essentially dependent on Haa1p, whereas expression of *PHM8* and *TPO3* is partially dependent on the Haa1p activation and *GRE1* is not expressed in response weak acids. Among Haa1p regulated genes *YGP1, TPO2* and *TPO3* and less significantly *YLR297w, YER130c* and *YRO2* are involved in yeast adaptation to acetic acid and propionic acid (Fernandes et al., 2005).

TPO2 and *TPO3* encode highly homologous putative multidrug transporter belonging to major facilitator superfamily (MFS). These putative drug:H^+ antiporters exhibit 12 predicted membrane spanning segments and are required for polyamine transport to alleviate polyamine toxicity (Tomitori et al., 2001; Tomitori et al., 1999). Deletion of *TPO2* and *TPO3* results in increased growth latency of yeast cells exposed to weak acids, though changes are less drastic as compared to *HAA1* deletion. Moreover, the *tpo3Δ* and *haa1Δ* strains show

(a)



(b)

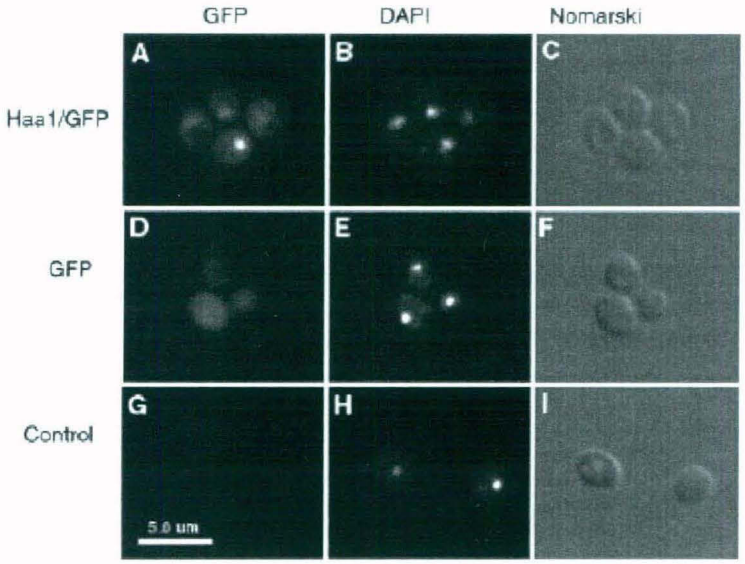


Figure 1.5: (a) Sequence comparison of the N-terminal segment of Haa1p and the DNA binding domains of Ace1p and **(b) Nuclear localization of Haa1p**. A GFP tagged Haa1p expressed from GAl1 promoter on galactose induction localizes to nucleus (Keller et al., 2001).

increased accumulation of radiolablled (1-^{14}C) acetic acid in cytosol compared to wildtype. These results indicate that Tpo2p and Tpo3p are possibly involved in active export of the counter-ion of weak acids towards which they provide protection (Figure-1.7). *YGP1* encodes highly glycosylated cell wall protein, which is induced in response to environmental stresses, like nutrient starvation, low external pH and hyperosmomic stress. In Fernandes et al (2005), studies the protective effect of Ygp1p was more evident for the lipophilic octanoic acid and benzoic acid, while negligible for acetic acid and propionic acid. As Ygp1p is involved in reducing cell wall permeability, thereby more protective towards lipophilic acids causing plasma membrane damage (Fernandes et al., 2005). Recent transcriptomic analysis of Haa1p regulated genes upon acetic acid treatment has shown that expression of *SAP30* and *HRK1* are essential for protection to acetic acid. *SAP30* encodes a subunit of a histone deacetylase complex and *HRK1* encode a protein kinase belonging to a family of protein kinases dedicated to the regulation of activity of plasma membrane transporters. Deletion of *HRK1* leads to increased accumulation of acetic acid into acetic acid stressed cells, so Hrk1p is proposed to be involved in activation of plasma membrane transporter by phosphorylation, to efflux acetate ions to reduce intracellular concentration. Sap30p is proposed to modulate the yeast transcriptional response to acetic acid by affecting the activity of transcription factor involved in this response (Figure-1.6) (Mira et al., 2010a).

Haa1p is also implicated in yeast transcriptional response to acetaldehyde stress. Transcriptional profiling of yeast cells after acletaldehyde shock has shown induction of *YRO2*, *TPO2, TPO3* and *TPO4*. The expression *YRO2*, *TPO2* and *TPO3* requires presence of functional Haa1p, since in *haa1Δ* cells expression of these genes is repressed (Aranda and del Olmo, 2004). The role of Haa1p in acetaldehyde tolerance in yeast should be indirect, as yeast cells have ability to metabolize acetaldehyde into acetic acid and activation of Haa1p may result from subsequent accumulation of acetic acid inside the yeast cells. A recent report suggests that Haa1p acts downstream of Yak1p to regulate acetic acid resistance and *FLO11* mediated adhesion in yeast (Figure 1.7). The Yak1p is a member of the family of dual-specificity tyrosine kinases and appears to be part of nutrient responsive signaling pathway that acts in parallel to cAMP-PKA pathway, in antagonistic manner (Garrett and Broach, 1989). Glucose starvation and rapamycin induced inhibition of TOR pathway results in nuclear localization of Yak1p (Moriya et al., 2001). Yak1p effects the expression of few known Haa1p regulated genes; these are *GRE1, PHM8, TPO2, YGP1* and *YRO1* and deletion of *YAK1* results into acetic acid sensitivity in yeast which can be suppressed by

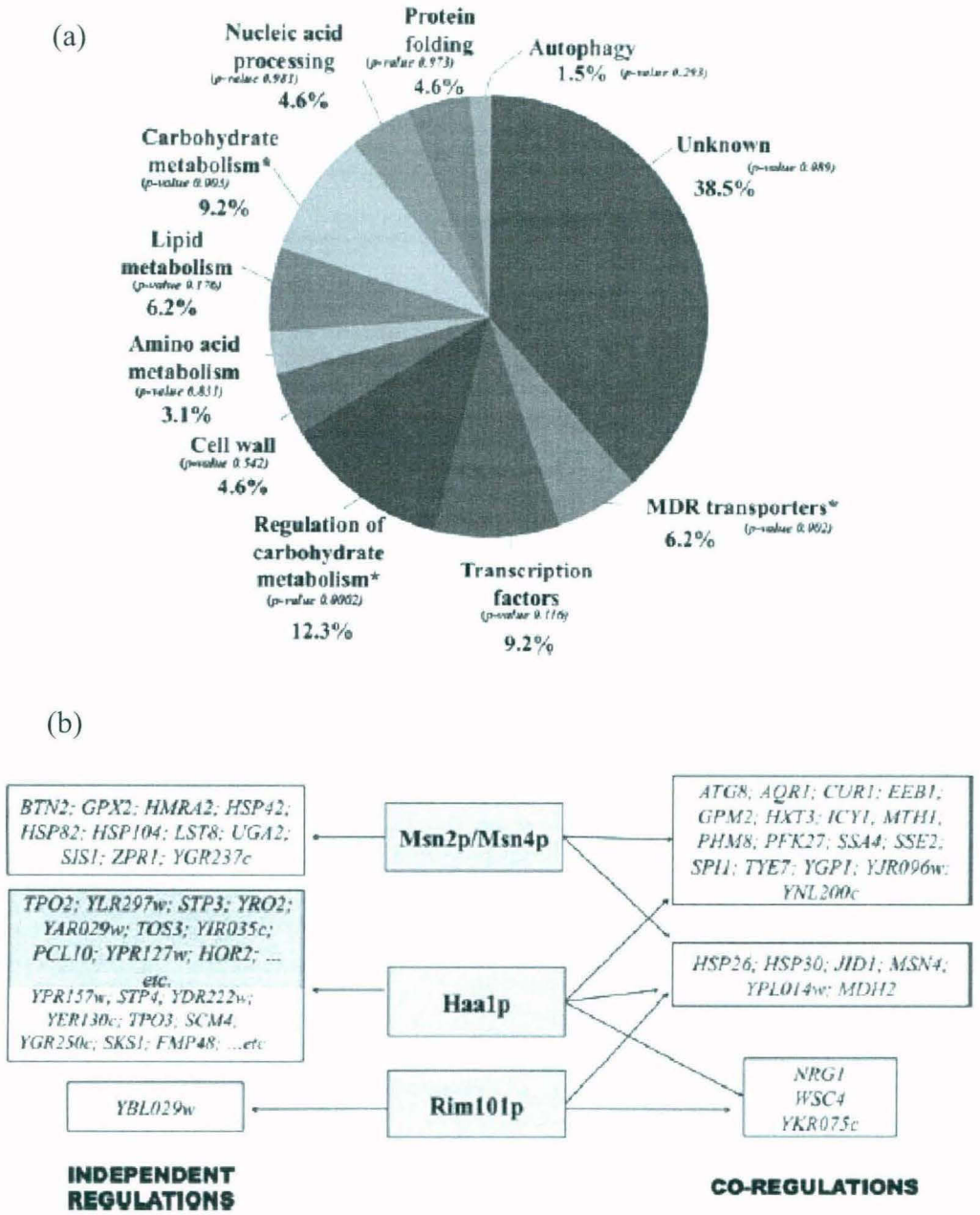


Figure 1.6: Haa1p mediated transcriptional response to acetic acid in yeast (Mira et al., 2010). (a) Clustering based on biological function, of genes activated in response to acetic acid stress in a Haa1p dependent manner and (b) schematic diagram showing the crosstalk between the regulatory systems controlled by the weak acid responsive transcription factors Haa1p, Rim101p, and Msn2p/Msn4p in yeast response to acetic acid.

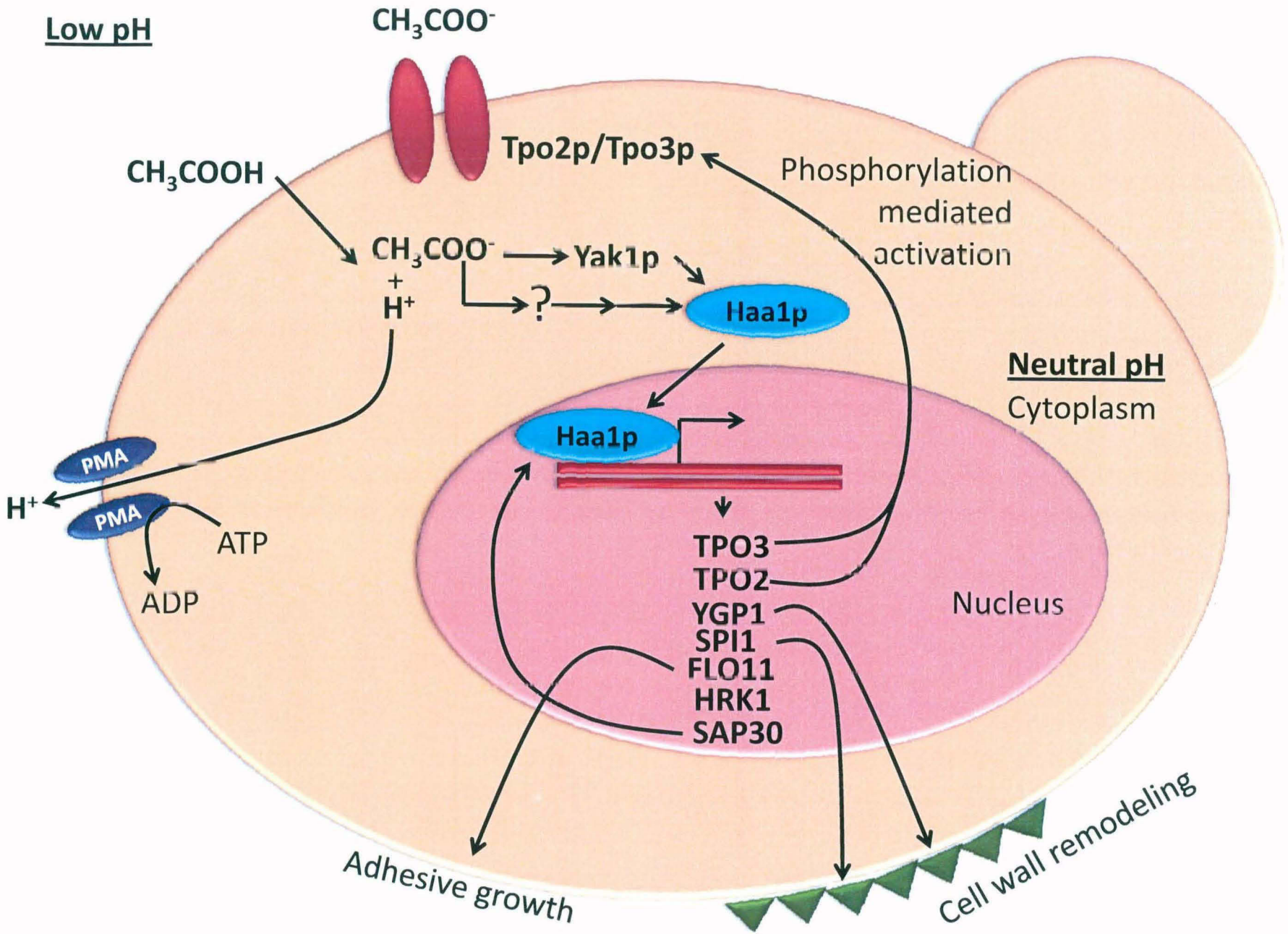


Figure 1.7: A schematic model for molecular mechanism Haa1p mediated acetic acid tolerance in yeast *S. cerevisiae*.

overexpression of *HAA1*. Moreover upon acetic acid stress, Haa1p activates expression of *FLO11* and possibly Flo11p mediated adhesion in Yak1p dependent manner (Malcher et al., 2011).

1.7.3 Haa1p mediated transcriptional response:

The transcriptional analysis of yeast cells upon acetic acid treatment to wildtype strain *BY4741* and *haa1Δ* strain by Mira et al (2010), have identified several novel target genes regulated by Haa1p that were not identified in earlier studies by Keller et al (2001) and Fernandes et al (2005). Genes transcritionally activated by acetic acid treatment belong to diverse functional classes, including stress response, signal transduction, regulation of carbohydrate metabolism and drug transport, whereas functional classes of genes downregulated include amino acid catabolism and ion transport. Activation of Haa1p is required for at least 80% (85 out of 112) of genes upregulated upon acetic acid shock. All genes previously reported to be regulated by Haa1p, like *TPO2, TPO3, YGP1* and *PHM8* etc. have reduced expression in acetic acid challenged *haa1Δ* cells, whereas 73 genes were identified as novel to Haa1p mediated acetic acid response in yeast. These novel set of Haa1p target genes regulated in response to acetic acid belongs to following functional classes: environmental stress response (*HOR2, SSE2* etc), carbohydrate metabolism (*NRG1, HRK1, TOS3, PCL10* etc), nucleic acid processing (*SYC1, SAP30* etc), lipid metabolism (*INM1, YPC1, SUR2* etc), cell wall and plasma membrane (*SPI1, PDR12, AQR1* etc), transcription factors (*MSN4, STP3/4, FKH2* etc), amino acid metabolism and at least 40% with unknown function (figure 1.6 (a)). Moreover YEASTRACT database analysis to classify acetic acid induced genes based on documented weak acid induced regulons, has shown that Haa1p specifically regulates almost 45% (51 out of 112 genes) of the acetic acid induced genes, although considerable overlap exists with Msn2/4p (23 genes), Rim101p (3 genes), War1p (1 gene) regulon/s and 6 genes appeared to be controlled by Haa1p, Msn2/4p and Rim101p regulons (Figure 1.6 (b)) (Mira et al., 2010a).

1.8 Stationary phase mediated acetic acid resistance in yeast:

1.8.1 Introduction:

Eukaryotic cell proliferation is controlled by specific growth factors and availability of essential nutrients. If either of these signals is absent, cells may enter a specialized non dividing resting state, known as stationary phase or G_0. The yeast *S. cerevisiae* utilizes

fermentable sugars such as glucose as preferable carbon source. When yeast cells are grown in liquid media containing glucose, they metabolize glucose by glycolysis and release ethanol in media. When glucose becomes limited, the yeast cells enter a diauxic shift characterized by decrease in growth rate and metabolic shift from glycolysis to aerobic utilization of ethanol. Once ethanol is depleted from media and no other carbon source is available, yeast cells enter quiescent or stationary phase G_0 (Figure-1.8). Similar manifestation of growth arrest is also seen when yeast cells are starved for nitrogen or phosphate (Zhang et al., 2009). Yeasts cells in diauxic shift or stationary phase are stressed by lack of nutrients and by accumulation of toxic metabolites from oxidative metabolism. Such cells are differentiated in such a way that cell viability is maintained under in nutrient limiting conditions. These cellular responses to stress are very much similar to cells undergoing stress response, such as induction of heat shock proteins and accumulation of trehalose (Gray et al., 2004).

1.8.2 Cellular manifestation:

The yeast cells in stationary phase are undividing, unbudded and contain unreplicated nuclear DNA. In such cells rate of transcription is reduced by 3-5 fold, whereas translation decreases to 0.3% of proliferating cells (Galdieri et al., 2010). Stationary phase cells exhibit thick and more rigid cell wall structure and increased resistance to cell wall perturbing agents (de Nobel et al., 2000). On approaching stationary phase, yeast cells accumulate storage sugars like glycogen, trahalose, triacylglycerol and polyphosphates. Accumulation of glycogen begins before depletion of glucose and peaks at diauxic shift; it is further utilized as storage polysachharide, whereas accumulated trehalose protects cells against stress. Entry into stationary phase also results in condensed chromosome structure, more abundant mitochondria and induction of autophagy (Gray et al., 2004). Stationary phase cells are also more resistant to heat shock, osmotic shock and toxic drugs and are able to induce transcriptional response to different stresses as exponentially growing cells (Galdieri et al., 2010). Most importantly stationary phase cells have ability to respond to nutrient availability and reenter the mitotic cycle of cell division (Martinez et al., 2004).

1.8.3 Regulation of entry into stationary phase:

Entry into stationary phase is highly regulated process and involves protein kinase A (PKA), TOR, Snf1p and Rim15p signaling pathways that signal availability of nutrients (Figure-1.9). PKA pathway plays inhibitory role in transition from exponential growth to diauxic shift and stationary phase. Cells with increased PKA activity fail to enter stationary phase and die early

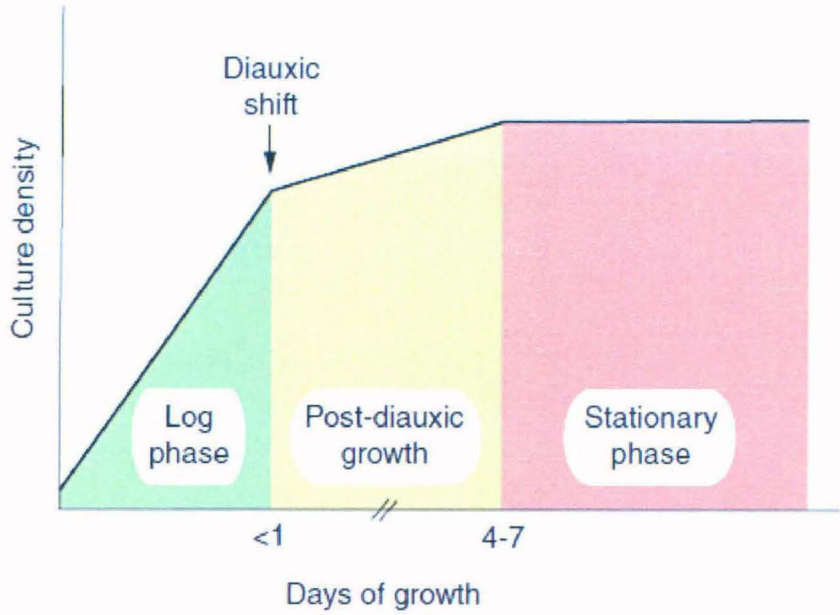


Figure 1.8: Growth phase exhibited by *S. cerevisiae* on glucose based media (Herman et al., 2002).

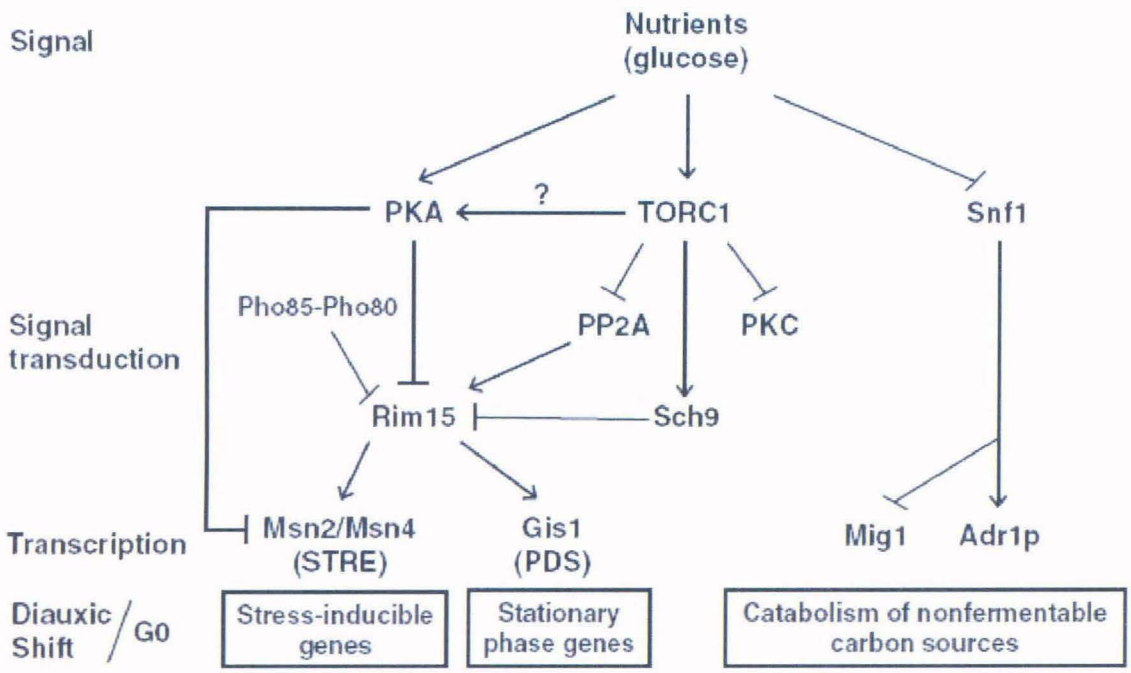


Figure1.9: Overview of signaling pathways that control transcription during transition from exponential growth to diauxic shift and stationary phase in yeast (Galdieri et al., 2010).

on nutrient limitation, whereas cells with decreased PKA activity shows stationary phase like features even when glucose is abundant (Zaman et al., 2008). In yeast, PKA signaling pathway is activated by Ras proteins (Ras1/2p) and it further inhibits Msn2p transcription factor and Rim15p protein kinase (Gorner et al., 2002; Reinders et al., 1998). TOR (target of rapamycin) pathway also inhibits transition into stationary phase. In yeast, Tor1p or Tor2p and three other proteins comprise TOR1C complex which responds to growth conditions and availability of nutrients. Inhibition of TOR1C by rapamycin or nitrogen starvation results in decreased protein synthesis, induction of autophagy and entry into G_0 state (Shamji et al., 2000). Inhibition of TOR1C results in activation of genes responsive to limitation of nitrogen and carbon by regulation of Gln3p and Gat1p transcription factors. On the other hand inhibition of TOR1C induces Sch9p (homolog of mammalian PKB/Akt kinase) dephosphorylation and inactivation, which in turn triggers nuclear localization of Rim15p that regulates entry into stationary phase (Swinnen et al., 2006). Snf1p and Rim15p dependent signaling pathways positively regulate entry into stationary phase. The Snf1p complex has Snf4p as regulatory subunit and Snf1p as catalytic subunit, in presence of glucose Snf4p binds Snf1p and inactivates its function. In absence of glucose Snf1p is released from Snf4p and results into activation of transcription via Mig1p and Adr1p. Mig1p is involved in transcriptional repression in presence of glucose and Snf1p mediated phosphorylation of Mig1p releases this repression. Adr1p is transcription factor involved in activation of genes involved in catabolism of nonfermentable carbon sources. Rim15p also positively regulates the entry into stationary phase and functional Rim15p is required for smooth transition from exponential to stationary phase of growth. Rim15p activates transcription factors Msn2p, Msn4p and Gis1p, which in turn regulates the expression of genes required for entry and survival into stationary phase (Galdieri et al., 2010).

1.8.4 Acetic acid tolerance:

Yeast cells on entering the stationary phase show resistance to many stress conditions, such as oxidative stress and heat shock. Recently Burtner et al (2009) have shown acetic acid acts as a mediator of cell death during stationary phase of growth. When yeast cells growing in minimal media (SC media) enter stationary phase, pH of media decreases to acidic range. Reduction of pH is further shown to result from release of acetic acid, malic acid, citric acid, pyruvic acid and oxalic acid in media from yeast cells. Among these acids accumulation of acetic acid at low pH causes reduced cell viability and chronological life span (CLS) of yeast

cells in stationary phase. When yeast cells are grown in media with dietary restriction (reduced glucose content, from 2% to 0.5% or 0.05%) reduction in pH by accumulation of acetic acid is not observed, thus yeast cells in stationary phase have increased viability and CLS. This increase in viability and CLS is also observed when yeast cells are grown in media with 2% glucose with some osmotic stabilizer, such as sorbitol or NaCl, though acidification of media occurs. These results indicate that viability of yeast cells in stationary phase can be increased by either reducing the accumulation of acetic acid or providing yeast cells resistance to oxidative stress caused by acetic acid. Furthermore, deletion of *SCH9* and *RAS2*, known to be involved in of progression of stationary phase, results in increased resistance to acetic acid. Resistance of *sch9Δ* to acetic acid depends on presence of Rim15p and Gis1p, which are known to be involved in yeast cell's entry and survival in stationary phase (Burtner et al., 2009). These results indicate that resistance to acetic acid in yeast cells is fundamental to survival in stationary phase. Therefore when yeast cells enter the stationary phase it activates several stress responsive mechanisms that result into resistance to acetic acid.

Chapter 2
Materials and Methods

2.1 Materials:

All chemicals and reagents used in the study were of analytical reagent grade and obtained from Sigma chemical Co., USA, USB, SRL, India or Merck, India. Acetic acid used in study was obtained from Merck, India. Other weak acids (propionic acid, benzoic acid, octanoic acid and benzoic acid) were obtained from Sigma. Oligonucleotides were from Biobasic Inc, Canada and Sigma-Genosys, India. Media components were obtained from Himedia, Sigma and Difco. Restriction endonucleases, other DNA modifying enzymes, thermopolymerases and ligase were from New England Biolabs, USA and Fermantas, Canada. Molecular weight ladders were from New England Biolabs, USA and Fermantas, Canada. Chemiluminescence kit was obtained from Millipore. Ni-NTA resins, Gel extraction and plasmid mini prep columns were obtained from Qiagen, USA. Monoclonal antibodies were from Cell Signaling, Sigma and Thermo. DNA sequencing Kits (ABI PRISM 310 with dye termination cycle sequencing ready reaction kit) was obtained from Perkin Elmer, USA.

2.2 Methods:

2.2.1 Growth and maintenance of yeast and *E.coli* strains:

S.cerevisiae strains were routinely maintained on YPD plates at 30°C or otherwise as specified, and *E.coli* strains were maintained on LB plates with appropriate antibiotic selection at 37°C. *DH5α* and *DH10B* were used as cloning hosts (Sambrook et al., 1989). For long term preservation strains were kept at -70°C in 15% glycerol.

2.2.2 Recombinant DNA technology:

All the molecular biology protocols were carried out using standard protocols (Gannon et al., 1988; Sambrook et al., 1989). DNA sequencing was done using ABI PRISM sequencer and reactions were carried out as described by the manufacturer of DNA sequencing kit (ABI PRISM 310 XL with dye termination cycle sequencing ready reaction kit)

2.2.3 Purification of DNA from agarose gel:

DNA extractions from agarose gel were done using QIAGEN gel extraction kit, following the instructions given by the manufacturer.

2.2.4 Polymerase chain reactions:

DNA fragments for cloning and yeast integration constructs were PCR amplified using Vent DNA polymerase (NEB). For analytical PCR Taq DNA polymerase was used. Appropriate plasmid or yeast genomic DNA was used as template and amplified fragments were purified after resolving in agarose gel.

2.2.5 Preparation of *E.coli* competent cells and *E.coli* transformation:

The $MgSO_4$ competent *E.coli* cells were prepared and used as described by Nishimura (Nishimura et al., 1990). Electrocompetent *E.coli* cells were prepared as described (Sambrook et al., 1989).

2.2.6 Yeast transformation:

Yeast transformations were carried out by the lithium acetate method (Gietz and Schiestl, 2007). Yeast strains were grown in YPD at 30°C, 200 rpm for 12-14 hours and reinoculated into fresh YPD at initial $O.D_{600}$ of 0.1. Cells were grown at 30°C for additional 3-4 hours till $O.D_{600}$ = 0.5 to 0.8. Cells were harvested at 6000 rpm for 5 minutes at 30°C and washed once with water and once with 100 mM Lithium acetate buffer (LiAc-TE). Cells were finally resuspended in LiAc-TE and 50µl aliquot in 1.5 ml microcentrifuge tube was used for each transformation. Approximately 300ng-500ng of plasmid or linear DNA construct in 5-10 µl, 5µl (10mg/ml stock) heat denatured salmon sperm carrier DNA and 300µl of 40% PEG (prepared in 100 mM LiAc-TE) were added, mixed well and incubated for 45 minutes at 30°C, followed by heat shock for 15 minutes at 42°C in Eppendorf thermomixer. Cells were pelleted down and resuspended in 100 µl of sterile water and plated on appropriate selection plates.

2.2.7 Dilution spotting:

For dilution spotting yeast strains were freshly revived on agar plate of appropriate medium. Primary cultures were grown for 16-20 hours at 30°C, with shaking at 200 rpm, and reinoculated into fresh medium at $O.D_{600}$=0.1 and grown till $O.D_{600}$ 0.5 to 0.8 was achieved. Cells were pelleted, washed with sterile water and resuspended in sterile water at $O.D_{600}$=1.0. Serial 10-fold dilutions ($O.D_{600}$=0.1, 0.01, 0.001 and 0.0001) were made and 5µl of each dilution was spotted on the selection plates. Plates were incubated at 30°C for 2-3 days.

2.2.8 Testing acetic acid sensitivity /tolerance by dilution spotting:

A 2X stock of YPD media containing various concentrations of acetic acid were prepared, pH of media was adjusted to 3 or 4.5 with 2 N HCl. Separately 2X stock of agar (4%) was prepared and autoclaved. After cooling to about 55-60°C, equal volumes of 2X YPD containing acetic acid and 2X agar were mixed and poured in Petri plates. Yeast grown in appropriate media to exponential or late growth phase (as mentioned in different experiments) were harvested, normalized to $O.D._{600} = 1.0$ after washing with sterile water, 10 fold serially diluted and spotted on acetic acid containing plates.

For other weak acids tested, acetic acid was replaced with these acids at desired concentrations and pH was adjusted to 4.0.

2.2.9 Expression and purification of recombinant protein:

Codon plus *E. coli* strain was used for the protein expression studies. Recombinant clones in Novagen pET vectors were introduced in *E. coli* and protein expression was induced by 0.25 mM IPTG at 25°C for 5 hours for Haa1p and H400p. Lysates of IPTG induced cells were analyzed by SDS-PAGE to monitor expression level. To purify the induced proteins, cells were pelleted at 4°C and resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl, 10% glycerol and 20 mM Imidazole, supplemented with protease inhibitors such as PMSF and orthovanadate) and lysed by sonication for 15 minutes with 30 second on and 30 second off cycles. Purification of the expressed proteins was carried out by Ni-NTA affinity chromatography. Cell lysates were passed through Ni-NTA column equilibrated with lysis buffer; the column was washed with 25 bed volume of lysis buffer and the bound proteins were eluted with elution buffer (200 mM Imidazole in lysis buffer). Different fractions were run on SDS-PAGE to check the protein quality; desired fractions with purified protein were pooled and dialyzed in lysis buffer without imidazole. Protein quantity was estimated by Bradford's assay (Bradford, 1976).

2.2.10 Gel filtration chromatography of purified recombinant proteins:

For gel filtration analysis, Ni-NTA purified proteins in 300 mM NaCl, 50 mM Tris-HCl, pH 8.0 and 10% glycerol was loaded on Superdex S200 gel filtration column (GE Health care). The column was run at 4-8°C at a flow rate of 0.5 ml/min in the same buffer and elution of the protein was monitored at 280nm. Protein fractions were collected and subsequently analysed by SDS-PAGE followed by CBB R-250 staining.

2.2.11 RNA isolation and Northern blotting:

The yeast total RNA was isolated by a modified hot phenol method as described (Mannan et al., 2009). Northern blotting was done with 15-20 µg of total RNA as per the protocol described (Gannon et al., 1988). The RNA samples were denatured at 65°C for 10 min in RNA loading dye (catalogue number R1386, Sigma), resolved by electrophoresis on 1.5% agarose gel containing 1X MOPS buffer and 0.23 M formaldehyde at 4°C at 70-80 Volts. The RNA was transferred to nylon membrane (Hybond N+, Amersham) in 10X saline sodium citrate (SSC) buffer pH 7.0. The blot was hybridized with [α–^{32}P] dATP labeled gene specific probe at 42°C for 12 h, in a buffer containing 50% formamide, 5X SSC, 5X Denhardt's solution and 0.5% SDS. The blot was first washed at room temperature for 5 min, followed by two washes at 42°C in 0.2X SSC containing 0.1% SDS for 15 min. The blot was later exposed to Fuji storage phosphor screen, image scanned with Fujifilm-FLA9000 imager and analyzed by Multigauge software.

2.2.12 Probe preparation by random primer labeling:

Gene specific probes were prepared by random primer labeling method. 50 ng template DNA was diluted to 10 µl in sterile water and denatured at 95°C and snap chilled on ice. Random nonamers, dNTPs without dATP, [α–^{32}P] dATP and dH_2O were added. Finally separate mix of Ecopol buffer and Klenow polymerase was added and reaction mix was incubated at 37°C for 45 min.

Template DNA	50 ng in 10 µl
Random nonamer	4 µl of 50 ng/µl
dNTPs-dATP	1 µl of 10 µM stock
H2O	29 ml
[α-^{32}P] dATP	1 µl of 10 µCurie
Ecopol Buffer	5 µl
Klenow DNA polymerase	1 µl of 5 U/µl
Total volume	50 µl

Efficiency of labeling was checked by Cerenkov counts. The probe was denatured just before use in hybridization.

2.2.13 Western blotting:

Western blotting was performed by electro blotting method in a mini trans-blot apparatus (Bio-Rad). The purified proteins or cell lysates were resolved by 10% SDS PAGE and transferred to Hybond ECL nitrocellulose membrane (GE Biosciences) in transfer buffer (submerged condition) at 125 mA for 2 h. Pre-stained protein molecular weight markers or Ponceau staining was used to check the efficiency of transfer. Blocking of blots was done in blocking solution (PBST with 10% skimmed milk), either overnight at 4°C or for 2 h at 37°C. The blots were then washed three times with PBST for 10 minutes each and incubated with primary anti-His (Cell signaling) or anti-HA (Sigma) antibody diluted in PBST with 5% skimmed milk (1:2500 dilution) and incubated for 2 h on rocking shaker at room temperature. Blots were then washed with PBST, and secondary anti mouse-IgG antibody was added (1:7500 dilution in PBST 5% skimmed milk) and incubated for 1 h. The blots were washed and transferred into 10 ml PBS and developed by chemiluminescences kit (Millipore, catalogue number P90719). Equal volumes of Solution A (Luminol reagent) and Solution B (peroxide solution) were mixed and added over the membrane and incubated in dark for 5 min, and membrane was exposed to X-ray film for 20 s to 2 min, depending upon signal strength.

2.2.14 Microscopic localization studies:

Yeast strains were grown to log phase, and 1.5 to 2.0 $O.D._{600}$ cells were harvested and fixed in 4% paraformaldehyde by incubating at 30°C and 100 rpm for 2 hours. Cells were washed with 100 mM potassium phosphate buffer (pH 6.5), followed by washing with the same buffer with 1.2 M sorbitol, and finally resuspended in phosphate sorbitol buffer. Spheroplasting was done by adding 10 µl of β–marcaptoethanol and 20 µl of 10 mg/ml zymolyase and incubating for 30 min at 30°C. Spheroplasts were washed with phosphate sorbitol buffer and finally resuspended in 100-200 µl of same buffer. Washed spheroplasts were adhered to poly-L-Lysine coated coverslips and permeabilized with 0.4% Triton-X in PBS for 2 min. Adhered spheroplasts were washed with blocking buffer (1% BSA in PBS), followed by blocking for 30 min in the same buffer. Mouse anti-HA primary monoclonal antibody (1:2000 dilution) was added to blocking buffer and incubated in moist chamber for overnight at 4°C. Primary antibody is removed by washing with blocking buffer for 10 times, Alexaflour 488 conjugated goat anti mouse secondary antibody (1:500 dilution) was added

and incubated for 2 hours at room temperature in dark. Spheroplasts were again washed 10 times with blocking buffer in the dark, and finally incubated with 0.1% DAPI in PBS and washed thrice with PBS. Spheroplast coated coverslips were mounted on glass slides after putting mounting media and the slides were examined under fluorescence (Ziess) microscope. DAPI fluorescence was detected with filter having excitation from 340-380 nm wavelengths and emission from 435-485 nm. Similarly, Alexa 488 filter has excitation from 465-495 nm and emission at 515-555 nm wavelengths.

Yeast cells expressing fluorescent tagged proteins were appropriately grown, washed with PBS and directly used for microscopic studies.

2.2.15 Electrophoretic mobility assay:

An increasing amount/conc. (0.5-5μM) of purified recombinant protein or cell lysate was mixed with 10 to 20 fmole (0.1-5 ng) of end labeled probe molecules in reaction mix and incubated for 30 min at 20°C.

10X binding buffer	2 μl
Labelled probe	1 μl (10-20 fmole)
ssDNA (sheared),10mg/ml	1 μl
H_2O	6 μl
Protein	10 μl (0.5-5 μM)
Total	20 μl

Reaction mix was mixed with 5X loading dye, and loaded on 5 % native PAGE gel. In unused lanes or lanes without protein loading dye with bromophenol blue was loaded as tracking dye. Gel was run for 5-6 hrs at 60-70 V in cold. The gel was separated from glass plates, dried in gel dryer and exposed to imaging screen. Signal was detected by scanning the imaging screen in phosphor imager.

2.2.16 Preparation of probe DNA by end labeling:

DNA of interest were PCR amplified, gel purified and quantified, end labeling reaction was set as shown below

PNK buffer (10X)	3 µl
γ–^{32} P -ATP	1.0 µl of 10 times diluted stock
Poly nucleotide kinase	1 µl (10U/µl)
H_2O	q.s.
Protein	10 µl (0.5-5 µM)
DNA	700 to 800 fmole (20-25 fmole/µl after purification)
Total	30 µl

Incubated at 37°C for 45 minutes and purified the labeled DNA from unincorporated γ–^{32} P -ATP by Qiagen nucleotide removal kit.

2.3 Media and buffer compositions:

All the buffers and media were prepared in Milli Q water or RO water.

2.3.1 Media Compositions:

1. LB (Luria-Bertani) medium **(per liter)**

Yeast extract	5g
Tryptone	10g
Sodium chloride	10g

Ampicillin was used at a final concentration of 50µg/ml and kanamycin was used at a final concentration of 25µg/ml. 2.0% agar was used as solidifying agent of the preparation of plates.

2. YPD medium **(per liter)**

Yeast extract	10g
Peptone	20g
Dextrose	20g

2.0% agar was used as solidifying agent of the preparation of plates.

3. SD (synthetic defined) medium **(per liter)**

Yeast nitrogen base	1.7g
(W/o amino acids and ammonium sulphate)	

Ammonium sulphate	5g
Dextrose	20g
Supplements	80mg each
(As per strain requirement)	

2.0 % agar was used as solidifying agent of the preparation of plates.

4. SC (synthetic complete) -Ura (per liter)

Yeast nitrogen base	1.7g
(W/o amino acids and ammonium sulphate)	
Ammonium sulphate	5g
Dextrose	20g
Yeast synthetic dropout medium	1.92g

5. Yeast synthetic dropout medium supplements (without uracil)

Final conc. of supplements per liter	
Adenine hemisulfate	18 mg
Alanine	76 mg
Arginine hydrochloride	76 mg
Asparagine monohydrate	76 mg
Aspartic acid	76 mg
Cysteine hydrochloride monohydrate	76 mg
Glutamic acid monosodium salt	76 mg
Glutamine	76 mg
Glycine	76 mg
Histidine	76 mg
myo-Inositol	76 mg
Isoleucine	76 mg
Leucine	380 mg
Lysine monohydrochloride	76 mg
Methionine	76 mg
p-Aminobenzoic acid potassium salt	8 mg
Phenylalanine	76 mg
Proline	76 mg
Serine	76 mg

Threonine	76 mg
Tryptophan	76 mg
Tyrosine disodium salt	76 mg
Valine	76 mg

6. SC (synthetic complete)

SC-ura medium was supplemented with 80mg/l uracil.

2.3.2 Buffer compositions:

2.3.2.1 Buffers for DNA work:

1. Solution I for plasmid isolation

Dextrose	50 mM
Tris-HCl	25 mM (pH 8.0)
EDTA	10 mM (pH 8.0)

Autoclave and stored at 4°C

2. Solution II for plasmid isolation

NaOH	0.2 N
Sodium dodecyl sulphate (SDS)	1% SDS

Freshly prepared.

3. Solution III for plasmid isolation (100 ml)

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

4. TE buffer

Tris-HCl	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)

5. TAE buffer

Tris-acetate	40 mM
EDTA	1 mM (pH 8.0)

6. Orange G dye (DNA loading dye, 6X)

Orange G	0.25%
Glycerol	30%

7. Yeast breaking buffer

TritonX 100	2% (v/v)
SDS	1% (v/v)
NaCl	100 mM
Tris-HCl	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)

2.3.2.2 Buffers for protein work:

1. 5X SDS PAGE sample buffer (10ml)

1.0 M Tris-HCl (pH 6.8)	1.5 ml
10% SDS	5 ml
Glycerol	2.5 ml
β-mercaptoethanol	1.25 ml
Bromophenol blue	(according to color)

2. 5X SDS PAGE running buffer (per liter)

Trizma base	15.1 g
Glycine	94 g

Dissolved in 900 ml of water, 50 ml of 10% SDS was added and final volume was made 1 litre with water. At 1X PH is around 8.3, not adjusted.

3. Staining solution (per litre)

Methanol	400 ml
Glacial acetic acid	100 ml
Water	500 ml
Coomassie blue R250	0.25 g

4. **Destaining solution**	**(per liter)**
Methanol	400 ml
Glacial acetic acid	100 ml
Water	500 ml

5. **Resolving gel buffer (4X)**	
Tris-HCl (pH 8.8)	1.5 M
SDS	0.4%

6. **Stacking gel buffer (4X)**	
Tris-HCl (pH 6.8)	0.5 M
SDS	0.4%

7. **Western blot transfer buffer**	**(per liter)**
Trizma base	2.45 g
Glycine	11.2 g
Dissolved in 800ml of water	
Methanol	200 ml

8. **10X PBS (Phosphate buffer saline)**	**(per liter)**
Sodium chloride	80 gm
Potassium chloride	2 gm
Potassium di hydrogen phosphate	2 g
Di sodium hydrogen phosphate	12 g
Adjust pH to 7.5	

9. **0.1% PBST (washing buffer)**	**(per liter)**
10X PBS	100 ml
Tween 20	1 ml
Water	899 ml

10. **10% Skim milk (blocking solution)**	**(50 ml)**
Skim milk	5 g

0.1% PBST	to make up volume to 50 ml

11. 5% skim milk (Antibody incubation buffer) (50 ml)

Skim milk	2.5 g
0.1% PBST	to make up volume to 50 ml

12. Yeast total cellular protein extraction buffer

Tris-HCl (pH 7.5)	20 mM
NaCl	20 mM
EDTA	1 mM
Glycerol	10%
NP-40	0.2%

2.3.2.3 Yeast transformation reagents:

1. Lithium acetate TE (LiAc-TE)

Lithium acetate	100 mM
Tris-HCl	10 mM (pH 7.5)
EDTA	1 mM (pH 8.0)

Adjusted to pH 7.5 with acetic acid and autoclaved.

2. 40% PEG (Poly ethylene glycol) 100 ml

PEG 3350	40 g

Dissolved in LiAc-TE buffer (final 1X) to make a final volume of 100 ml, autoclaved

3. Salmon sperm DNA (10 mg/ml)

100 mg of salmon sperm DNA dissolved in 10 ml of TE buffer by vigorous pipetting or vortexing at intervals and keeping on ice. After dissolving it completely, samples were denatured by boiling, followed by snap chilling in ice.

2.3.2.4 RNA isolation and northern blotting reagents:

1. AE buffer

Sodium acetate	50 mM (pH 5.5)
EDTA	10 mM (pH 8.0)

2. **Acidic phenol, pH 4.5 obtained from SRL, Mumbai**

3. **CI (Chloroform and Isoamyl alcohol in 49:1 ratio)**

4. **3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0)**

MOPS	0.2 M
Sodium acetate	100 mM
EDTA	10 mM

5. **20X SSC**

NaCl	3.0 M
Sodium citrate	0.3 M

6. **Denhardts Reagent (100X)**

BSA (Bovine serum albumin)	2%
Ficoll (400)	2%
PVP (Poly vinyl pyrrolidone)	2%

7. **RNA sample loading buffer**

Deionized formamide	62.5% (V/V)
Formaldehyde	1.14 M
Bromophenol blue	200 µg/ml
Xylene cyanole	200 µg/ml
MOPS buffer	1.25X

All the reagents used in RNA isolation were prepared in water that was DEPC treated (0.1% final conc.) and autoclaved.

2.3.2.5 Electrophoretic mobility shift assay (EMSA) reagents:

1. **10X TBE buffer**

Tris base	890 mM
Boric acid	890 mM
EDTA	20 mM (pH 8.0)

2. 40% (w/v) Acrylamide bis acrylamide solution

Acrylamide	38 g
bisAcrylamide	2 g
Dissolve in dH_2O to final volume of 100 ml	

3. 10X Binding reaction buffer

Tris-HCl	100 mM (pH7.5)
EDTA	10 mM
KCl	1 M
DTT	1 mM
Glycerol	50%

4. PAGE loading dye

Ficoll	30%
Tris-HCl	10 mM (pH 7.5)
EDTA	1 mM (pH 8.0)

2.3.2.6 Immunofluorescence and microscopy reagents:

1. 100 mM Potassium phosphate buffer (pH 6.5)

Stocks of KH_2PO_4 and K_2HPO_4 mixed as follow to make 100 mM concentration.

Volume	KH_2PO_4, 400 mM	K_2HPO_4, 400 mM	dH_2O
100 ml	34.5 ml	15.75 ml	50 ml
50 ml	17.125 ml	7.875 ml	25 ml
25 ml	8.5625 ml	3.9375 ml	12.5 ml

2. 100 mM Potassium phosphate buffer (pH 6.5) with 1.2 M sorbitol

To make 50 ml buffer, 10.932 g sorbitol was added to phosphate buffer constituents and final volume was adjusted to 50 ml.

3. Paraformaldehyde (4% w/v) in 100 mM Potassium phosphate buffer (pH 6.5)

To make 10 ml buffer, 0.4 g of paraformaldehyde was added to phosphate buffer constituents and final volume is adjusted to 10 ml.

4. 1% Poly-L-Lysine

10 mg of Poly-L-Lysine is dissolved in 1 ml dH_2O and stored at -20°C.

5. Dabco mounting media

0.1 mg/ml of 1,4-diazabicyclo (2,2,2) octane (DABCO, Sigma) was dissolved in 50% glycerol in 1X PBS.

6. DAPI solution

1mg/ml of 4',6-diamidino-2-phenylindole (DAPI, Sigma) dissolved in sterile water.

2.4 List of strains used:

Table 2.1 Parental yeast strains and strains constructed in this study:

Strain Name	Organism	Genotype	Reference
FY3	*S.cerevisiae*	*MATa ura3-52*	Brachmann,1998
FY4	*S.cerevisiae*	*MATa* (prototroph)	Brachmann,1998
BY4741	*S.cerevisiae*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura 3Δ0*	Brachmann,1998
BY4742	*S.cerevisiae*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura 3Δ0*	Brachmann,1998
EGY48	*S.cerevisiae*	*MATα trp1 his3Δ1 ura 3Δ0 leu2::6lexAop-LEU2*	Origene
FY3-pRS	*S.cerevisiae*	FY3 strain with pRS306 integrated at *ura3-52* locus	Puria, 2008
FYGY4, 5 and 11	*S.cerevisiae*	*MATa ura3-52,URA3*-P_{GPD}-*YLR297w*	This study
FPG1 and 2	*S.cerevisiae*	*MATa ura3-52,YLR297w*-CFP-HYG	This study
G11G1 and 2	*S.cerevisiae*	*MATa ura3-53,URA3*-P_{GPD}-*YLR297w*-CFP-HYG	This study
FPY1 and 2	*S.cerevisiae*	*MATa ura3-54,YLR297w*-YFP-KAN	This study
G11Y1 and 2	*S.cerevisiae*	*MATa ura3-53,URA3*-P_{GPD}-*YLR297w*-YFP-KAN	This study
ngg1Δ haa1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR176w::kanMX4, HAA1::HIS3*	This study
HAA1-TAP *ngg1Δ*	*S.cerevisiae*	S288C;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, HAA1*-TAP-*HIS3MX, YDR176w::kanMX4*	This study
ygp1Δ haa1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL160w::kanMX4, HAA1::HIS3*	This study
tpo2Δ haa1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGR138c::kanMX4, HAA1::HIS3*	This study

Table 2.2 Yeast strains procured from external sources:

Strain Name	Organism	Genotype	Source
HAA1-TAP	*S.cerevisiae*	S288C;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, HAA1*-TAP-*HIS3MX*	Open Biosystems
ylr297wΔ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR297w::kanMX4*	Euroscarf
haa1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPR008w::kanMX4*	Euroscarf
ygp1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL160w::kanMX4*	Euroscarf
tpo2Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGR138c::kanMX4*	Euroscarf
tpo3Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPR156c::kanMX4*	Euroscarf
msn5Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR335w::kanMX4*	Euroscarf
ngg1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDR176w::kanMX4*	Euroscarf
ste20Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YHL007w::kanMX4*	Euroscarf
spi1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER150w::kanMX4*	Euroscarf
fus1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YCL027w::kanMX4*	Euroscarf
tda6Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPR157w::kanMX4*	Euroscarf
com2Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER130c::kanMX4*	Euroscarf
yro2Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR054w::kanMX4*	Euroscarf
phm8Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER037w::kanMX4*	Euroscarf

vma1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL185w::kanMX4*	Euroscarf
vph1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR270c::kanMX4*	Euroscarf
mnt4Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNR059w::kanMX4*	Euroscarf
atp14Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR295c::kanMX4*	Euroscarf
ylr296wΔ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR296w::kanMX4*	Euroscarf
war1Δ	*S.cerevisiae*	BY4742; *MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YML076c::kanMX4*	Euroscarf
hrk1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR267c::kanMX4*	Euroscarf
sap30Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YMR263w::kanMX4*	Euroscarf
pho80Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOL001w::kanMX4*	Euroscarf
pcl10Δ	*S.cerevisiae*	CEN.PK; *MATa; ura3-52; his3Δ1; leu2-3_112; trp1-289; YGL134w(3, 1297) ::kanMX4*	Euroscarf
nto1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPR031w::kanMX4*	Euroscarf
yak1Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL141c::kanMX4*	Euroscarf
gln3Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YER040w::kanMX4*	Euroscarf
pho85Δ	*S.cerevisiae*	BY4741; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPL031c::kanMX4*	Euroscarf

Table 2.3 *E. coli* strains used in this study:

Strain Name	Organism	Genotype
DH5α	*E.coli*	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d lacZΔM15 Δ(*lacZYA-argF*)*U169*, *hsdR17*(r_K^- m_K^+), λ^-
DH10B	*E.coli*	F⁻ *endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ^-
BL21DE3	*E.coli*	F⁻ *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])
Codon plus	*E.coli*	*E. coli* B F⁻ *ompT hsdS*(r_B^- m_B^-) dcm^+ *Tetr gal* λ(DE3) *endA Hte* [*argU* proLCam^r] [*argU ileY leuW* Strep/Spec^r]

2.5 List of oligos used:

Table 2.4 oligos used for cloning:

HAA1 deletions cloning	**5' ⟶ 3'**
HAA1-SpeI S1	ATTACGACTAGTATGGTCTTGATAAATGGCAT
HAA1-SpeI S2 (N50)	ATTACGACTAGTATGAAAAACAAGAATGCAAATCC
HAA1-SpeI S2 (N100)	ATTACGACTAGTATGTATCATGCTCAAAAGAGAC
HAA1-EcoRI A1	ACCTAAGAATTCTCATAACGAAGACATGAAATTATC
HAA1-EcoRI A2 (500)	ACCTAAGAATTCTCATCGAGTGAAAGGTCTGTCAG
HAA1-EcoRI A2 (400)	ACCTAAGAATTCTCAAAGCGTTGAAATCGACGATG
HAA1-EcoRI A2 (200)	ACCTAAGAATTCTCATTGTGGTATACTGAAATTTTG
HAA1-EcoRI A2 (50)	ACCTAAGAATTCTCATCGAAGTTGTTTACAATAGTCG
HAA1 deletions His and HA tag in yeast cloning	
HAA1-400HA-EcoRI	ACCTAAGAATTCTCAAGCGTAATCCGGAACATCATACGGGTAAA GCGTTGAAATCGACGA
HAA1-HA-EcoRI	ACCTAAGAATTCTCAAGCGTAATCCGGAACATCATACGGGTATAA CGAAGACATGAAATT
HAA1-HisEcoRI	ACCTAAGAATTCTCAATGGTGATGGTGATGGTGATGGTGTAACGA AGACATGAAATTATC
HAA1-500-HisEcoRI	ACCTAAGAATTCTCAATGGTGATGGTGATGGTGATGGTGTCGAGT GAAAGGTCTGTCAG
HAA1-400-HisEcoRI	ACCTAAGAATTCTCAATGGTGATGGTGATGGTGATGGTGAAGCGT TGAAATCGACGATG
HAA1 deletion clones *E. coli* expression cloning	
HAA1-NdeI-S1	ACGTACCATATGGTCTTGATAAATGGCA
HAA1-N100-NdeI-S2	ACGTACCATATGTATCATGCTCAAAAGAGAC
HAA1-N50-NdeI-S3	ACGTACCATATGAAAAACAAGAATGCAAATCC
HAA1-XhoI-R1	ACCTAACTCGAGTAACGAAGACATGAAATTATC
HAA1-500-XhoI-R2	ACCTAACTCGAGTCGAGTGAAAGGTCTGTCAG
HAA1-400-XhoI-R2	ACCTAACTCGAGAAGCGTTGAAATCGACGATG
HAA1 promoter cloning	
HAA1 Up SacI S1	CTACGGAGCTCTGCAGAATATTTGTAATATTAATC
HAA1 Up SacI S2	CTACGGAGCTCGAAAAATGTGCGAAATAG
HAA1 Up Sac1 S3	CTACGGAGCTCCTCAATTGTAAAAATAAG
HAA1 Up Xba1 A1	ACGTACTCTAGATATTTTTTAGGTTTTTTTTTTC
HAA1 Up Ty del C	TTATTTTTACAATTGAGGATATTATACATTACCAA
HAA1 Up Ty del W	TGGTAATGTATAATATCCTCAATTGTAAAAATAAG

NGG1 deletions cloning	
NGG1 (N' 1) BamHI	ACTACGGGATCCATGCCTAGACATGGAAGAAG
NGG1 (N' 364) BamHI	ACTACGGGATCCATGTATAAAAAACCTTCACCATA
NGG1 (N' 452) BamHI	ACTACGGGATCCATGACATTATCTCAGGAGAAC
NGG1 (N' 214) BamHI	ACTACGGGATCCATGAAAATGGAAAACGATCCAAC
NGG1 (C' 702) EcoRI	ACCTAAGAATTCTTAATTTAGTTCCACGTCCT
NGG1 (C' 671) EcoRI	ACCTAAGAATTCttaGGGGATTCTCTTCATAATTT
NGG1 (C' 346) EcoRI	ACCTAAGAATTCttaGCCTAGTTTCGGTATAAT
NGG1 C (274-307)	TTGTCATCACTAAGATCATCTTTCAAATCATTTGT
NGG1 W (274-307)	CAAATGATTTGAAAGATGATCTTAGTGATGACAAC
NGG1 W (308-373)	TGGAAAATTTTTTCAAAGCATCTACTATACTACCA
NGG1 C (308-373)	GGTAGTATAGTAGATGCTTTGAAAAAATTTTCCAC
TPO2 and YGP1 clonings	
YGP1-SacI-S1	ACCTAAGAGCTCTCCTAATGGAGAGGCTCAG
YGP1-SacI-S2	ACCTAAGAGCTCGTCGAAACTTTTGCTTACC
YGP1-SacI-S3	ACCTAAGAGCTCGGGCAAATTTAGGAATGTC
TPO2- SacI -S1	ACCTAAGAGCTCATGCACTGTAATGCCACTG
TPO2- SacI -S2	ACCTAAGAGCTCTCTCCTCCCGAGGGACATAA
TPO2- SacI -S3	ACCTAAGAGCTCCCTATGCAAAAACCCTTCC
YGP1-PstI-A	ACCTAACTGCAGTCATGGGAAAATGCTTTC
TPO2-PstI-A	ACCTAACTGCAGTTACATCTCAACACTGTC
TPO2-UP-S (696-715) SacI	CTATAGGGCGAATTGGAGCTCTATGTTCTTTTTTCCTGAAG
TPO2-UP-S (788-806) SacI	CTATAGGGCGAATTGGAGCTCCTTCTCCTACTATATCAAG
YGP1-UP-S (421-442) SacI	CTATAGGGCGAATTGGAGCTCGAAGATCCGGCGGGCGAAAAAG
YGP1-UP-S (520-540) SacI	CTATAGGGCGAATTGGAGCTCCACTGTCTAAATAGTTTACC
YGP1-UP-S (710-729) SacI	CTATAGGGCGAATTGGAGCTCATGCTACAGTTCAACATCG

Table 2.5 Oligos for northern blot probes and diagnostic PCR:

YLR297w-S2	CCTTCTTCTTTGCTCTATCGTG
YLR297w-A2	CCAAGTAGTAAAACTCCAACCAC
YLR297w-S3	ATGGTCGAAGGTGATTTTG
YGP1-S1	ATGAAGTTCCAAGTTGTTTT
YGP1-A1	ACTGGAATGGCATAAGCGG
TPO3-S1	ATGAACAGACAGGAATCCAT
TPO3-A1	GGATCGCCAGTCACAAAGG
TPO2-S1	ATGAGTGATCAAGAATCTGT
TPO2-A1	CATGAGGGCCAATTGTGAG
HAA1-S2	AGCAAAATAAGACCCCGTG
HAA1C-F(1741-1759)	CCTAAAACTGGAAGTCGCC
His 3-PA1	CTGACTAATGCCGTGTTC
M13 forward	GTTTTCCCAGTCACGACG
M13 Reverse	AGCGGATAACAATTTCACACAGGA
GPD-P	GACGGTAGGTATTGATTGTAATTCTG
pBS-GFP-A	TCCATCTTCAATGTTGTGTC
CYC1-T1	CCTTTTCGGTTAGAGCG
Sch9-Up-S1	CCCACTCTCACATAATCACC

Table 2.6 Oligos for EMSA probes:

TPO2-UP-A1	ATTTGTTTTGTGTATTATTTTTG
TPO2-UP-S1	ATGCACTGTAATGCCACTG
TPO2-UP-A2	GAAACCTCAAAAGGGTGTAT
TPO2-UP-S2	TCTCCTCCCGAGGGACATAA
TPO2-UP-A3	GATGAATTTAACGGCTATGG
TPO2-UP-S3	CCTATGCAAAAACCCTTCCC
YGP1-UP-A1	TTTCTATTACTGTATTACTTAAC
YGP1-UP-S1	TCCTAATGGAGAGGCTCAGG
YGP1-UP-A2	GCTTCACTCCCTACTGAGAT
YGP1-UP-S2	GTCGAAACTTTTGCTTACCC
YGP1-UP-A3	GGATCTTCCGCGGATGAC
YGP1-UP-S3	GGGCAAATTTAGGAATGTCT
YGP1 UPRT R1	AACATGCCAAAAGGGCAG
YGP1 UPRT R2	CAAGCTTTTTATATTTCAG
YGP1 UPRT S1	ATATAAAAAGCTTGATAG
YGP1 UPRT S2	GTACTCTATTGCATCTTC
TPO2 UPRT R1	TTTGTGAGTTGAATGAAAC
TPO2 UPRT S1	GTTTGAATTTTAATTTTAAC
TPO2 UPRT R2	TAAAATTAAAATTCAAACCG
TPO2 UPRT S2	ATACACCCTTTTGAGGTTTC

Table 2.7 Oligos for gene disruption, promoter replacement and tagging in yeast:

Oligos Name	Sequence	Application
HAA1mcherry F	TCAAGGATTTGCGGATTTGGATAATTTCATG TCTTCGTTACCGCTAGCGCTACCGGTC	Amplify mCherry tag for insertion at C terminus of HAA1 cloned in p416TEF/HAA1 by gap repair
HAA1mcherry R	ACTCGAGGTCGACGGTATCGATAAGCTTGAT ATCGAATTCTCACTTGTACAGCTCGTCCATG	
HAA1-GFP-F	TCAAGGATTTGCGGATTTGGATAATTTCATG TCTTCGTTACGATACCGTCGACCTCG	Amplify GFP tag for insertion at C terminus of HAA1 cloned in p416TEF/HAA1 by gap repair
HAA1-GFP-R	ACTCGAGGTCGACGGTATCGATAAGCTTGAT ATCGAATTCTCATTTGTATAGTTCATCC	
HAA1 Del HIS3 S	TATAATAAAAGAGCCAAGACAAAACGTGGC AGGGAAGCTTCGGCATCAGAGCAGATTG	Amplify HAA1 deletion cassette with HIS3 selection marker
HAA1 Del HIS3 A	ACTACAGTTACAGAGAAGCAAGAGACGAAA AGCAAATTTACTACATAAGAACACCTTTGG	
NGG1 Del HIS3 S	ATAACAAAGACGGAGCGACGAGAAGTATTG GACAGGACATCGGCATCAGAGCAGATTG	Amplify NGG1 deletion cassette with HIS3 selection marker
NGG1 Del HIS3 A	CGTTATTATGCTACGTATTTTTCCTTAGAGTT CGTATATTCTACATAAGAACACCTTTGG	
YLR297w-GPD-A1	AATGTTCGATTGCTCATCGACAAAATCACCT TCGACCATCGTCGAAACTAAGTTCTGGTG	Amplify GPD promoter with URA3 selection marker from pGV8 for insertion at upstream of YLR297w coding sequence
YLR297W-URA3-S1	GAGTAGTAATATATAAAAGAGAAATCGTATA AGGACCGTGCGGCATCAGAGCAGATTG	
YLR-GFP-S1	GTCCAACGATGAAGAAAAGCAAGCACTTGCT GAAAAAGCAGGTCGACGGATCCCCGGG	Amplify CFP or YFP tag from pBS7 or 10 vector for insertion at C terminus of YLR297w
YLR-GFP-A1	GGCGTGAAGAGCGGTGCCATCTATTATATCT ATTTTTATTTAGTGGATCTGATATCATCG	
NGG1 UP S	GTTGGAAAAACATAACAAAG	Amplify NGG1 deletion cassette with KanMX from ngg1D strain
NGG1 UP A	GATAGTTATCCAAATTGTTAG	

2.6 List of plasmids used:

Table 2.8 Plasmids taken from other sources:

Clone Name	Description	Source
pFL44L	pUC19 based yeast multicopy vector having *URA3* selection marker, STB gene and 2 micron ORI	Bonneaud, 1991
pGV8	Yeast integrative vector with *URA3* selection marker and GPD promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Sharma, 1998
pBS10	Yeast integrative vector with YFP (venus variant) and Kanamycin (G418) selection marker	Nagai et al ,2002
p416TEF	Yeast centromeric vector derived from pRS416, having *URA3* selection marker and TEF promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Mumberg et al, 1995
p426TEF	Yeast multicopy vector derived from pRS426, having *URA3* selection marker and TEF promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Mumberg et al, 1995
p426GPD	Yeast multicopy vector derived from pRS426, having *URA3* selection marker and GPD promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Mumberg et al, 1995
p426GAL	Yeast multicopy vector derived from pRS426, having *URA3* selection marker and *GAL1* promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Mumberg et al, 1994
p426ADH	Yeast multicopy vector derived from pRS426, having *URA3* selection marker and *ADH1* promoter and CYC terminator flanking *Xba*I and *Xho*I sites in MCS respectively	Mumberg et al, 1995
pRS315	Yeast centromeric vector having *LEU2* selection marker	Sikorski and Heiter, 1989

Table 2.9 Plasmids constructed in this study:

Clone Name	Description	Source
p416ADH	ADH promoter from p426ADH was cloned in p416TEF replacing TEF promoter at *Sac*I and *Xba*I sites	This study
p315ADH	*Sac*I-P_{ADH}-MCS-T_{CYC}-*Kpn*I(Blunt end) was cloned in pRS315 at *Sac*I and *Sal*I (end filled), *Sal*I and onwards sites repeat in MCS	This study
p416HAA1(S1A1)	*HAA1* promoter region from -1 to -1639 bp cloned in p416TEF replacing TEF promoter at *Sac*I and *Xba*I sites	This study
p416HAA1(S2A1)	*HAA1* promoter region from -1 to -1173 bp cloned in p416TEF replacing TEF promoter at *Sac*I and *Xba*I sites	This study
p416HAA1(S3A1)	*HAA1* promoter region from -1 to -844 bp cloned in p416TEF replacing TEF promoter at *Sac*I and *Xba*I sites	This study
p416HAA1(-Ty)	*HAA1* promoter region from -1 to -1639 bp with internal deletion from -1172 to -845 cloned in p416TEF replacing TEF promoter at SacI and XbaI sites	This study

Table 2.10 *HAA1* and its deletion clones constructed in this study:

Clone Name	Description	Source
p416TEF+ HAA1	Full-length *HAA1* gene cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ H500	H500 deletion of *HAA1* encoding first 500 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ H200	H200 deletion of *HAA1* encoding first 200 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ H50	H50 deletion of *HAA1* encoding first 50 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ N50	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ N100	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ N504	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p416TEF+ N1004	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ HAA1	Full-length *HAA1* gene cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ H500	H500 deletion of *HAA1* encoding first 500 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ H200	H200 deletion of *HAA1* encoding first 200 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study

p426TEF+ H50	H50 deletion of *HAA1* encoding first 50 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ N50	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426TEF+ N100	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in p426TEF at *Spe*I and *Eco*RI sites	This study
p426GAL+ HAA1-His	Full-length *HAA1* gene cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ H500-His	H500 deletion of *HAA1* encoding first 500 amino acids cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ H400-His	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ N50-His	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ N100-His	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ N504-His	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p426GAL+ N1004-His	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acid cloned in p426GAL at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416HAA1 (S1A1)+ HAA1	Full-length *HAA1* gene cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI	This study
p416HAA1 (S1A1)+ H500	H500 deletion of *HAA1* encoding first 500 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study

p416HAA1 (S1A1)+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study
p416HAA1 (S1A1)+ N50	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study
p416HAA1 (S1A1)+ N100	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study
p416HAA1 (S1A1)+ N504	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study
p416HAA1(S1A1) +N1004	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acid cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites	This study
pET23a+ HAA1	Full-length *HAA1* gene cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ H500	H500 deletion of *HAA1* encoding first 500 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ H200	H200 deletion of *HAA1* encoding first 200 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ N50	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ N100	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study

pET23a+ N504	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET23a+ N1004	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acid cloned in pET23a at *Nde*I and *Xho*I sites, C terminal His tag	This study
pET28c+ HAA1	Full-length *HAA1* gene cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ H500	H500 deletion of *HAA1* encoding first 500 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ H200	H200 deletion of *HAA1* encoding first 200 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ N50	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ N100	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ N504	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
pET28c+ N1004	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acid cloned in pET28c at *Nde*I and *Eco*R1 sites, N terminal His tag	This study
p416HAA1 (S2A1)+ HAA1	Full-length *HAA1* gene cloned in p416HAA1(S2A1) at *Spe*I and *Eco*RI	This study
p416HAA1 (S3A1)+ HAA1	Full-length *HAA1* gene cloned in p416HAA1(S3A1) at *Spe*I and *Eco*RI	This study

p416HAA1 (-Ty) +HAA1	Full-length *HAA1* gene cloned in p416HAA1(-Ty) at *Spe*I and *Eco*RI	This study
pEG202+ HAA1	Full-length *HAA1* gene cloned in pEG202 at *Eco*RI and *Xho*I sites	This study
pJG45+ HAA1	Full-length *HAA1* gene cloned in pJG4-5 at *Eco*RI and *Xho*I sites	This study
pEG202+ H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in pEG202 at *Eco*RI and *Xho*I sites	This study
pJG4-5 + H400	H400 deletion of *HAA1* encoding first 400 amino acids cloned in pJG4-5 at *Eco*RI and *Xho*I sites	This study
p416TEF+ HAA1-HA	Full-length *HAA1* gene cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal HA tag	This study
p416TEF+ H400-HA	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal HA tag	This study
p416HAA1 (S1A1)+ HAA1-HA	Full-length HAA1 gene cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites with C terminal HA tag	This study
p416HAA1 (S1A1)+ H400-HA	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites with C terminal HA tag	This study
p416TEF+ HAA1-mCherry	Full-length *HAA1* gene cloned in p416TEF at *Spe*I and *Eco*RI sites, C terminal mCherry tag added to *Eco*RI digested p416TEF+HAA1 by gap repair in yeast	This study
p416TEF+ HAA1-GFP	Full-length *HAA1* gene cloned in p416TEF at *Spe*I and *Eco*RI sites, C terminal GFP tag added to *Eco*RI digested p416TEF+HAA1 by gap repair in yeast	This study
p416HAA1 (S1A1)+ HAA1-mCherry	Full-length *HAA1* gene cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI sites, C terminal mCherry tag added to *Eco*RI digested p416HAA1(S1A1)+HAA1 by gap repair in yeast	This study

p416HAA1 (S1A1)+ HAA1-GFP	Full-length *HAA1* gene cloned in p416HAA1(S1A1) at *Spe*I and *Eco*RI, C terminal GFP tag added to *Eco*RI digested p416HAA1(S1A1)+HAA1 by gap repair in yeast	This study
p416TEF+ HAA1-His	Full-length *HAA1* gene cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ H500-His	H500 deletion of *HAA1* encoding first 500 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ H400-His	H400 deletion of *HAA1* encoding first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ N50-His	N50 deletion of *HAA1* encoding protein lacking firsts 50 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ N100-His	N100 deletion of *HAA1* encoding protein lacking firsts 100 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ N504-His	N504 deletion of *HAA1* lacking firsts 50 amino acids and encoding upto first 400 amino acids cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study
p416TEF+ N1004-His	N1004 deletion of *HAA1* lacking firsts 100 amino acids and encoding upto first 400 amino acid cloned in p416TEF at *Spe*I and *Eco*RI sites with C terminal His Tag	This study

Table 2.11 Other clones constructed in this study:

Clone Name	Description	Source
pFL44L+ YLR297w	*Xho*I and *Eco*RV digested 1267 bp long fragment from insert region of clone3807-2, carrying *YLR297w* with upstream regulatory regions cloned in pFL44l at *Sma*I site after end filling	This study
p416ADH+ NGG1	Full-length *NGG1* gene encoding 702 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (1-346)	*NGG1* deletion encoding 1-346 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (1-671)	*NGG1* deletion encoding 1-671 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (214-702)	*NGG1* deletion encoding 214-702 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (214-346)	*NGG1* deletion encoding 214-346 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (274-307)	*NGG1* with in frame deletion encoding 1-273/308-702 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
p416ADH+ NGG1 (308-373)	*Bam*HI and *Eco*RI with in frame deletion encoding 1-307/374-702 amino acids cloned in p416ADH at *Bam*HI and *Eco*RI sites	This study
pJG4-5+ NGG1	Full-length *NGG1* gene cloned in pJG4-5 at *Eco*RI site	This study
p315ADH+ MSN5	Full-length *MSN5* cloned in p315ADH at *Spe*I and *Sma*I sites	This study
p315+YGP1 (YS1)	*YGP1* gene with 390 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+YGP1 (YS2)	*YGP1* gene with 680 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study

p315+YGP1 (YS3)	*YGP1* gene with 980 bp upstream region cloned in p315ADH replacing ADH promoter at SacI and PstI sites	This study
p315+TPO2 (TS1)	*TPO2* gene with 405 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+TPO2 (TS2)	*TPO2* gene with 640 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+TPO2 (TS3)	*TPO2* gene with 1000 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+YGP1 (YS4)	*YGP1* gene with 579 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+YGP1 (YS5)	*YGP1* gene with 480 bp upstream region cloned in p315ADH replacing ADH promoter at *SacI and PstI* sites	This study
p315+YGP1 (YS6)	*YGP1* gene with 290 bp upstream region cloned in p315ADH replacing ADH promoter at *Sac*I and *Pst*I sites	This study
p315+TPO2 (TS4)	*TPO2* gene with 304 bp upstream region cloned in p315ADH replacing ADH promoter at *SacI and PstI* sites	This study
p315+TPO2 (TS5)	*TPO2* gene with 212 bp upstream region cloned in p315ADH replacing ADH promoter at *SacI and PstI* sites	This study

Chapter 3
Screening for genes conferring resistance to acetic acid in yeast *Saccharomyces cerevisiae*

3.1 Introduction:

Earlier studies have identified several genes involved in acetic acid tolerance in yeast *S. cerevisiae*, but the mechanism of adaptation to acetic acid stress is not fully understood. Moreover, deletion of most of these genes results in moderate or marginal growth defect in presence of acetic acid, with the exception of *HAA1* and few other genes. Thus identifying additional genes involved in tolerance to acetic acid was taken up as the initial objective of this work, considering their possible use in development of strains tolerating high concentration of acetic acid.

Earlier studies to identify genes involved in resistance to acetic acid and other weak acids mostly relied on either screening of gene deletion strains for sensitivity/resistance to weak acid or transcriptional response to weak acids in yeast. These genome level studies have identified several genes, belonging to distinct functional classes of yeast, to play role in tolerance to acetic acid. Genes identified based on either loss of phenotype upon deletion or transcriptional responses to acetic acid cannot be readily used in strain improvement, since the effect of modulating the expression of the individual genes need to be further studied. Thus in this study, we directly selected genes involved in acetic acid tolerance based on higher resistance to acetic acid imparted by overexpression of such genes, by screening a multicopy genomic library of *S. cerevisiae*.

3.2 Yeast Multicopy Genomic Library:

A multicopy genomic library of yeast was kindly gifted by Pierre Thuriaux, C.E.A Saclay, France. This library was made in yeast 2μ *ori* (origin of replication) based multicopy plasmid pFL44L (Figure-3.1), by cloning 4-10 kb genomic DNA fragments of *S. cerevisiae* generated by partial digestion with Sau3AI restriction endonuclease. The library represents about 10^7 ampicillin resistant clones (Stettler et al., 1993). An aliquot of plasmid DNA isolated from pooled clones was provided to us. This plasmid pool was transformed into *E. coli* strain DH10B by electroporation and approximately 10^8 ampicillin resistant clones were pooled and plasmid was isolated from this pool. Heterogeneity of insert in individual *E. coli* clones was also checked by restriction digestion of 24 individual clones with *Sph*I and *Kpn*I and size of insert DNA fragments varied from 3-10 kb, indicating that the complexity has been retained in the library (Figure-3.2). The plasmid DNA of this genomic library was transformed into *S. cerevisiae* strain *FY3*, using large scale high efficiency lithium acetate based yeast

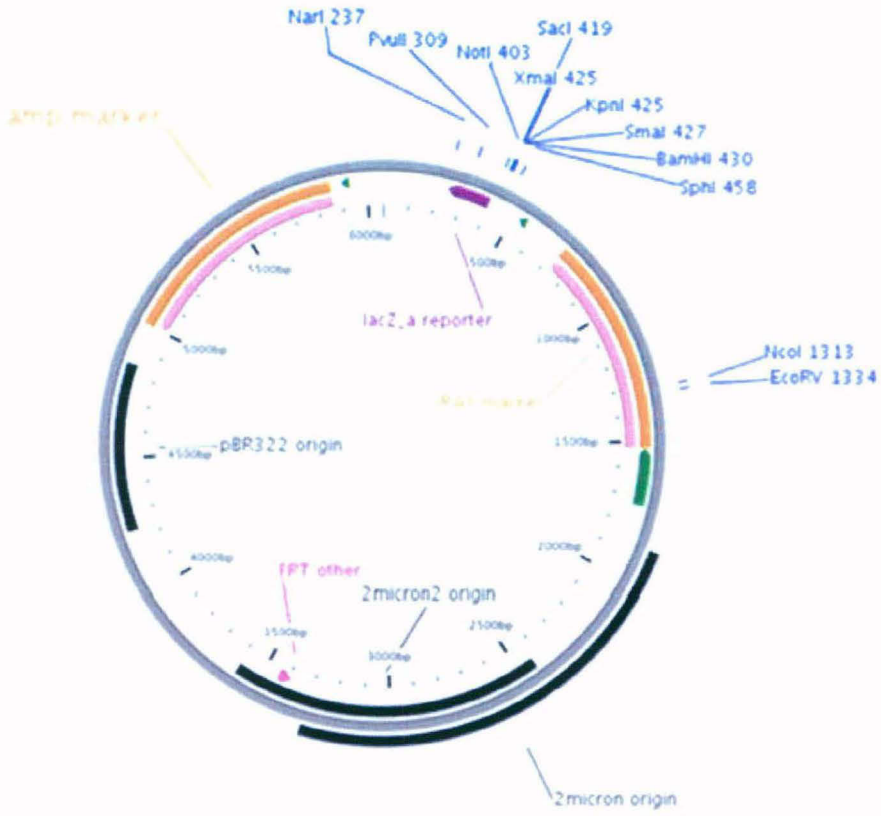
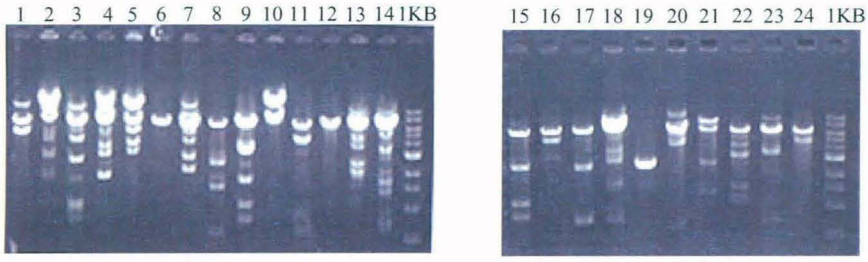


Figure 3.1: Map of vector pFL44L (6063 bp), generated using plasmapper (Dong et al., 2004).



(a) (b)

Figure 3.2: Restriction digestion of yeast genomic library clones in pFL44L plasmid. (a-b) plasmids from 24 randomly picked clones were subjected to double digestion with *Sph*I and *Kpn*I enzymes.

transformation protocol (Gietz and Schiestl, 2007). Transformants were selected on four 245 mm X 245 mm size rectangular plates containing synthetic dextrose (SD) 2% medium with agar media. Approximately 10^5 yeast transformants were selected, pooled by scraping and stored for further use. Transformation efficiency was determined by plating serial dilutions of yeast transformation mix.

3.3 Strategy for screening acetic acid tolerant clones:

The toxic effect of weak acid on yeast is seen at pH below its pKa value, where acid predominantly exists in undissociated form. Undissociated weak acid can enter the yeast cells by simple diffusion and exerts its toxic effect through acidification and anion accumulation in cytosol. The pKa value of acetic acid is 4.78, so most acetic acid sensitivity experiments in yeast are performed at pH below 4.78. Initially we chose to perform acetic acid sensitivity experiments at two different pH values, pH 4.5 and 3.0. Varying concentration of acetic acid at pH 3.0 and pH 4.5 were earlier used to check tolerance/sensitivity to acetic acid of yeast genes identified to be involved in fermentation stress tolerance in yeast (Puria et al., 2009). The wildtype strain *FY3*-pRS (FY3 strain with vector pRS306 integrated at *ura3-52* locus) can grow well on YPD agar plates containing 50 mM acetic acid at pH 3.0 and 100 mM acetic acid at pH 4.5 up to four serial dilutions, but not with 100 mM acetic acid at pH 3 and 200 mM at pH 4.5 (Figure-3.3). Thus, we screened yeast transformants carrying plasmids from multicopy genomic library of yeast on YPD agar plates containing acetic acid at pH 3.0 (50, 60, 70, 80, 90 and 100 mM acetic acid) and pH 4.5 (100, 120, 140, 160, 180 and 200 mM acetic acid). A range of acetic acid concentrations were used to select genes conferring resistance to as high a concentration of acetic acid as possible. Pool of cells representing 10^5 yeast transformants of genomic library were grown to exponential phase and 10^6 and 10^7 cells representing genomic library clones at 10-fold and 100-fold respectively, were plated on YPD agar plates containing acetic acid at concentrations and pH as mentioned above. Wildtype stain *FY3*-pRS cells were also plated on YPD plates containing similar concentration of acetic acid, to determine the frequency of spontaneous resistant clones. Yeast clones obtained at acetic acid concentrations where wildtype cells are unable to grow were further selected as acetic acid resistant clones (Table-3.1). A schematic outline of the strategy used for this screening is shown in Figure-3.4.

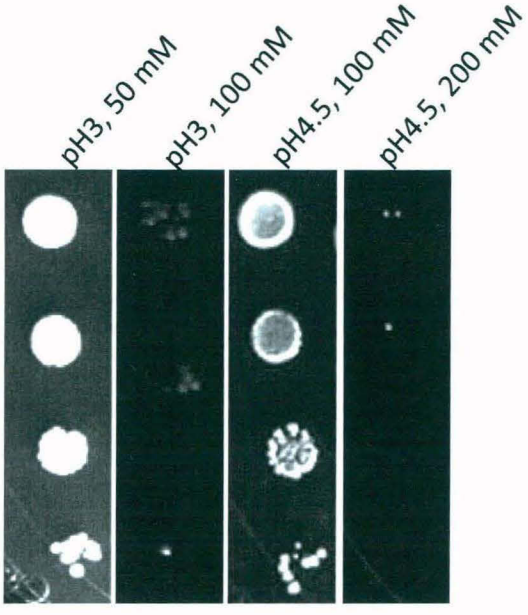


Figure 3.3: Acetic acid sensitivity of wildtype yeast strain. *S. cerevisiae* strain *FY3-pRS* was grown to exponential phase ($O.D._{600}$= 0.5-0.6), 10 fold serially diluted from 1 $O.D._{600}$ normalized suspension and 5 µl each was spotted on YPD agar plates containing 50 mM and 100 mM acetic acid, at pH 3.0 and 100 mM and 200 mM acetic acid at pH 4.5. Plates were incubated at 30°C for 60 hours and photographed.

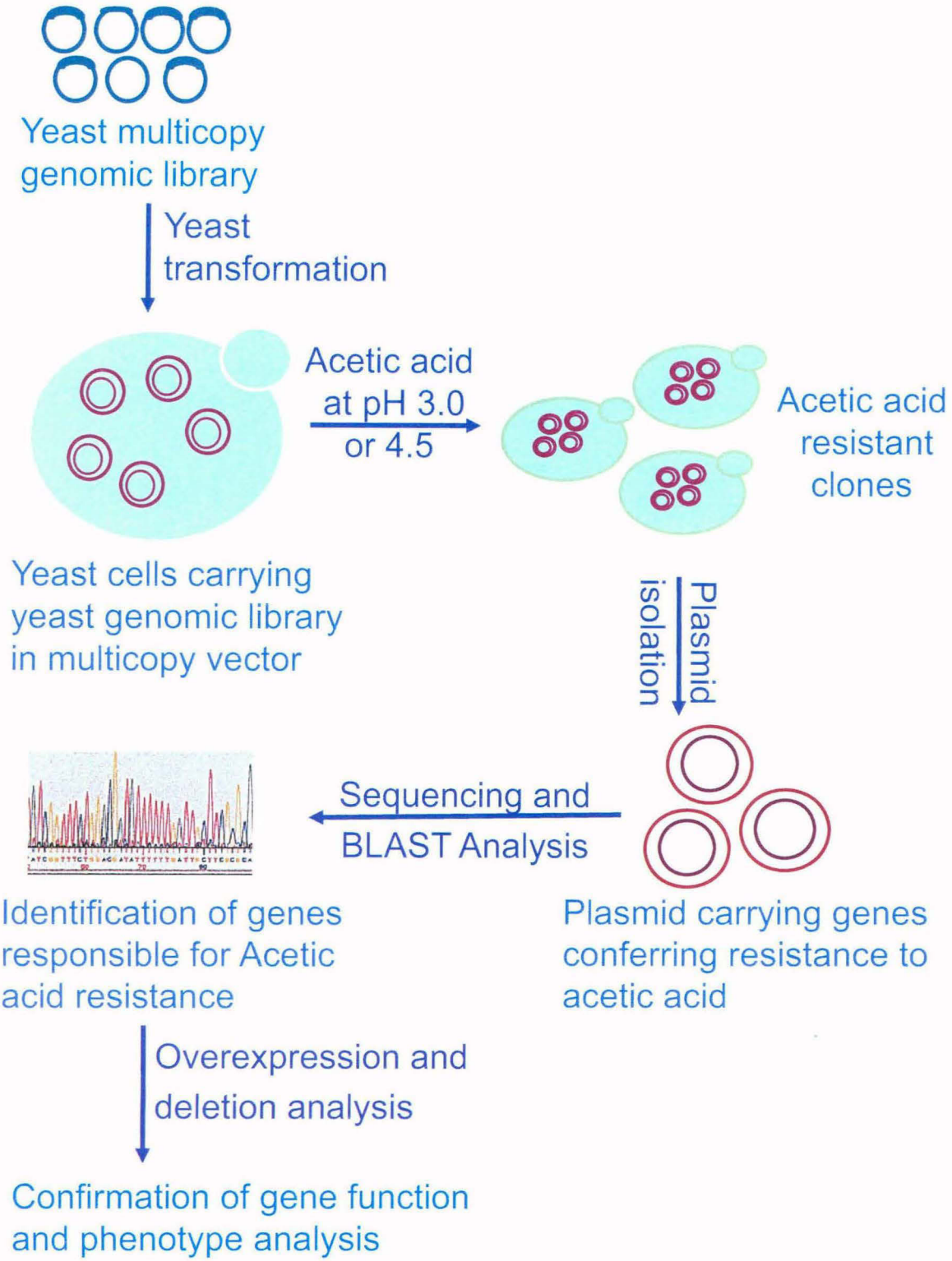


Figure 3.4: Schematic outline of steps involved in screening for genes enhancing acetic acid tolerance from yeast cells transformed with yeast genomic library.

Acetic acid	FY3pRS		Library	
pH 3.0	10^7 cells	10^6 cells	10^7 cells	10^6 cells
0 mM	Lawn	Lawn	lawn	Lawn
50 mM	Lawn	Lawn	lawn	10000s
60 mM	1000s	200-500	10000s	1000- 2000
70 mM	10s	no	1000s	100s
80 mM	no	no	100s	100- 200
90 mM	no	no	10 to 20	no
100 mM	no	no	no	no

(a)

Acetic acid	FY3pRS		Library	
pH 4.5	10^7 cells	10^6 cells	10^7 cells	10^6 cells
0 mM	Lawn	Lawn	lawn	Lawn
100 mM	Lawn	Lawn	lawn	10000
120 mM	5000-10000	1000-2000	5000-10000	1000-2000
140 mM	10 to 20	10 to 20	1000-2000	200- 400
160 mM	no	no	80-120	2 0- 40
180 mM	no	no	no	no
200 mM	no	no	no	no

(b)

Table 3.1: Conditions used for screening of acetic acid resistant yeast clones from yeast transformants with multicopy yeast genomic library, (a) at pH 3.0 and (b) pH 4.5. Wildtype (FY3-pRS) cells were also plated under similar conditions to identify conditions yielding true acetic acid resistant clones rather than spontaneous mutants. Acetic acid concentrations shown in **bold** indicate the conditions from which acetic acid resistant clones were further selected.

3.4 Outcomes of acetic acid screening:

After incubation for 48 hours yeast clones growing on acetic acid containing plates were selected. Higher number of colonies was obtained with transformants carrying genomic library compared to wildtype on YPD plates with 60, 70, 80 and 90 mM acetic acid at pH 3.0 and 140 and 160 mM acetic acid at pH 4.5 (Table-3.1). Putative acetic acid tolerant yeast clones were patched on SD agar plates and replica plated on YPD plates containing inhibitory concentration of acetic acid at pH 3.0 and pH 4.5 to confirm the tolerance. Clones successively showing resistance to acetic acid were further selected as resistant clones. Plasmids were isolated from these yeast clones and transformed into *E. coli* strain DH10B by electroporation. Plasmid isolated from respective *E. coli* transformants were sequenced with vector specific M13 reverse and M13 forward primers to identify insert region. Nine plasmids were sequenced with both M13R and M13F primers (Table-3.2), whereas 17 were sequenced with M13R primer only (Table-3.3). Four out of nine bidirectionally sequenced clones and three out of rest of 17 sequences carries *HAA1* gene, a gene already implicated in adaptation to acetic acid, thereby validating our screen. However we didn't identify any other gene, that provide as much or higher tolerance provided by *HAA1*. The other clones identified in the screen showed tolerance to at most 10 mM additional acetic acid and marginal growth advantage of just one or two dilutions (Data not shown).

3.5 Effects of media and supplements on acetic acid resistance:

In our screening FY3pRS strain was used as wildtype which has single copy of pRS306 (URA3 selection marker) plasmid integrated at *ura3-52* locus. However the yeast genomic library was constructed in multicopy plasmid, pFL44L, copy number ranges from 40-60 in yeast cells. The possibility that yeast cells transformed with single copy or multicopy plasmids, having differences in acetic acid sensitivity was tested. *FY3* cells transformed with pFL44L plasmid have similar acetic acid sensitivity as *FY3*-pRS, though slight advantage was seen for pFL44L transformed cells. Furthermore, effect of pregrowth media and supplements on acetic acid sensitivity of yeast cells was checked. Yeast strain *FY4* (*MAT*a, without any auxotrophy) and *BY4741* (*MAT*a *his3Δ leu2Δ met15Δ ura3Δ*) were checked for acetic acid sensitivity after pregrowth in SD, SC, YPD and YPDS (YPD with supplements added to SC medium). We observed that *FY4* cells were more resistant to acetic acid than *BY4741* cells, after pregrowth in respective media. Moreover yeast cells pregrown in SD and

S.N.	Clone name	Region of insert	Genes present	Acetic acid resistance
1	3606-1	chrXVI:570749..575058	*HAA1*	resistant
2	3606-3	chrXVI:572121..576682	*HAA1*	resistant
3	3707-1	chrXVI:570410..574461	*HAA1*	resistant
4	3807-2	chrXII:721968..726586	*ATP14, YLR297w* and *YHC1*	resistant in late phase of growth
5	451406-2	chrXVI:570705..574431	*HAA1*	resistant
6	90-4	chrXIII:371819..382924	*STB2, FAR1* and *YMR052C-a*	moderately resistant in late phase of growth
7	140-56	chrXIV:736079..741067	*MNT4* and *FRE4*	no benefit
8	80-3	chrIX:146572..151000	*NUP2* and *POR2*	no benefit
9	160-20	chrIX:180964..183926	*FYV1* and *YIL096C*	moderately resistant

Table 3.2: Genomic DNA regions and gene present in acetic acid resistant clones. Genomic DNA regions present in resistant clones were identified by sequencing both ends of insert with vector specific primers and by comparing the sequence to the yeast genome database by BLAST analysis. Genes present within sequenced region and subsequent acetic acid resistance provided upon retransformation of these clones in yeast is also shown. **Resistant**- 3-4 serial dilution growth benefit over wildtype strain upon 20 mM increment in acetic acid conc. at pH 3.0. **Moderately resistant**- 1-2 serial dilution growth benefit over wildtype strain upon 10 mM increment in acetic acid conc. at pH 3.0. **No benefit**- acetic acid sensitivity similar to wildtype strain.

S.N.	Clone name	Starting coordinate of insert from sequenced end	Genes present within insert region	Insert size	Acetic acid resistance
1	3706-1	chrXV:220183	*YOL057w*	+3.6 kb	not documented
2	3706-2	chrVIII:510721	*SKN7*	+4.0 kb	not documented
3	3706-3	chrII:763308	*SAF1*, and *DUG2*	+3.5 kb	not documented
4	3706-4	chrX:545980	*PTK2* and *CBF1*	+5.5 kb	not documented
5	3707-2	chrXVI:570720	*HAA1*	+6.0 kb	resistant
6	3807-1	chrXVI:570720	*HAA1*	+6.0 kb	resistant
7	451606-2	chrXVI:570419	*HAA1*	+4.5 kb	resistant
8	80-1	chrX:327868	*BIT61, YHC3* and *BNA3*	-6.0 kb	not documented
9	80-2	chrIV:835282	*YDR186c* and *UPS3*	-3.7 kb	not documented
10	80-4	chrI:72,151	*YAL037w, FUN12* and *RBG1*	+5.5 kb	not documented
11	80-5	chrX:326,780	*BIT61, IKS1* and *ZAP1*	+6.0 kb	not documented
12	90-1	chrXIII:383,692	*STV1*and *BUB1*	+4.0 kb	no benefit
13	90-3	chrIV:1,134,893	*SWR1*	+5.5 kb	moderately resistant in late phase of growth
14	90-4	chrXII:784,149	*NMA1* and *REC102*	+4.0 kb	moderately resistant in late phase of growth
15	90-5	chrXVI:137,150	*PCL8, SAR1* and *BMS1*	+3.8 kb	moderately resistant
16	160-1	chrXII:629,870	*IRC20*	+3.6 kb	moderately resistant
17	160-2	chrIV:1,335,558	*PPZ2, GPI19, THI74* and *LSR4*	+5.0 kb	moderately resistant

Table 3.3: Genomic DNA regions present in acetic acid resistant clones identified by sequencing one end of insert with M13 reverse primer. Insert length was determined by digestion of plasmids with with *Sac*I and *Sal*I, genes present within insert region are documented, (+) and (-) signs indicate sense and antisense orientation from starting coordinate, respectively. Resistant, moderately resistant and no benefit are as described in table 3.2 and **Not documented-** clones which were enriched as acetic acid resistant, but subsequently no benefit was seen, so not studied further.

SC media show more resistance than cells pregrown in YPD and YPDS media (Figure-3.5). To further understand the effect of media on acetic acid sensitivity of yeast cells, pH of spent media was determined and it was found that pH of minimal media (SD and SC) drastically reduced to 2.0 to 3.0 after 24 hours of growth. Such reduction of pH in YPD and YPDS media were slow and less significant. Thus differences in acidification of media, depending on their buffering capacity, may cause this difference in acetic acid sensitivity of yeast cells pregrown in respective media. Furthermore it was observed that sensitivity of cells to 50 to 100 mM acetic acid at pH 3.0 was comparable to 100 to 200 mM acetic acid at pH 4.5. Thus, in subsequent experiments acetic acid sensitivity/resistance was routinely tested at pH 3.0.

3.6 *YLR297w* and late growth phase specific acetic acid resistance:

While identifying the novel targets for resistance to acetic acid from yeast clones identified from multicopy genomic library screening, it was found that a clone initially named as 3807-2 showed significant resistance and growth on acetic acid containing YPD agar plates when streaked from a patch on SD agar plates stored at 4°C (Figure-3.6 (a)). But, when the same clone were grown to exponential phase in SD medium and dilution spotted on acetic acid containing YPD agar plates no significant resistance was observed (Figure-3.6 (b)). This was seen repeatedly in multiple sets of experiments, and thus it was initially hypothesized that gene/s expressed from clone 3807-2 may play a role in acetic acid resistance only in certain phase of growth, more specifically in late phase of growth. To further understand the growth phase dependent role of clone 3807-2 in acetic acid resistance, yeast cells carrying 3807-2 clone were grown to exponential phase (7-8 hours of growth; OD_{600} 0.5-0.6) and late phase (24 hours of growth; OD_{600} 6.0-7.0) in SD media and acetic acid resistance was checked by dilution spotting. In these experiments *FY3* strain carrying pFL44L plasmid (*FY3*-pFL) was used as vector control strain and a *HAA1* expressing clone 3707-1 was used as positive control. *HAA1* expressing clone 3707-1 was resistant to acetic acid in both pregrowth conditions, while FY3-pFL was sensitive. Yeast cells transformed with 3807-2 clone were as sensitive as *FY3*-pFL to acetic acid after exponential phase of growth; however they were more resistant when pregrown to late phase, which was comparable to resistance conferred by *HAA1* clone, 3707-1 (Figure-3.7).

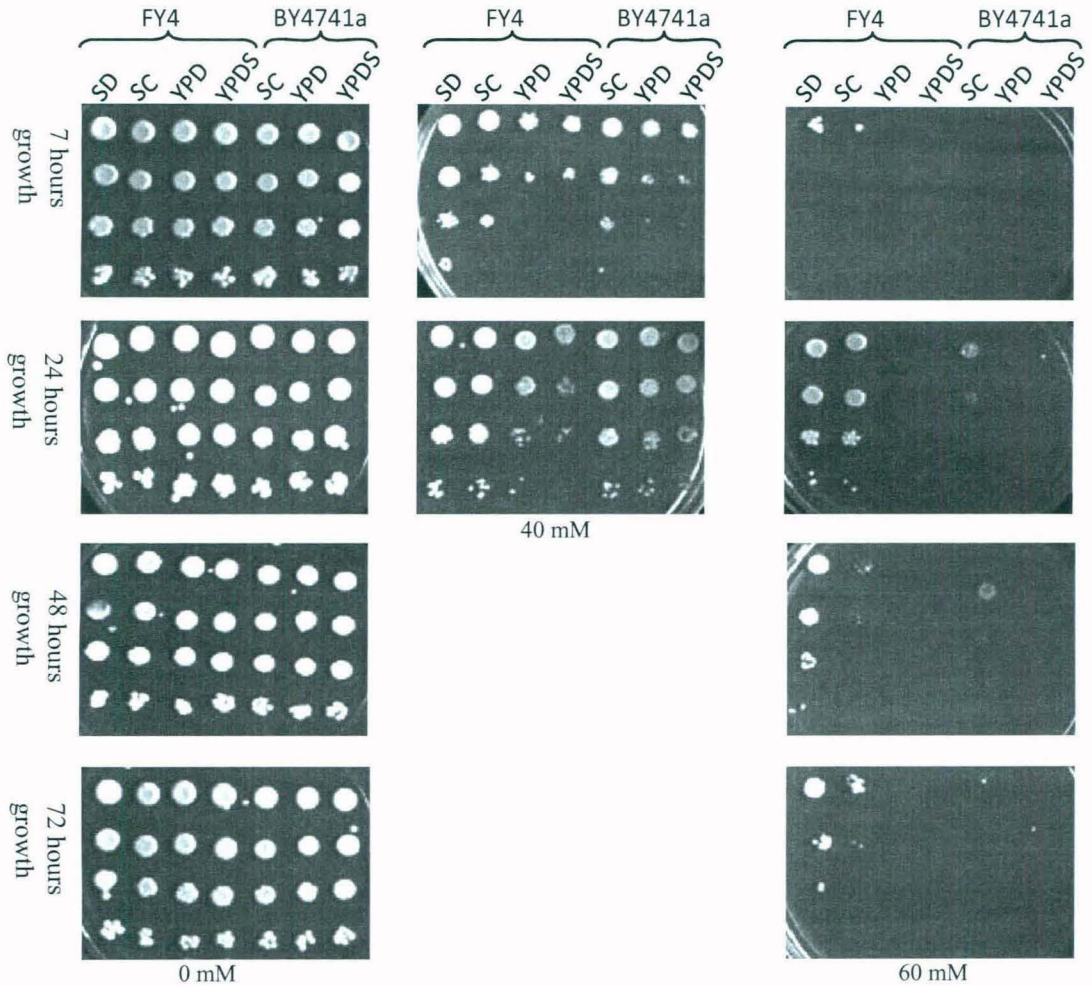
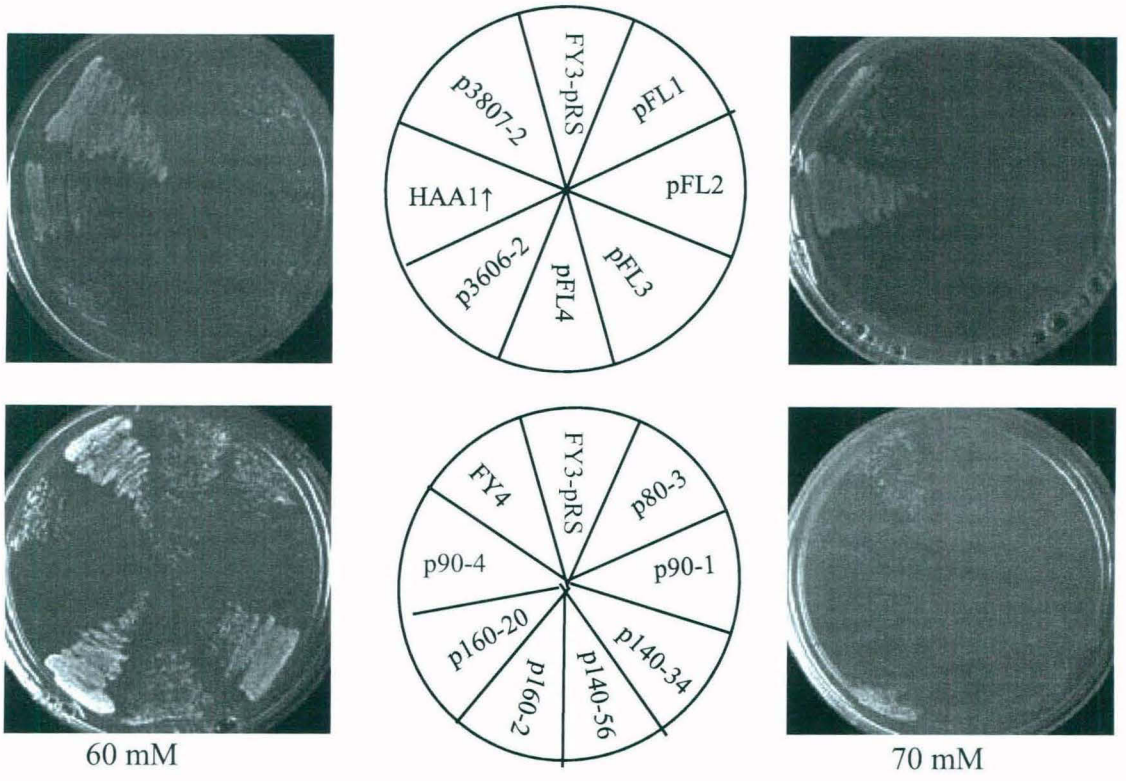
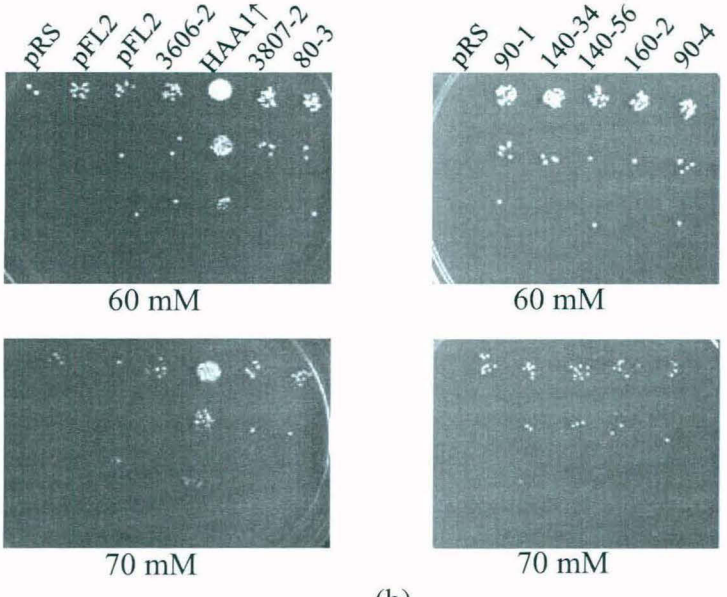


Figure 3.5: Effect of pregrowth media on acetic acid sensitivity of wildtype strains. *S. cerevisiae* strains *FY4* and *BY4741a* were grown in SD (synthetic dextrose), SC (synthetic complete), YPD and YPDS (YPD supplemented with amino acids and other essential nutrients). Acetic acid sensitivity was checked after 7, 24, 48 and 72 hours of growth by dilution spotting on YPD agar plates containing indicated concentration of acetic acid at pH 3.0.



(a)



(b)

Figure 3.6: Rechecking of acetic acid tolerance of selected clones by (a) streaking and (b) by dilution spotting after exponential growth in SD medium, on YPD plates containing acetic acid at pH 3.0 (60 mM or 70 mM) . pRS is FY3pRS, pFL1 and pFL2 are independent transformants of *FY3* stain transformed with pFL44L. HAA1↑ is *FY3* strain transformed with *HAA1* overexpressing clone p3707-1. 3606-1, 3807-2, 80-3, 90-1, 140-34, 140-56, 160-2, 160-20 and 90-4 are *FY3* transformants of multicopy library clones enriched for acetic acid resistance.

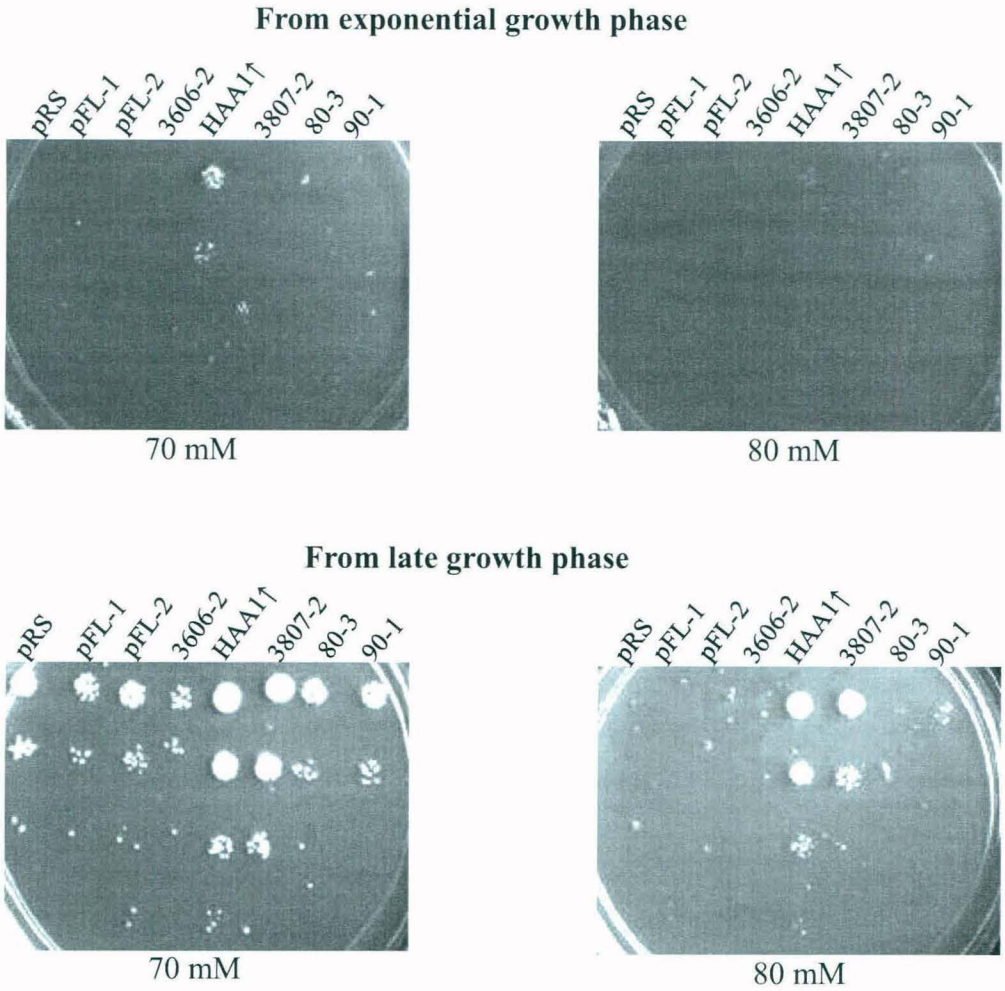


Figure 3.7: Growth phase dependent acetic acid tolerance. Few selected clones obtained in acetic acid screening were grown to exponential phase (OD_{600}=0.5-0.6) or late growth phase (OD_{600}=6.0-7.0) in SD medium and dilution spotted on YPD agar plates containing 70 mM or 80 mM acetic acid at pH 3.0. *HAA1* overexpressing clone 3707-1 is used as positive control. pRS is FY3pRS, pFL1 and pFL2 are independent transformants of *FY3* with pFL44L. 3807-2, 80-3 and 90-1, are selected *FY3* transformants of multicopy library clones enriched for acetic acid resistance.

3.6.1 Characterization of 3807-2 clone:

The 3807-2 clone is one of the acetic acid resistant clones selected from 10^7 yeast cells plated on YPD agar plate containing 80 mM acetic acid at pH 3.0. The insert region of clone 3807-2 corresponds to 4618 bp fragment from chromosome XII (chrXII:721968..726586) with four genes namely *ATP14, YHC1, YLR296w* and *YLR297w* (Figure-3.8). *ATP14* encodes subunit h of the F0 factor of mitochondrial F1F0 ATP synthase. *YHC1* encodes a component of the U1 snRNP complex required for pre-mRNA splicing. *YLR297w* is an uncharacterized ORF encoding putative protein predicted to localize in vacuole, whereas *YLR296w* is dubious ORF unlikely to code any protein (www.yeastgenome.org). To identify which gene among these confer acetic acid resistance, each ORF present within the clone 3807-2 were subcloned into pFL44L along with 500 to 600 bp upstream sequences. For subcloning, plasmid DNA of clone 3807-2 was simultaneously digested with *Hind*III, *Nde*I, *Eco*RV and *Xho*I. Unique fragments 1267bp (*YLR297w*), 922bp (*ATP14*), 934bp (*YHC1*) and 736 bp (*YLR296w*) were cloned into plasmid pFL44L (Figure-3.9). These were transformed into yeast strain *FY3* and transformants were checked for acetic acid resistance. While in exponentially growing cells, none of them were resistant to acetic acid, subclones expressing *YLR297w* (1267bp) were showing resistance to acetic acid in late growth phase comparable to 3807-2 (Figure-3.10).

3.6.2 *YLR297w:*

YLR297w is a 390 bp long uncharacterized ORF which encodes a 129 amino acids long protein with a predicted transmembrane domain of 23 amino acids between 77-99 residues. It does not have any known function, but genome wide GFP localization studies have shown its possible localization in yeast vacuole (Huh et al., 2003). It also does not have any similarity to other protein sequence in yeast or other organisms. Although no function is as yet assigned to *YLR297w*, its expression is enhanced in late phase of growth and by a variety of stress conditions in yeast. *YLR297w* expression is induced by exposure of yeast cells to DNA damaging agent like 8-methoxypsoralen and UVA irradiation (Caba et al., 2005; Dardalhon et al., 2007), peroxide, salt, and acid stress (Causton et al., 2001) and nitrogen depletion, stationary phase, diauxic shift and heat shock (Gasch et al., 2000). Expression of *YLR297w* is also reported to be induced by acetic acid stress in yeast in Haa1p dependent manner, but no significant role for *YLR297w* is predicted in adaptation to acetic acid (Fernandes et al., 2005; Mira et al., 2010a). Expression of *YLR297w* is regulated by at least 21 transcription factors as

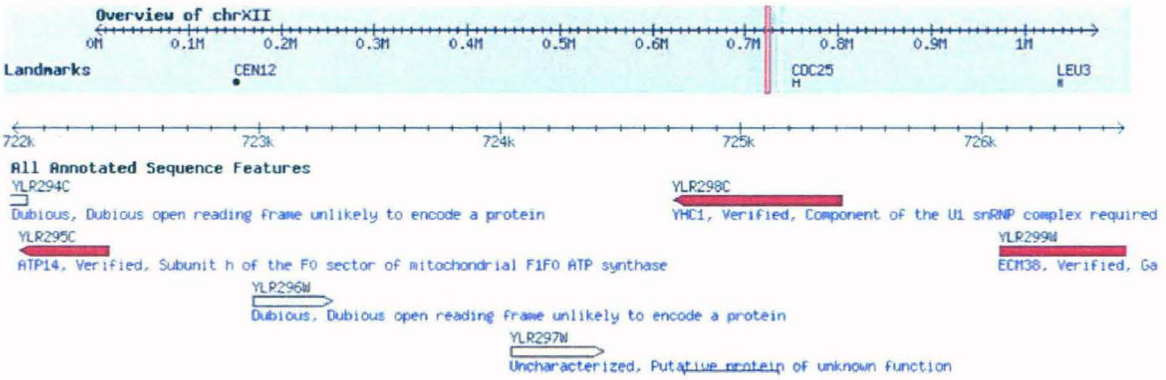


Figure 3.8: Chromosome coordinates of insert region of clone p3807-2. Insert region of this clone was determined by sequencing with vector specific M13 Reverse and M13 Forward primers. Insert region shown is from *S. cerevisiae* chromosome XII and represents chromosome coordinates chrXII:721968..726586. Figure taken from SGD (www.yeastgenome.org).

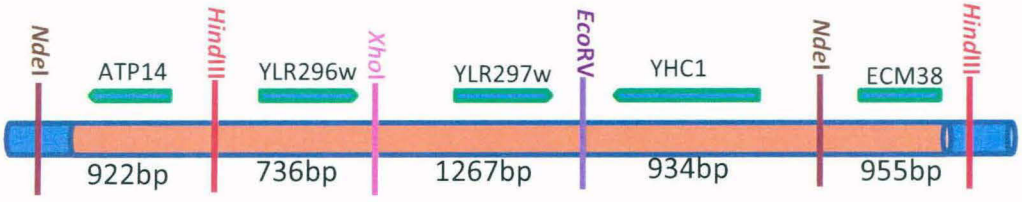


Figure 3.9: Subcloning of genes present in insert region of clone p3807-2. p3807-2 plasmid was simultaneously digested with *Hind*III, *Nde*I, *Eco*RV and *Xho*I and subcloned into pFL44L. All genes were subcloned with approximately 400 to 500 bp upstream regulatory sequences, except *ECM38* which had only N-terminal region. Orange bar indicates insert region and its blue flanks indicate the vector sequences. Figure is not drawn to scale.

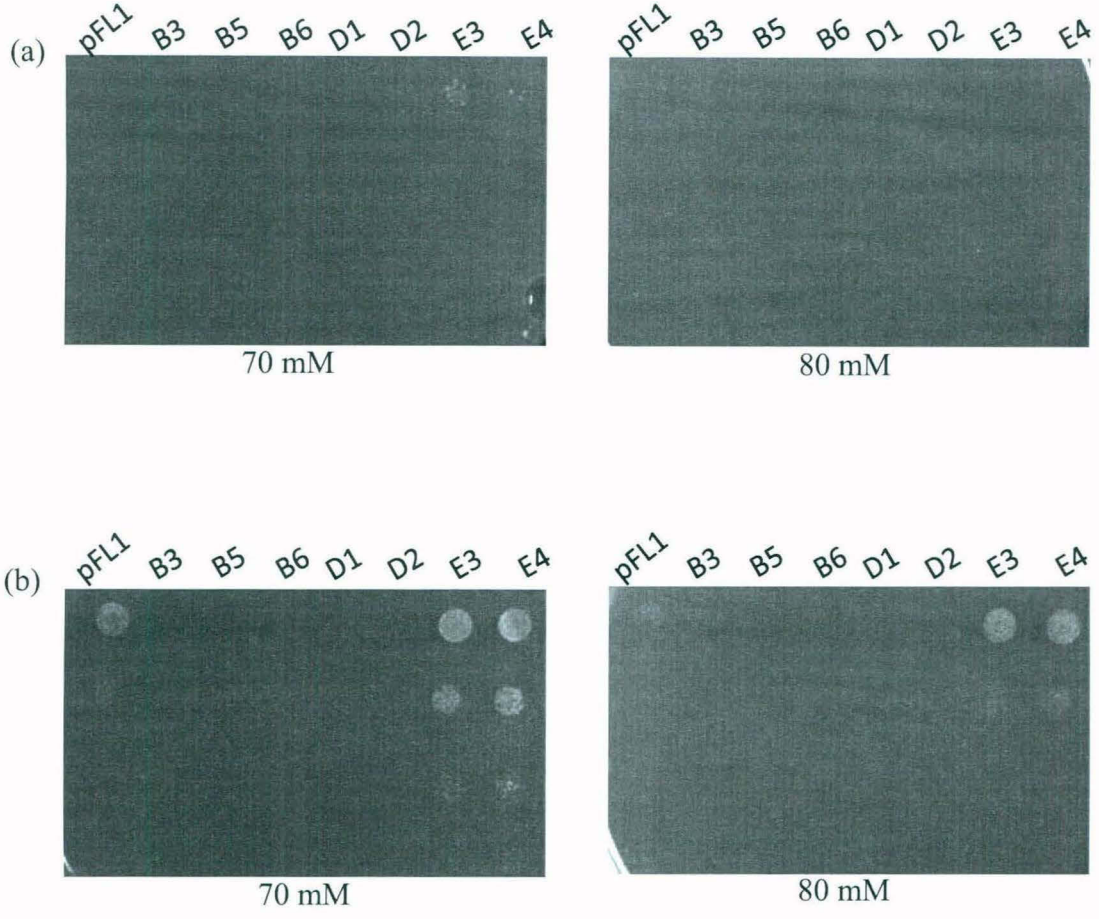


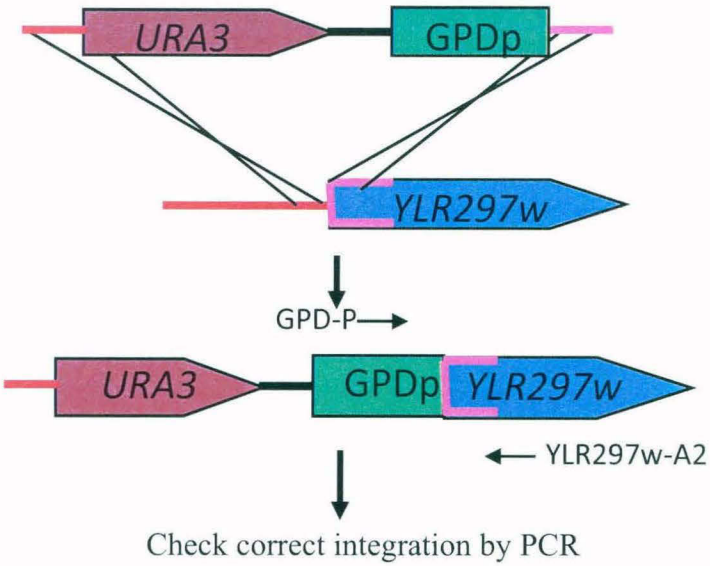
Figure 3.10: Acetic acid tolerance of subclones of p3807-2. Yeast strains were grown to (a) exponential phase (OD_{600}=0.5-0.6) or (b) late growth phase (OD_{600}=6.0-7.0) in SD medium and acetic acid tolerance was checked by dilution spotting on YPD agar plates with 70 mM or 80 mM acetic acid at pH 3.0. pFL1 is vector control. B3, B5 and B6 are subclones carrying *YHC1* or *ATP14*. D1 and D2 are subclones carrying *YLR296w* and E3 and E4 are subclones carrying *YLR297w*.

per the data available at Yeastract database; these include mainly stress induced transcription factors such as Haa1p, Yap1p, Yap5p, Msn2p, Msn4p and Pdr1p (Teixeira et al., 2006).

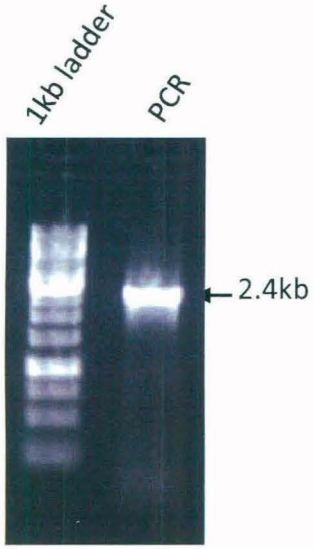
3.6.3 *YLR297w* contributes to late growth phase specific acetic acid resistance:

The late growth phase specific acetic acid tolerance conferred by *YLR297w* might be due to its restricted expression mainly during this phase. If so, then its constitutive overexpression might make the cells resistant to acetic acid during exponential growth phase as well. To test this, yeast strain overexpressing *YLR297w* from a strong and constitutive GPD promoter were constructed. For this purpose, GPD promoter along with *URA3* auxotrophic selection marker was PCR amplified from pGV8 (Sharma, 1998) plasmid with 40 bp homology at both ends of start codon of *YLR297w* for integration at just upstream of coding sequence. This PCR generated cassette was gel purified and transformed into yeast strain *FY3* and transformants were selected on SD agar plates. Integration of cassette at the correct locus was confirmed by diagnostic PCR using primers specific for GPD promoter and *YLR297w* coding sequence (Figure-3.11). Yeast strains with *YLR297w* under the control of GPD promoter were then checked for acetic acid resistance along with *FY3*-pRS strain as wildtype control. Constitutive overexpression of *YLR297w* resulted in resistance to acetic acid only in late phase of growth and no resistance was seen for cells harvested from exponential phase of growth (Figure-3.12). These observations indicate that Ylr297wp function in acetic acid resistance is specific to stationary or late growth phase and may depend on protein(s) that are expressed only during late growth phase.

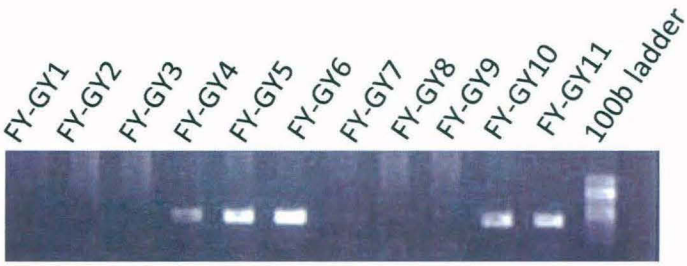
Since acetic acid resistance caused by overexpression of *YLR297w* is seen only in late phase of growth, the time course of activation of acetic acid resistance was studied. Yeast strain *FY3* carrying plasmid pFL44L (vector control), p3707-1 (HAA1 expressing clone), p3807-2 and pFL44L subclone expressing *YLR297w* were grown in SD media. Cells were harvested after 12 hours, 15 hours, 18 hours and 24 hours of growth and dilution spotted on acetic acid containing YPD agar plates. *YLR297w* overexpressing cells from 18 hours old culture could tolerate 70m mM acetic acid and cells from 24 hours old culture could tolerate up to 80 mM acetic acid at pH 3.0 (Figure-3.13). The growth rate of these strains was comparable and maximum cell density was reached in SD medium by 18 hours (Figure-3.14). Thus, the role of Ylr297wp in acetic acid tolerance becomes obvious only in saturated cultures, whereas Haa1p function is not growth phase dependent.



(a)



(b)



(c)

Figure 3.11: Constitutive overexpression of *YLR297w* under GPD promoter. (a) Schematic outline of strategy for replacement of *YLR297w* promoter with GPD promoter. The position of primers GPD-P and YLR297w-A2 used for confirming correct integration of promoter are also shown. (b) Agarose gel showing 2.4 kb band of PCR amplified *YLR297w* promoter replacement construct. (c) Diagnostic PCR to check integration of GPD promoter upstream of *YLR297w* coding sequence.

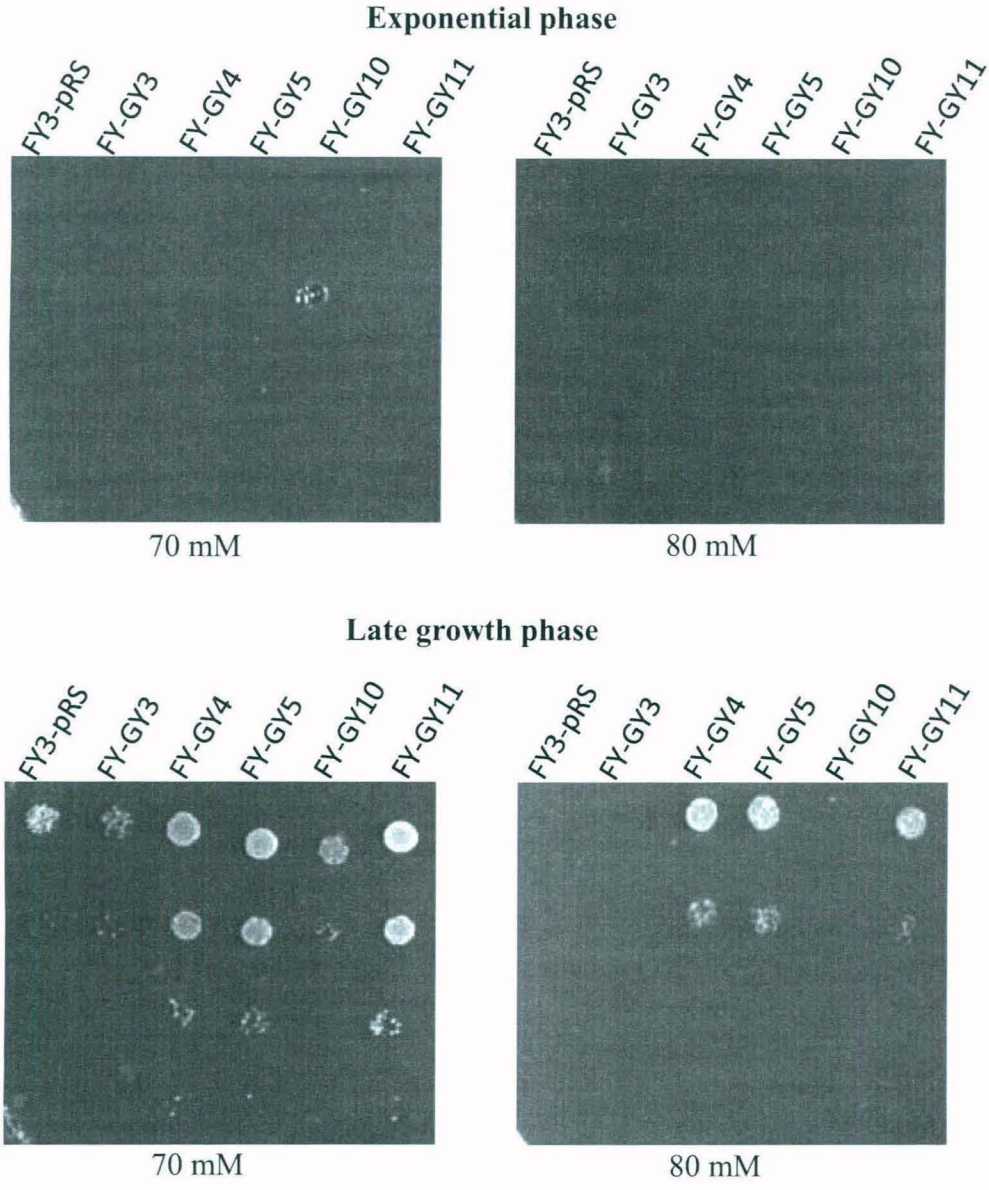


Figure 3.12: Acetic acid tolerance upon constitutive overexpression of *YLR297w*. Yeast strains were grown to exponential phase (OD_{600}=0.5-0.6) or late growth phase (OD_{600}=6.0-7.0) in SD medium and acetic acid tolerance was checked by dilution spotting on YPD agar plates containing 70 mM or 80 mM acetic acid at pH 3.0. FY3-pRS is *FY3* transformed with pRS306. FY-GY4, FY-GY-5, FY-GY10 and FY-GY11 are *FY3* strain transformants in which *YLR297w* promoter was replaced with GPD promoter and confirmed by diagnostic PCR. FYGY3 was not confirmed for promoter replacement and served as negative control.

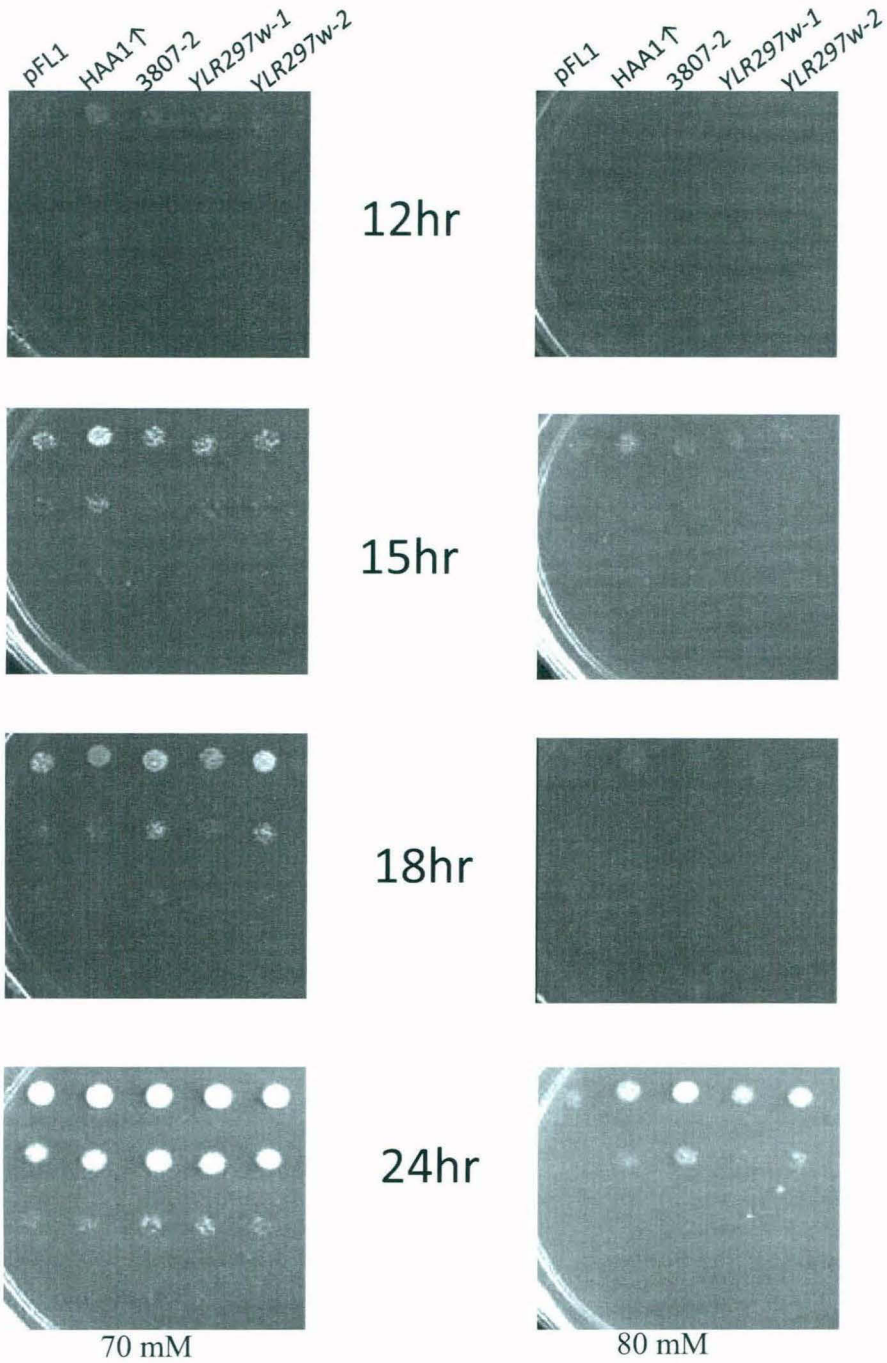


Figure 3.13: Time dependent acetic acid tolerance of *YLR297w* overexpressing strains. Yeast strains were grown in SD media and acetic acid tolerance was checked by dilution spotting on YPD agar plates containing acetic acid at pH 3.0 after 12, 15, 18 and 24 hours of growth. The results of 70 mM and 80 mM are shown. *HAA1* overexpressing clone 3707-1 is used as positive control. pFL1 is *FY3* transformed with pFL44L; 3807-2 is *FY3* transformed with *YLR297w* overexpressing plasmid p3807-2 and *YLR297w*-1 and -2 are independent transformnats of *FY3* with *YLR297w* coding region subcloned in pFL44L along with its native promoter.

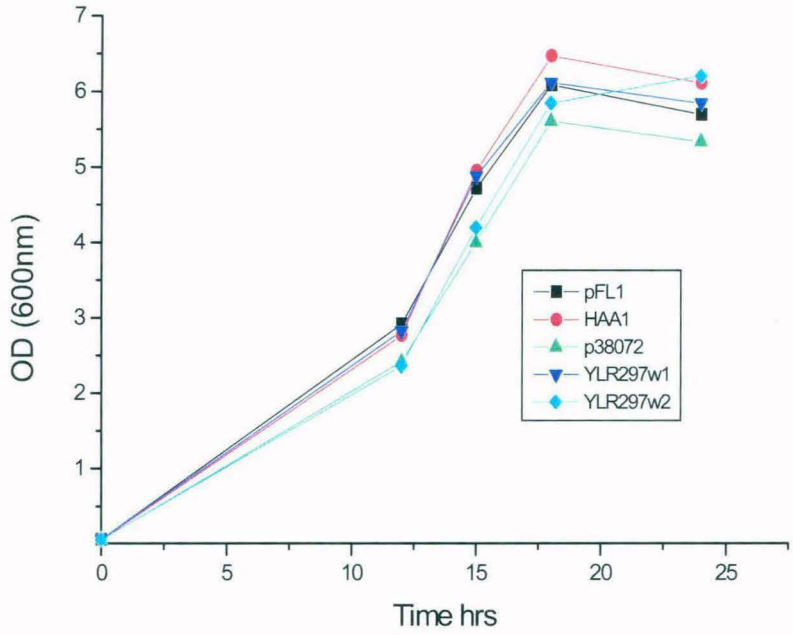


Figure 3.14: Growth curves of *YLR297w* overexpressing strains. Yeast strains were inoculated in SD (synthetic dextrose) media at 0.05 $O.D_{600}$ and growth was monitored by $O.D._{600}$, at time points corresponding to dilution spotting for examining time dependent acetic acid tolerance of *YLR297w* overexpressing strains in figure 3.13. Strains are same as listed in figure 3.13.

3.6.4 *YLR297w* is not essential for acetic acid resistance, but overexpression of *YLR297w* leads resistance to wide range of weak acids:

HAA1 is required for adaptation of yeast cells to relatively hydrophilic weak acids such as acetic acid and propionic acid, but not for adaptation to lipophilic acids such as octanoic acid and benzoic acid. Expression of *YLR297w* is also regulated by Haa1p, although role of *YLR297w* in adaptation to acetic acid stress is not shown (Fernandes et al., 2005). The role of *YLR297w* in resistance to lipophilic acids was examined by dilution spotting yeast strains overexpressing *YLR297w* on YPD plates containing inhibitory concentrations of lipophilic acids at pH 4.0. Besides providing resistance to acetic acid, *YLR297w* overexpression in yeast also results in resistance to propionic acid and lipophilic acids like octanoic acid and benzoic acid in late phase of growth. Yeast strain overexpressing *HAA1* was resistant to propionic acid but not to lipophilic octanoic and benzoic acids (Figure-3.15). These results indicate that *YLR297w* has much wider role in adaptation of yeast cells to broad range of weak acids, whereas Haa1p functions only in adaptation to hydrophilic acids.

Earlier studies have shown that deletion of *HAA1* and its target genes *TPO2* and *TPO3*, render yeast cells to weak acid stress, a marginal sensitivity on deletion of *YLR297w* is also shown (Fernandes et al., 2005). To further examine the role of *YLR297w* expression in yeast cells adaptation to acetic acid in stationary phase of growth, *ylr297wΔ* strain, along with *haa1Δ* and *tpo3Δ* strains, was checked for acetic acid sensitivity by dilution spotting after exponential and 24 hours of growth. The *ylr297wΔ* strain showed marginal to no sensitivity towards acetic acid, whereas as previously reported *haa1Δ* and *tpo3Δ* were found to be sensitive to acetic acid (Figure-3.16 (a)). Sensitivity of these strains to lipophilic weak acids benzoic acid, octanoic acid and propionic acid was also checked (Figure-3.16 (b-d)). The *ylr297wΔ* strain shows marginal sensitivity towards propionic acid as well as lipophilic weak acids tested both in exponential and late growth phase. On the other hand *haa1Δ* strain was sensitive to propionic acid, marginally sensitive to benzoic acid and not sensitive to octanoic acid. The *tpo3Δ* strain showed moderate sensitivity for benzoic acid, but no sensitivity towards propionic acid and octanoic acid. *YLR297w* deletion showed only marginal sensitivity to acetic acid and other weak acids tested even in late growth phase, possibly because the role of Ylr297wp can be either bypassed or performed by alternative mechanisms. Nevertheless, an important role for *YLR297w* cannot be ruled out as its overexpression increases tolerance of yeast cells to multiple weak acids.

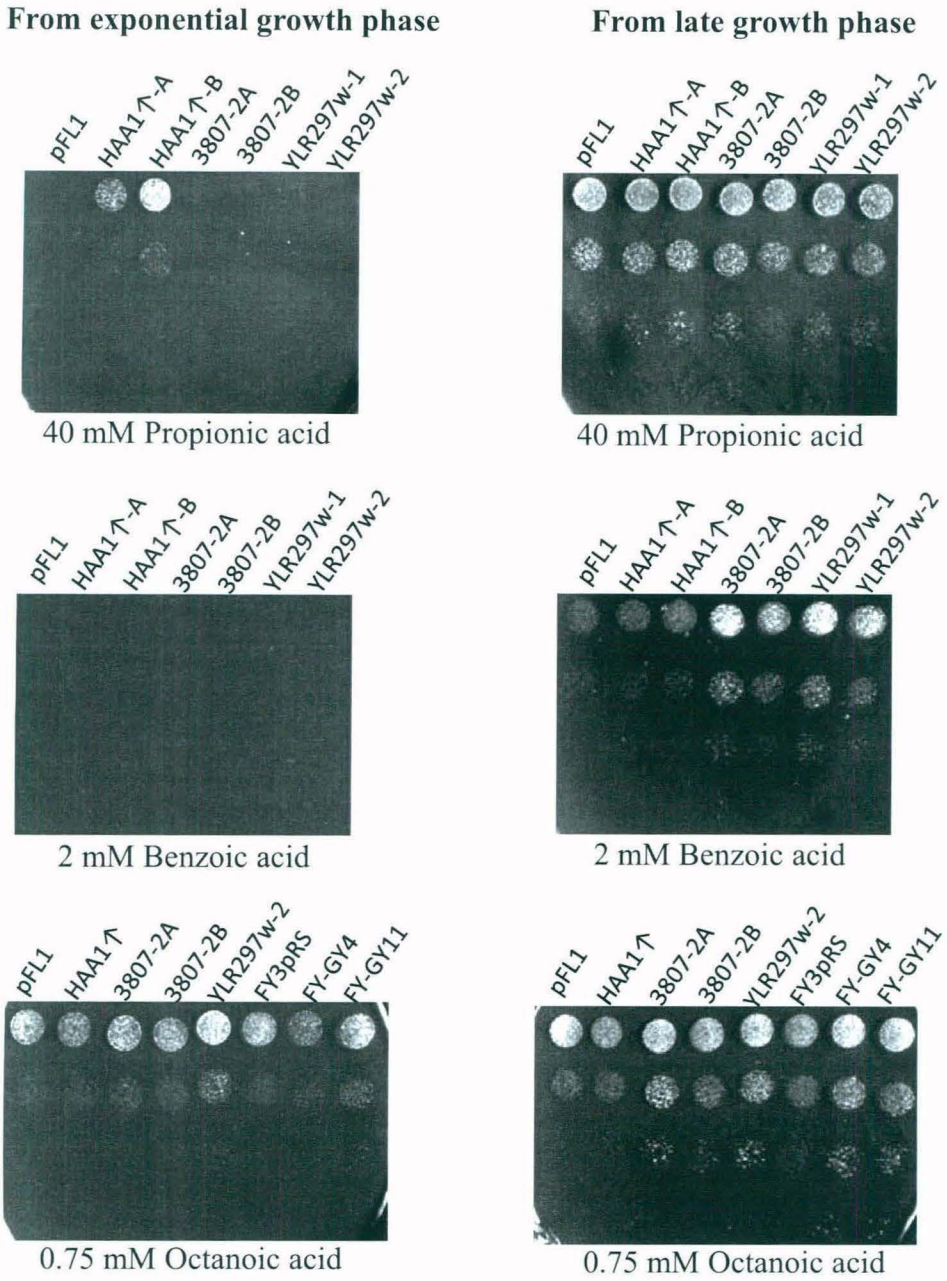


Figure 3.15: Weak acid tolerance upon overexpression of *YLR297w*. Yeast strains were grown to exponential phase (OD_{600}=0.5-0.6) or late growth phase (OD_{600}=6.0-7.0) in SD media and dilution spotted on YPD agar plates containing indicated concentrations of propionic acid, octanoic acid and benzoic acid at pH 4.0. pFL1 is *FY3* transformed with pFL44L, HAA1↑-A and B are *FY3* independent transformants of *HAA1* overexpressing clone p3707-1. 3807-2A and B are *FY3* independent transformants of *YLR297w* overexpressing clone p3807-2 in FY3. *YLR297w*-1 and -2 are *FY3* independent transformants of *YLR297w* coding region subcloned in pFL44L along with native promoter. FY3-pRS is *FY3* transformed with pRS306. FY-GY4 and FY-GY11 are transformants of *FY3* in which *YLR297w* promoter is replaced with GPD promoter.

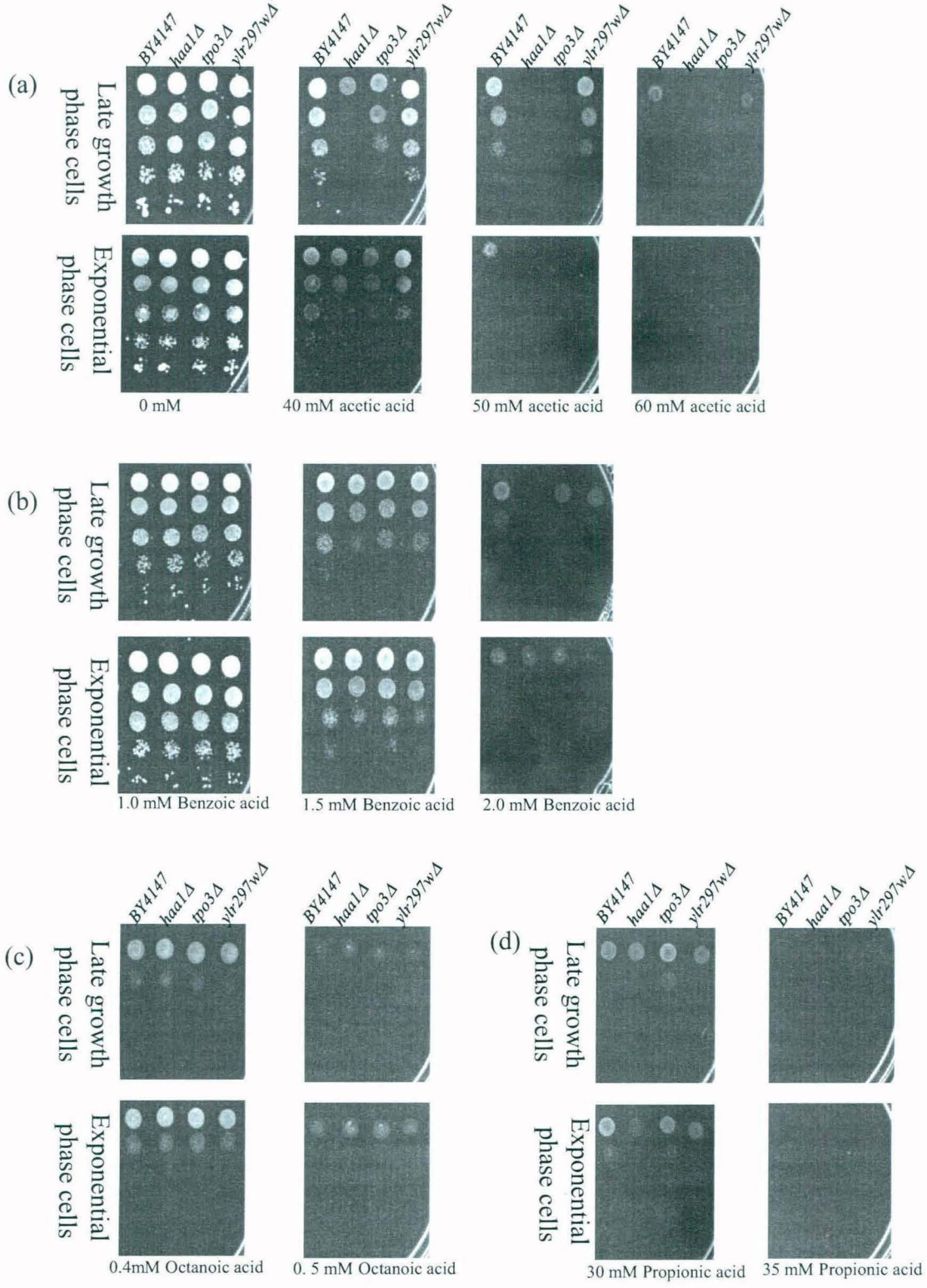


Figure 3.16: Weak acid sensitivity of *ylr297wΔ* strain, Yeast strains were grown to exponential (OD_{600}=0.5-0.6) or late growth phase (OD_{600}=6.0-7.0) in SD media supplemented with histidine, uracil, methionine and leucine and dilution spotted on YPD agar plates containing (a) acetic acid at pH 3.0, and (b) Benzoic acid, (c) octanoic acid and (d) propionic acid at pH 4.0.

3.6.5 Localization of Ylr297wp:

To precisely check if overexpression of *YLR297w* effects its localization in yeast cells, YFP (Yellow fluorescent protein) construct was integrated at the 3' terminal end of *YLR297w* coding sequence. YFP (Venus) fluorescent tag along with G418 resistant selection marker was PCR amplified from pBS7 plasmid using primer set having 40 bp overhangs homologous to 3' terminal end of *YLR297w* coding sequence. This PCR product was transformed into yeast strain *FY3*-pRS (*YLR297w* expressing under its own promoter) and FYGY11 (*YLR297w* expressing under strong GPD promoter). Positive transformants were selected on G418 drug selection plates and further confirmed by unique PCR product obtained from *YLR297w* and tag specific primers. Fusion protein is functional since YFP tagged Ylr297wp expressed under GPD promoter conferred acetic acid resistance in late phase of growth. These strains were either grown up to exponential phase or late phase of growth, cell were harvested, washed with PBS and directly used for microscopic observation. Slides were prepared by adhering cells to poly-L-lysine coated coverslip followed by washing away unadhered cells and mounting coverslip on slide with mounting media. Prepared slides were visualized by Ziess confocal microscope at 100X magnification. In yeast strains expressing *YLR297w* under its own promoter very faint fluorescent signal was observed in cytosol from exponential as well as stationary phase cells (Figure-3.17a & b). Similar but more intense fluorescent signal was observed in discrete location of cytosol from yeast strains expressing *YLR297w* under constitutive GPD promoter (Figure-3.17c & d). This indicates that Ylr297wp localizes to some internal compartment of cell and possibly on vacuolar membrane.

3.6.6 *YLR297w* mediated acetic acid adaptation requires acidification and presence of acetic acid in pregrowth media:

The chronological life span of yeast, which is measured as the survival time of populations of nondividing cells, has been used successfully for the identification of key pathways responsible for the regulation of aging. Recently it is shown that acetic acid produced by yeast cell in stationary phase decreases chronological life span and it can be increased either by dietary or calorie restrictions (reduced glucose content in pregrowth media) that reduces acetic acid production or by providing resistance to acetic acid (Burtner et al., 2009). In one of our experiments we have observed that acetic acid tolerance caused by *YLR297w* overexpression is seen after pregrowth in SD media only but not when cells were pregrown in YPD media. In the same experiment *HAA1* overexpression results in acetic acid tolerance

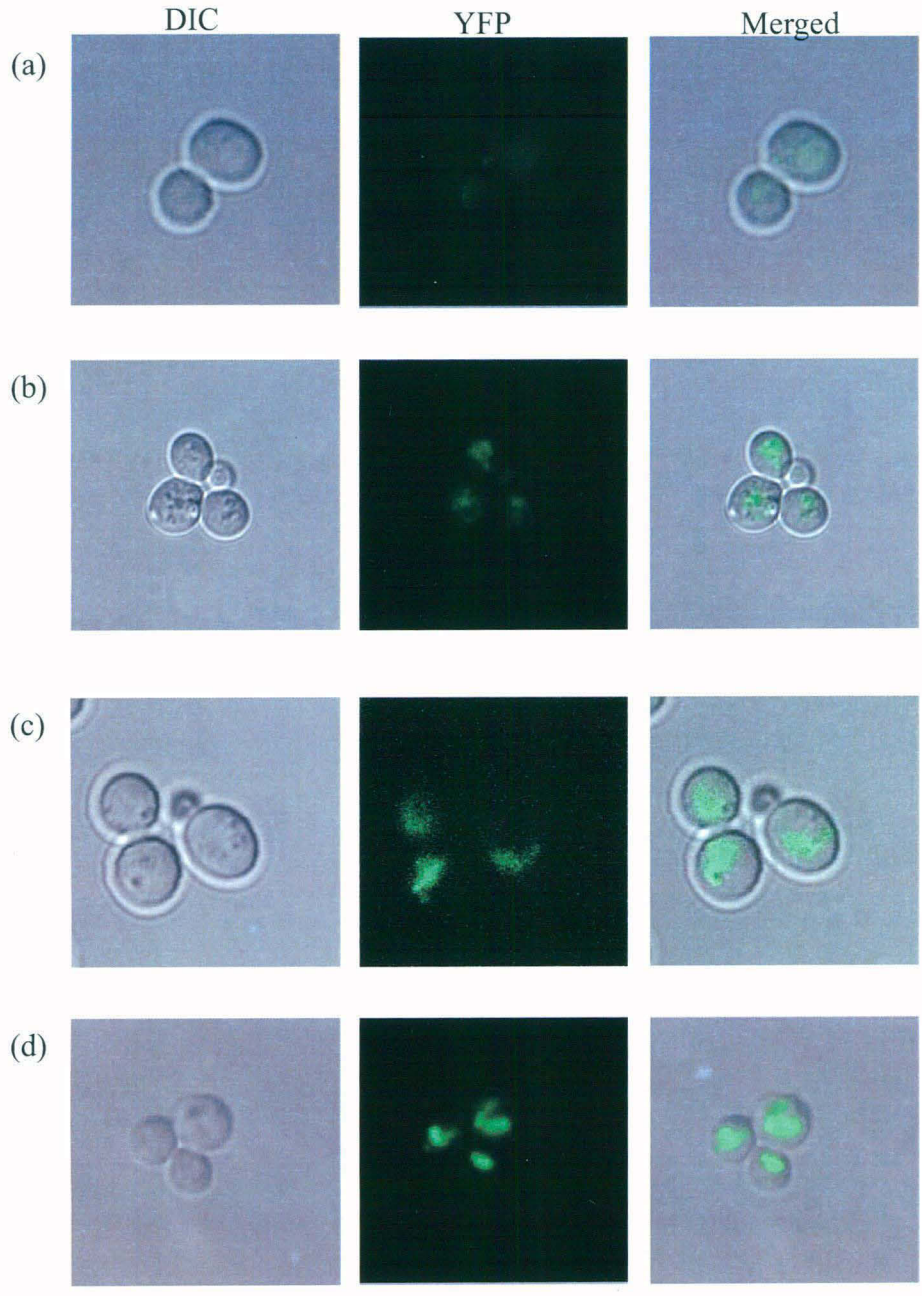


Figure 3.17: Localization of Ylr297wp-YFP. YFP tagged Ylr297wp expressed from own promoter (a & b) or GPD promoter (c & d) and observed in exponentially growing cells (a & c) and in late growth phase cells (b & d). For each set of figures DIC, YFP fluorescence and merged images are shown.

after pregrowth in both SD and YPD media (Figure-3.18). Moreover pH of media was checked after growth in respective media and acidification of media was seen after 24 hours growth in SD media whereas no such acidification was observed in YPD media. So we checked the effect of glucose content, pH and presence of acetic acid in pre-growth media on acetic acid resistance. Yeast cells overexpressing *YLR297w* either from multicopy vector pFL44L or under constitutive GPD promoter were checked for acetic acid resistance with respect to their control strains. These strains were pregrown in SD media with 2% glucose, 0.5% glucose, 0.05% glucose or with 2% glucose with pH adjusted to 6.0 with citrate phosphate buffer. After 24 hours of growth in various media we observed that pregrowth in SD media with 2% glucose with or without pH adjustment $O.D_{600}$ for different strains tested reaches 6.0-7.0, whereas in SD media with 0.5% glucose $O.D_{600}$ reaches to 2.5 to 3.0 and in media with 0.05% glucose it reaches to 1.5 to 2.0. Acetic acid resistance of these strains was checked after exponential growth and late phase of growth (24 hours growth). Acetic acid resistance was not observed on overexpression of *YLR297w* in yeast cells harvested from any of the above mentioned media in exponential phase of growth. On the other hand *YLR297w* overexpression in stationary phase results in acetic acid resistance after pregrowth in SD media with 2% glucose or with 0.5% glucose, whereas pregrowth in SD media with 0.05% glucose or pH buffered to 6.0 does not confer any acetic acid resistance (Figure-3.19). To examine the effect of acidification, the pH of these pregrowth media was also checked after 24 hours. In SD medium with 2% glucose pH reduces to 2.7 to 2.8, in media with 0.5% glucose pH reduces to 3.4-3.5, whereas in media with 0.05% glucose (4.9-5.0) and media with pH adjusted to 6 (5.2-5.3) no such acidification is seen. This observation is consistent with the report that when yeast cells growing in SC media with 2% glucose or with 0.5% glucose approach stationary phase, pH of media become highly acidic whereas growth in media with 0.05% glucose or pH buffered to 6.0 such acidification is not observed (Burtner et al., 2009). These observations suggests that acidification of pregrowth media is prerequisite for *YLR297w* mediated acetic acid resistance.

Meanwhile we checked the effect of acetic acid produced by yeast cells in late phase of growth on acetic acid resistance caused by *YLR297w* overexpression. Burtner et al. (2009) have shown that yeast grown in SC media (2% glucose), glucose is consumed by 12 hours of growth, and significant acidification is observed after 24 hours and pH finally reaches 2.82 after 96 hours. Moreover accumulation of acetic acid up to 10 mM was reported after 50 hours growth SC media that causes chronological aging in yeast (Burtner et al., 2009). In our

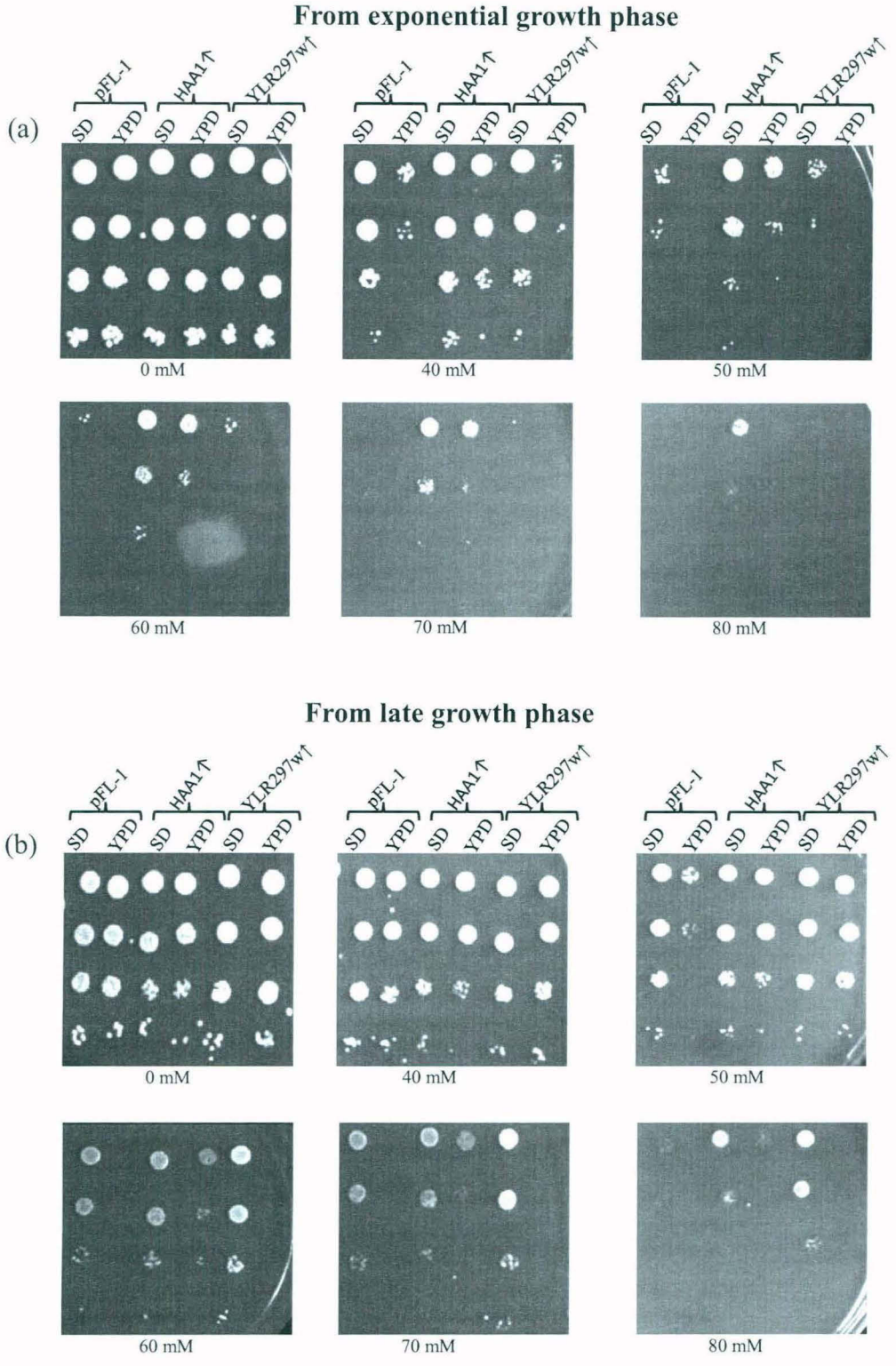
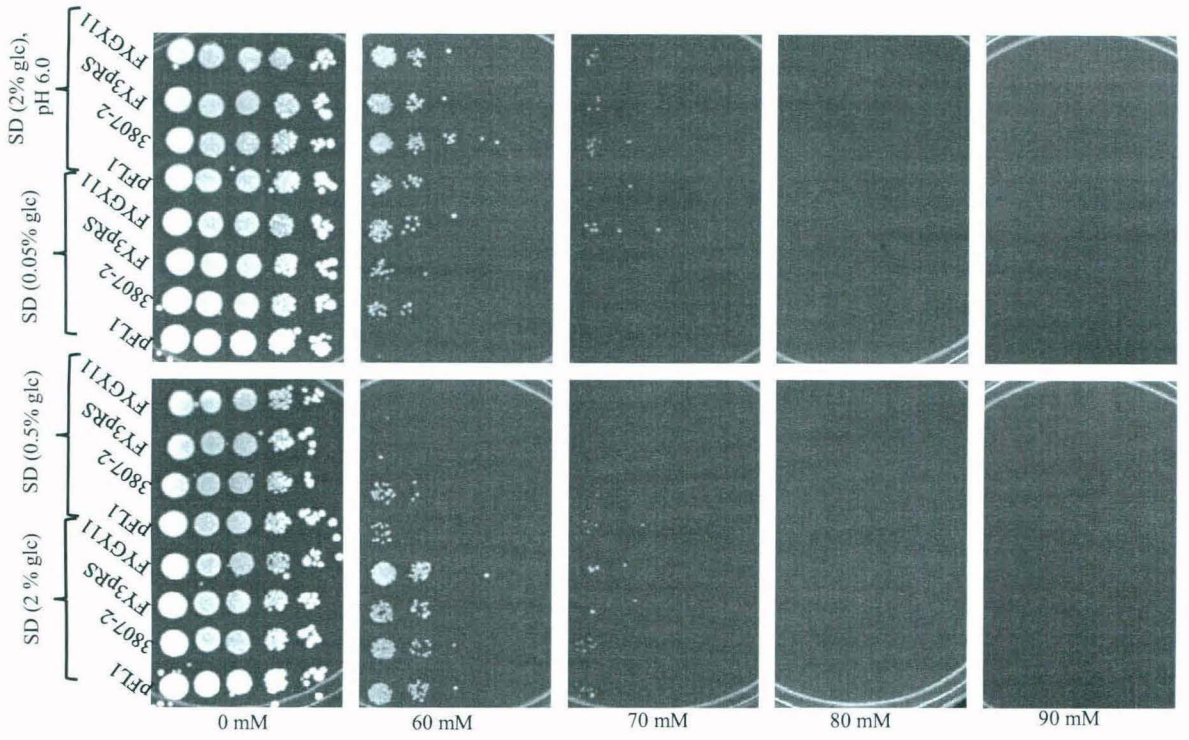
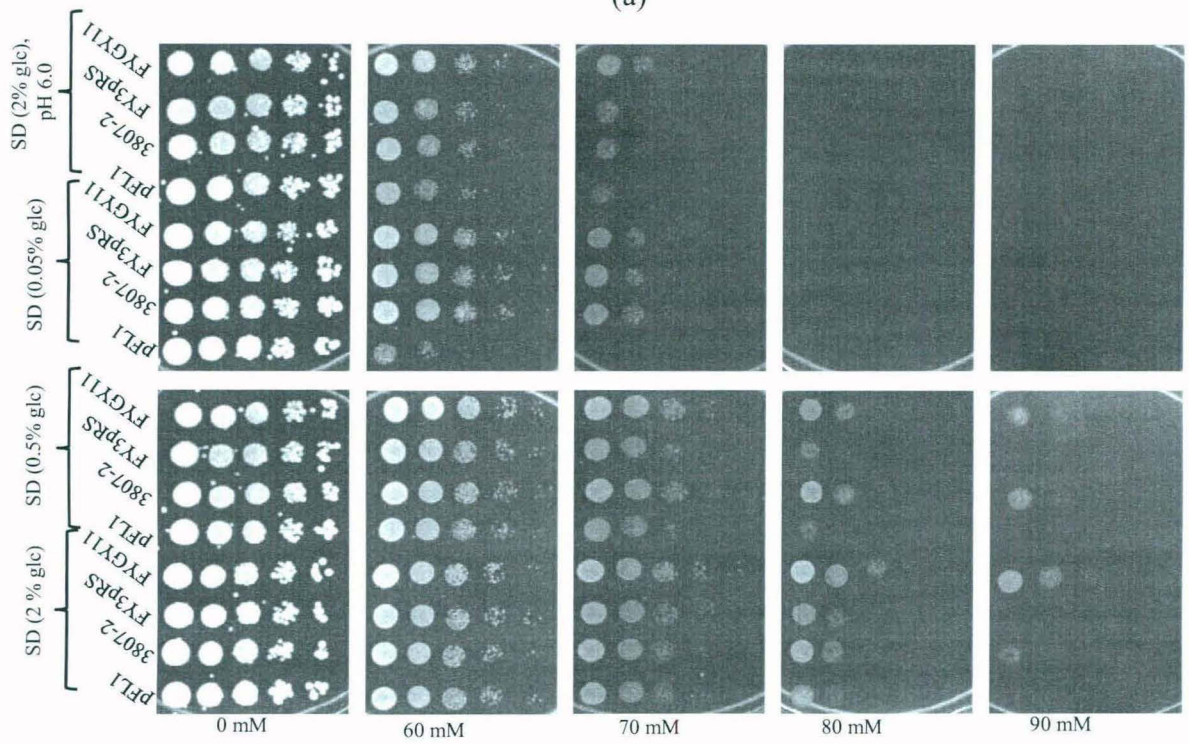


Figure 3.18: Effect of pregrowth media on acetic acid tolerance mediated by *YLR297w*. Yeast strains were grown in SD or YPD to (a) exponential ($O.D._{600}$ 0.5-0.6) or (b) late growth phase ($O.D._{600}$ 6.0-7.0) and dilution spotted on acetic acid containing YPD plates at pH 3.0. pFL1 is *FY3* transformed with pFL44L, HAA1↑ is FY3 transformed with *HAA1* overexpressing clone p3707-1. YLR297w↑ is *FY3* transformed with *YLR297w* overexpressing clone p3807-2.



(a)



(b)

Figure 3.19: Acidification of pregrowth media is essential for acetic acid resistance caused by *YLR297w* overexpression in stationary phase of growth. *YLR297w* overexpressing and control strains were grown in SD media with 2%, 0.5%, 0.05% glucose and 2% glucose with pH buffered to 6.0. Cells were harvested in (a) exponential phase (OD_{600}=0.5-0.6) or (b) late growth phase (OD_{600}=6.0-7.0) and dilution spotted on YPD agar plates containing indicated conc. of acetic acid at pH, 3.0.

experiments we have seen that pregrowth for 24 hours in SD media (2% glucose) results in acidification and pH falls to 2.8. Based on these findings SD media with 0.05% glucose and pH buffered to 2.6 with citrate buffer were supplemented with 10 mM acetic acid and it was formulated as spent medium composition that induces apoptotic manifestation in yeast cells in stationary phase as described by Burtner et al. Since growth in such media was very slow with very long lag phase, it was difficult to further study acetic acid resistance caused by *YLR297w* overexpression in such media. Yeast cells overexpressing *YLR297w* either from multicopy vector pFL44L or under GPD promoter were grown to exponential phase in SD media. Cells were harvested and further incubated in SD media (0.05% glucose and pH 2.6) with or without acetic acid at cell density corresponding to stationary phase in SD media (6-7 OD_{600}). Acetic acid resistance was checked after 0, 2, 4 and 18 hours of incubation in respective media. It was observed that immediately after incubation at 0 hour stage *YLR297w* overexpression from multicopy vector or GPD promoter does not cause any acetic acid resistance. With longer time *YLR297w* overexpression results in acetic acid resistance in yeast cells incubated with 10 mM acetic acid. On the other hand, in yeast cells incubated without acetic acid at low pH, very moderate or no acetic acid resistance is conferred by *YLR297w* overexpression (Figure-3.20a). Thus, acetic acid resistance caused by *YLR297w* overexpression in stationary phase requires acidification of pregrowth media with acetic acid. Next we checked if *YLR297w* mediated acetic acid resistance requires higher cell density as in late growth phase. A similar experiment was performed where exponentially grown yeast cells were further incubated in SD media (0.05% glucose and pH 2.6) with or without acetic acid at cell density corresponding to late growth phase (OD_{600}= 6.0-7.0) or exponential growth (OD_{600}=1.0) in yeast. Yeast cells incubated at 1.0 OD_{600} in acetic acid containing media were equally resistant to acetic acid as cells incubated at higher cell density upon *YLR297w* overexpression (Figure-3.20b). Thus, rather than higher cell density or late growth phase growth conditions, *YLR297w* overexpression in presence of acetic acid at low pH is critical for increased acetic acid resistance of yeast cells.

3.7 Discussion:

Acetic acid is one of the inhibitors generated during acid pretreatment of lignocellulisic biomass, which is further used for ethanolic fermentation. Acetic acid and other weak acids are also used as food preservatives. Acetic acid is also implicated in induction of programmed cell death in *S. cerevisiae* (Ludovico et al., 2001) and acts as a mediator of cell

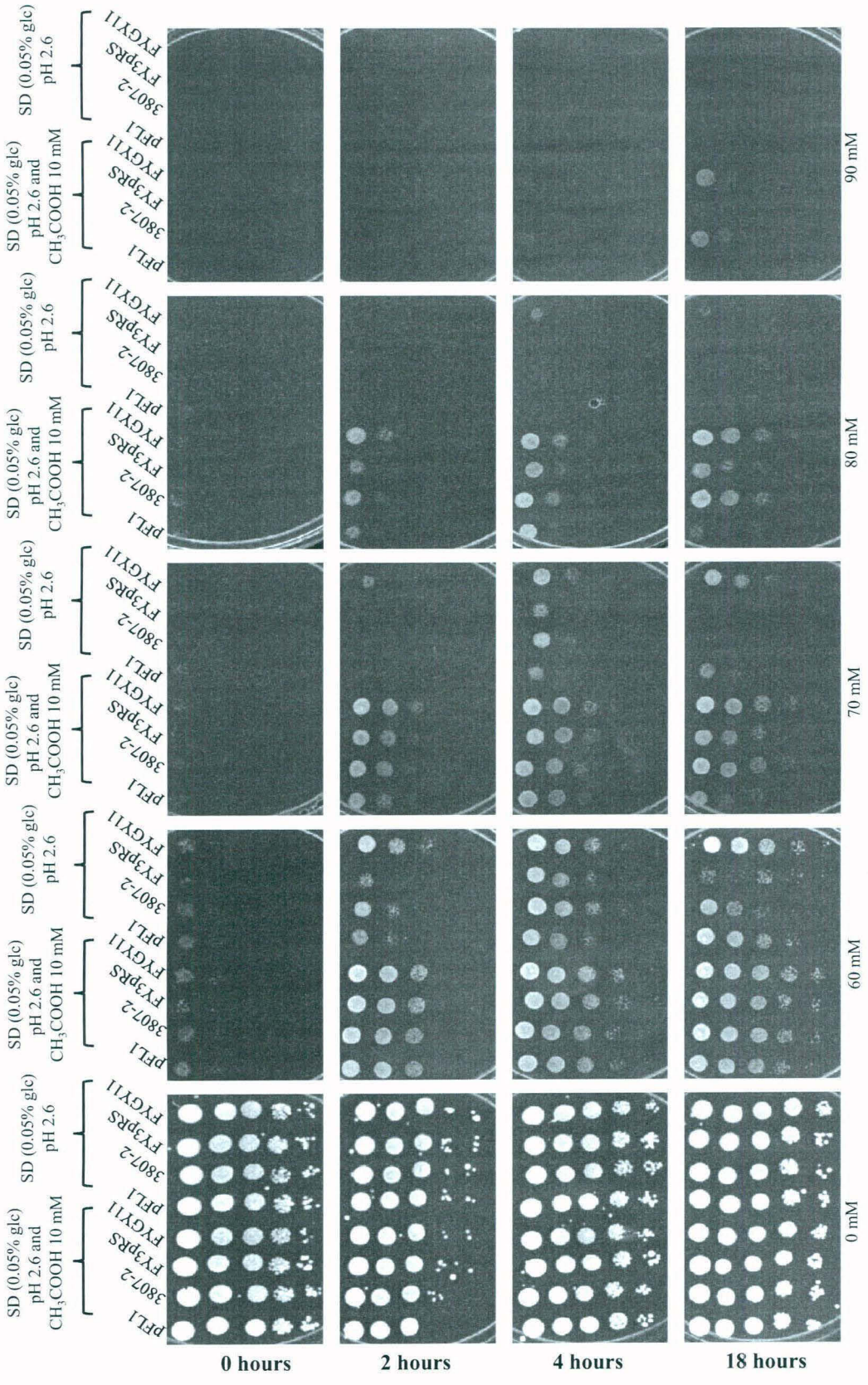

0 mM
60 mM
70 mM
80 mM
90 mM
0 hours
2 hours
4 hours
18 hours
SD (0.05% glc) pH 2.6 and CH3COOH 10 mM
SD (0.05% glc) pH 2.6
pFL1
3807-2
FY3pRS
FYGY11

Figure 3.20 (a)

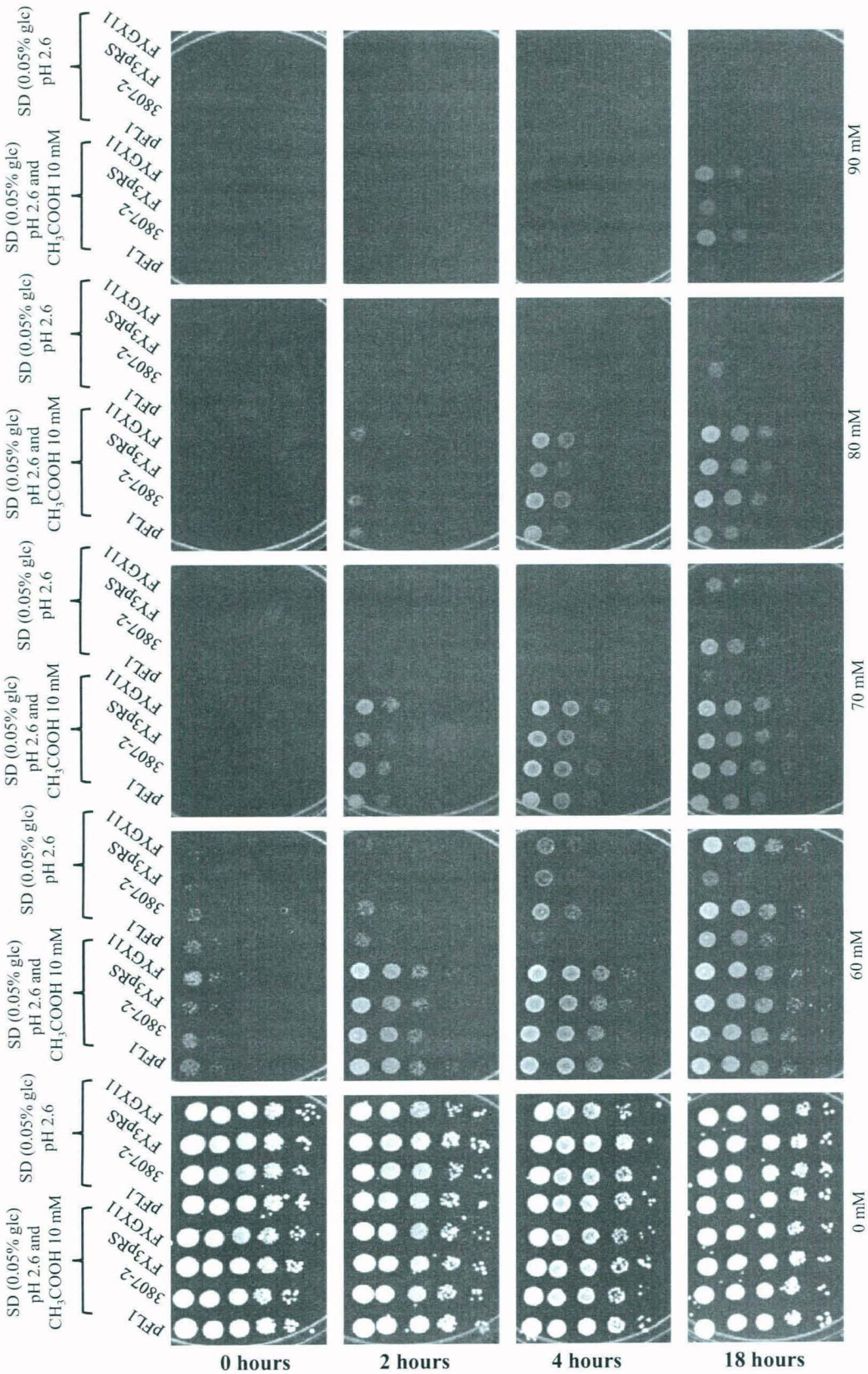

SD (0.05% glc) pH 2.6
SD (0.05% glc) pH 2.6 and CH3COOH 10 mM
pFL1
3807-2
FY3pRS
FYGY11
0 mM
60 mM
70 mM
80 mM
90 mM
0 hours
2 hours
4 hours
18 hours

Figure 3.20 (b)

Figure 3.20: Presence of acetic acid in pregrowth media induces acetic acid resistance caused by *YLR297w* overexpression in stationary phase of growth. Exponentially grown cells were harvested and resuspended in SD media (0.05% glucose) at pH 2.6 with 10 mM acetic acid or without acetic acid. After incubation at 30°C for of 0, 2, 4 and 18 hours acetic acid tolerance was checked by dilutions spotting on YPD agar plates containing indicated conc. of acetic acid at pH 3.0. pFL1 is *FY3* transformed with pFL44L, 3807-2 is FY3 transformed with *YLR297w* overexpressing clone p3807-2, FY3pRS is FY3 transformed with pRS306 and FYGY11 is FY3 strain in which *YLR297w* promoter was replaced with GPD promoter. (a) exponentially grown cells were resuspended at 6 OD_{600} and (b) exponentially grown cells were resuspended at 1 OD_{600}.

death during chronological aging (Burtner et al., 2009). Acetic acid induced cell death in yeast displays typical apoptotic features such as chromatin condensation, DNA fragmentation and cytochrome C release (Ludovico et al., 2002). Thus, adaptation to acetic acid stress in yeast *S. cerevisiae* plays important role in survival when yeast cell encounter inhibitory concentration of acetic acid in growth media.

To better understand the mechanism of acetic acid tolerance in yeast and to identify novel genes responsible for acetic acid tolerance; we screened a yeast genomic library cloned in multicopy vector pFL44L. Screening for genes by overexpression may have advantages over other genome wide strategies since here gain of function can be identified by positive selection pressure i.e. survival at higher concentration of acetic acid. Moreover, this strategy has the advantage of identifying genes providing only moderate fitness; deletion of such genes may not show definitive phenotype possibly due to genetic redundancy. Our screening yielded multiple yeast clones showing better tolerance to acetic acid compared to wildtype strain. Acetic acid resistance provided by such clones was confirmed by retransformation of the plasmids recovered from these clones into yeast *FY3* strain and further testing. The insert regions present within the plasmids were sequenced to identify the genes providing the resistance. Our screening identified multiple clones harboring the *HAA1* gene, a transcription regulator known to be involved in adaptation of yeast cells to acetic acid stress, thus validating our screen. Most of the other clones were found to provide only one or two serial dilutions benefit when acetic acid tolerance of such clones were checked by dilution spotting. Thus genes expressed from these clones contribute only moderately to acetic acid tolerance in yeast. However, several such genes together would increase acetic acid resistance substantially and most of them are possibly regulated by transcription factor such as Haa1p. Haa1p was recently reported to regulate up to 80% of the genes regulated upon acetic acid stress (Mira et al., 2010a). Moreover, individual deletion of as many as 650 genes result in some sort of sensitivity to acetic acid, whereas upon acetic acid shock yeast cells upregulate the expression of at least 112 genes and downregulate the expression of 83 genes by at least 1.5 fold (Mira et al., 2010b; Mira et al., 2010c). Thus adaptation of yeast to acetic acid seems to require involvement of multiple gene products from different functional classes.

When yeast strains are grown in SD medium, growth gets saturated after 24 hours and glucose is consumed by this time. Thus we considered yeast growth after 24 hours as late phase of growth or early stationary phase. We identified *YLR297w* from a multicopy library

clone, which upon overexpression provides acetic acid resistance in late phase of growth. Overexpression of *YLR297w* even under constitutive GPD promoter does not provide acetic acid resistance in exponentially growing yeast cells and acetic acid resistance is seen only after 24 hrs growth. Thus *YLR297w* seems to play role in yeast cells adaptation to acetic acid only in stationary phase of growth. Expression of *YLR297w* is known to be induced by stationary phase of growth, peroxide stress, DNA damaging agents etc., but no function is attributed to this gene so far. Expression of *YLR297w* is also upregulated upon acetic acid shock in yeast in Haa1p dependent manner (Fernandes et al., 2005; Mira et al., 2010a). Deletion of *YLR297w* causes moderate sensitivity to acetic acid, octanoic acid and benzoic acid stress even in stationary phase. Moreover, overexpression of *YLR297w* in stationary phase increases resistance to more lipophilic weak acids such as octanoic acid and benzoic acid. On the other hand, overexpression of *HAA1* which is known to regulate early adaptation to acetic acid and propionic acid does not enhance the resistance to octanoic acid and benzoic acid. Ylr297wp tagged with YFP fluorescent tag was found to localize in internal compartment of cell and it has a predicted transmembrane domain, thus possibly localizes to vacuolar membrane as earlier proposed in genome scale localization studies (Huh et al., 2003). However this remains to be confirmed by colocalization of Ylr297wp with marker specific to vacuole.

Our results indicate that Ylr297wp must be playing an important role in adaptation to a broad range of weak acids in stationary phase of growth in yeast. *YLR297w* is likely function as part of complex or machinery which is specific to stationary phase, although function of Ylr297wp seems to be dispensable by similar protein or it can be bypassed by cellular machinery. Since Ylr297wp is presumably localized in vacuole it may be associated with role of vacuolar machinery in weak acid adaptation. Vacuolar ATPases and transporters have been implicated in yeast adaptation to weak acids; moreover trafficking of proteins to vacuole is known to be effected by weak acid stress (Marcantoni et al., 2007; Mira et al., 2009). Ylr297wp must be associated with one of these vacuolar functions in acetic acid and other weak acid adaptations.

Acetic acid is normal end product of fermentation in yeast, moreover ethanol produced by fermentative growth in yeast may undergo respiratory metabolism to produce acetic acid. Yeast cells growing in defined SC media undergo chronological aging in stationary phase, such reduction in lifespan is further associated with reduction in pH and production of acetic acid. It has been reported that dietary restriction, i.e. growth on reduced glucose

concentration or growth on nonfermentable carbon sources increases chronological life span by reducing acetic acid production. Acetic acid induced aging is associated with generation of oxidative stress and inhibition of cell cycle arrest in stationary phase. Therefore, addition of osmotic stabilizer such as 18% sorbitol or 300 mM NaCl or deletion of *SCH9* or *RAS2* genes, both of which inhibit progression of cells to stationary phase, increase chronological life span and resistance to acetic acid. Resistance of *sch9Δ* to acetic acid depends on presence of Rim15p and Gis1p, proteins known to be involved in yeast cell's entry and survival in stationary phase (Burhans and Weinberger, 2009; Burtner et al., 2009).

As the role of *YLR297w* in acetic acid resistance in stationary phase of growth is demonstrated here, we checked *YLR297w* function in survival of yeast cells subjected to acetic acid induced chronological aging. *YLR297w* overexpressing strain grown in SD media with 2% or 0.5% glucose can acquire acetic acid resistance after 24 hours of growth. On the other hand, when cells are grown in YPD medium or SD media with 0.05% glucose or 2% glucose with pH adjusted to 6.0, no increase in acetic acid resistance mediated by overexpression of *YLR297w* is seen. These results correlate with the finding that acidification of media occurs when yeast cells are grown for 24 hours in SD media with 2% or 0.5% glucose, whereas acidification is not observed upon growth in SD media with 0.05% glucose or YPD medium. We also found that acidification and presence of acetic acid in pregrowth media are critical for acetic acid tolerance mediated by overexpression of *YLR297w*. Activation of *YLR297w* during yeast stationary phase may support the survival yeast cells against stress caused by acetic acid and other weak acids. Thus *YLR297w* must have a definitive role in increasing the yeast cells chronological life span and in survival in apoptotic response generated by acetic acid.

Chapter 4
Insights into molecular mechanism of Haa1p mediated acetic acid resistance

4.1 Introduction:

Adaptation to weak acid stress, in particular due to acetic acid involves, modulation of expression of several genes belonging to diverse functional classes in *S. cerevisiae.* Transcriptional response to acetic acid stress involves interplay of several transcription factors, such as Haa1p, Rim101p and Msn2/4p. Each of these transcription factors activates set of target genes involved in adaptation to acetic acid stress and significant cross talk is also reported (Mira et al., 2010a). Haa1p is implicated in early adaptation of yeast cells to acetic acid and propionic acid stress, and *haa1Δ* strain is sensitive to acetic acid and propionic acid and marginally sensitive to benzoic acid (Fernandes et al., 2005). Haa1p regulates the expression of at least 80% of the acetic acid induced genes in *S. cerevisiae* that are induced immediately upon exposure to acetic acid (Mira et al., 2010a). Among Haa1p target genes, *TPO2, TPO3, YGP1, HRK1* and *SAP30* possibly play important role in adaptation to acetic acid since deletion of these genes render the cells sensitive to acetic acid (Fernandes et al., 2005; Mira et al., 2010a). Haa1p also upregulates the expression of *FLO11* and adhesive growth in acetic acid shocked yeast cells in Yak1p dependent manner (Malcher et al., 2011).

HAA1 gene encodes a 694 amino acid protein having significant similarity to the N terminal region of copper regulated transcription factor Ace1p. However, the mechanism for activation of target genes by Haa1p is reported to be different from Ace1p (Keller et al., 2001). Thus, one of our objectives is to understand the molecular mechanism of Haa1p mediated acetic acid resistance in *S. cerevisiae.*

4.2 Haa1p is major regulator of acetic acid resistance in *S. cerevisiae:*

In our screen of multicopy yeast genomic library for genes conferring acetic acid resistance, 7 out of 26 resistant clones sequenced were found to have *HAA1* gene, validating the screen as well as the importance of Haa1p in acetic acid tolerance. Out of 7 clones having *HAA1* gene, four clones were sequenced from both ends of insert region. Clones named 3606-1 and 3606-3 carried full length *HAA1* gene with varying lengths of promoter region; clone 3606-1 begins from *REC8* gene situated upstream of *HAA1* gene and carries possibly all upstream regulatory elements, whereas clone 3606-3 has approximately 800 bp regulatory sequence upstream of *HAA1* coding sequence. In two other clones (3707-1 and 451406-2), upstream region was from *REC8* gene but they had incomplete *HAA1* coding sequence. Though the

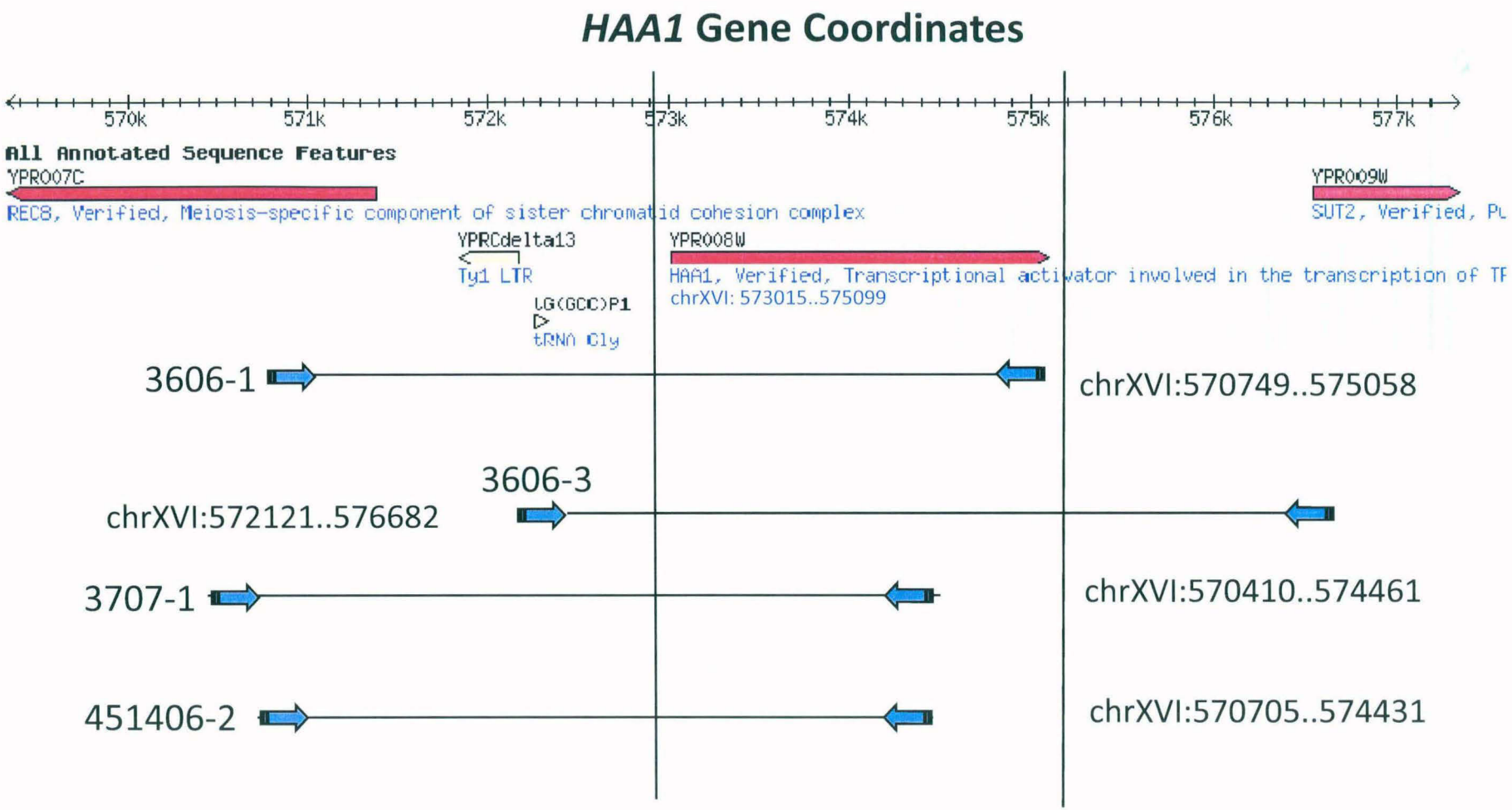


Figure 4.1: Chromosome coordinates of four *HAA1* carrying clones. identified by screening of yeast transformants carrying genomic library cloned in multicopy vector for acetic acid resistance. Different lengths of *HAA1* coding region and flanking regions are present in different clones, shown below the *HAA1* region taken from SGD page for *HAA1* (Position of *HAA1* gene in respective clones is marked by vertical lines).

encoded Haa1p would be missing about 220 amino acids from the carboxyl terminus, still they were conferring acetic acid tolerance and thus were studied in depth (Figure-4.1).

4.3 *HAA1* expression from truncated clones can complement acetic acid sensitivity of *haa1Δ* strain:

Wild type yeast strain *FY3* transformed with different *HAA1* overexpressing genomic library clones were checked for acetic acid tolerance by dilution spotting. The yeast strains carrying truncated clones were providing equal or even better acetic acid resistance as compared to strains overexpressing full length *HAA1* gene (Figure-4.2 (a)). Strains deleted for *HAA1* are impaired in acetic acid tolerance and also show increased lag phase of growth in media containing acetic acid (Fernandes et al., 2005). To check the ability of truncated *HAA1* clones to complement *HAA1* deletion, the *haa1Δ* strain was transformed with different *HAA1* clones and checked for acetic acid resistance. The *haa1Δ* strain expressing *HAA1* from library clones can grow on YPD plates containing 70 mM acetic acid at pH 3.0, whereas control strain is unable to grow at moderate 40 mM acetic acid concentration. More importantly clones with truncated copy of *HAA1* were also able to complement the acetic acid sensitivity of *haa1Δ* strain (Figure-4.2 (b)), clearly indicating Haa1p protein deleted at the C terminus can complement for Haa1p function, at least for adaptation to acetic acid stress.

Expression of *HAA1* mRNA was checked from *HAA1* clones transformed in *haa1Δ* strain by northern blot analysis. PCR amplified 500 bp fragment of *HAA1* gene from 5' terminus of coding sequence was used to prepare the probe for detecting *HAA1* mRNA. The *haa1Δ* strain transformed with pFL44L vector did not show any *HAA1* transcripts, as expected. Clone 3606-1 which has the entire regulatory region present between *HAA1* and upstream *REC8* gene (Figure-4.1), was showing *HAA1* mRNA of same size as wildtype strain (*BY4741*) but expression was significantly higher as it is expressed from a multicopy vector. Clones 3707-1 and 451406-2 with *HAA1* gene truncated in the 3' region were expressing shorter mRNA than full length *HAA1* as expected. Surprisingly *HAA1* expression from 3606-3 clone was aberrant as two transcripts were observed and expression was very high (Figure-4.3). Clone 3606-3 lacks part of regulatory regions upstream of *HAA1* coding sequence, possibly resulting in increased transcription of *HAA1*. This finding also supports the possibility that *HAA1* expression is regulated by repressors that bind to upstream sequences deleted in clone3606-3.

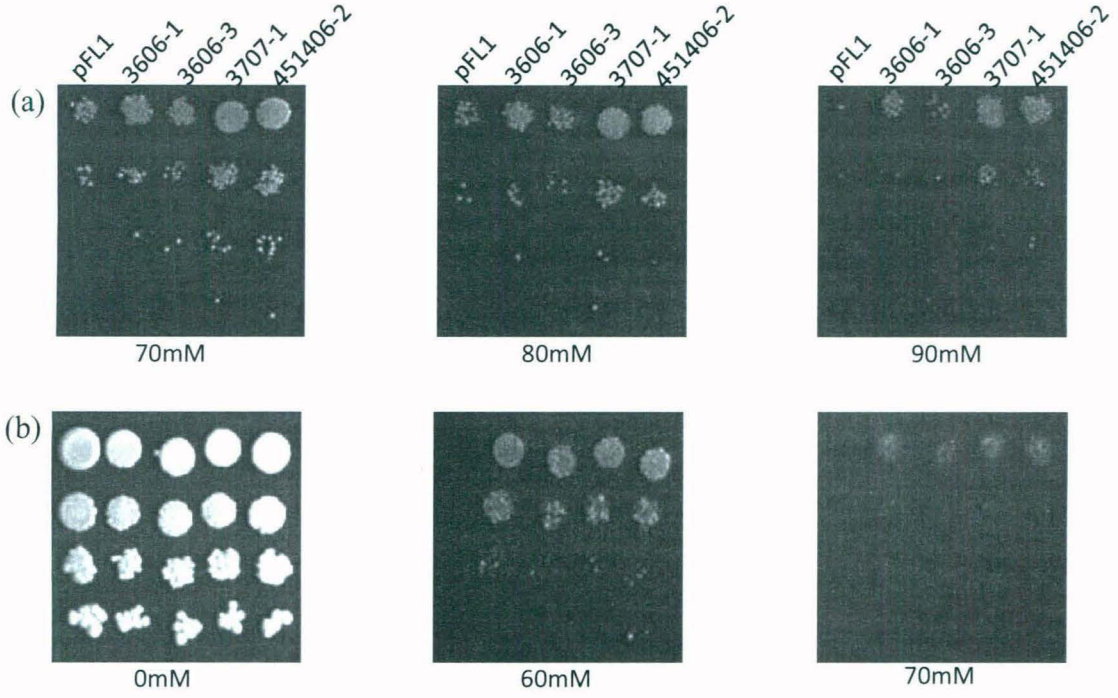


Figure 4.2: Acetic acid tolerance of *HAA1* overexpressing clones. *S.cerevisiae* strains transformed with *HAA1* overexpressing multicopy library clones and vector pFL44L were grown to exponential phase and dilution spotted on YPD agar plates containing indicated conc. of acetic acid at pH 3.0. (a) transformants of strain *FY3* and (b) transformants of *haa1Δ* strain.

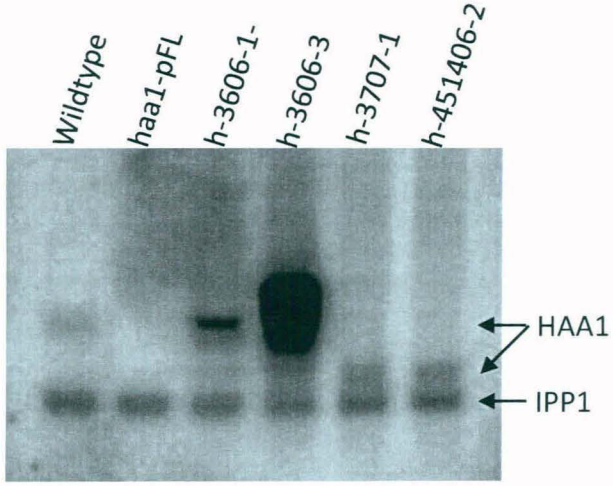


Figure 4.3: Expression of *HAA1* from *HAA1* overexpressing multicopy library clones. The *haa1Δ* strain transformed with *HAA1* overexpressing multicopy library clones and vector pFL44L were checked for *HAA1* expression by northern blot analysis. Wildtype is the *BY4741* strain transformed with pFL44L. Total RNA was isolated, resolved on denaturing agarose gel , transferred to nylon membrane and hybridized with (α-^{32}P-ATP) labelled *HAA1* probe. mRNA level of *IPP1* was detected and used as loading control.

4.4 *HAA1* domain mapping and construction of deletion clones:

As truncated *HAA1* gene was able to complement *HAA1* function in *haa1Δ* strain, it was interesting to identify the exact regions or domains of *HAA1* important for acetic acid adaptation in yeast. BLAST (Basic Local Alignment Search Tool) analysis of Haa1p showed significant similarity of N terminal 100 amino acid sequence both with other *S. cerevisiae* proteins as well as proteins encoded by other fungal species such as *A. gossypii, D. hansenii, Z. rouxii, K. lactis* etc. Haa1p sequence following this initial stretch of 100 amino acid residues shows no or very less similarity with other protein sequences. Moreover close homologs of Haa1p N terminal region Ace1p (225 aa) and Mac1p (417 aa) in *S. cerevisiae*, with N terminal DNA binding domain and C terminal activation domain, are quite smaller than Haa1p. Based on sequence similarity Haa1p has both Zn module and copper regulatory domain present in N terminal DNA binding domains of Ace1p and Mac1p. Activity of Ace1p and Mac1p is regulated by copper status of cell, whereas copper regulation of Haa1p is not found, although it is proposed that presence of tetracopper structure may stabilize the DNA binding domain of Haa1p (Jungmann et al., 1993; Keller et al., 2001). To understand the role of Haa1p in acetic acid adaptation in yeast, DNA binding ability of N terminal region of Haa1p and other important regulatory regions present in Haa1p need to be characterized. Thus, various deletions of Haa1p were characterized for their role in acetic acid adaptation of yeast.

Construction of *HAA1* deletion clones to identify regions of importance for Haa1p function was based on (1) presence of DNA binding domain in the N terminal region and (2) our observation that truncated clone missing the C-terminal region from 466 to 685 aa was able to complement *haa1Δ* strain in acetic acid adaptation. To further delineate the function of C-terminal region, C-terminal deletions of *HAA1* gene were made, encoding first 500 or 400 amino acids (further referred as H500 and H400), as around this length of truncated protein expressed from truncated clones were sufficient to complement Haa1p function in *haa1Δ* strain. C-terminal deletions encoding only the first 200 or 50 amino acid protein were also constructed as Haa1p homolog Ace1p has both DNA binding domain and activation domain in 225 aa sequence and initial 50 amino acids represent possible Zn domain in Haa1p (further referred as H200 and H50 respectively). Similarly N-terminal deletions lacking initial 50 or 100 residues were also constructed, missing N-terminal Zn domain (1-40 residues) and Zn domain along with tetracopper domain (41-80) respectively (further referred as N50 and

N100). Later, based on phenotypic studies of these clones, two more deletions having both N and C terminal deletions were also constructed. These deletion clones have coding sequence for upto 400 residues and lack either 50 or 100 amino acid at N terminus (further referred as N504 and N1004 respectively) (Figure-4.4).

Haa1p full-length gene and its deletions as designed above were PCR amplified, and cloned in yeast expression vectors under the control of strong and constitutive GPD or TEF promoter in both multicopy and single copy vectors. For these clonings, unique restriction sites *Spe*I and *Eco*RI introduced at 5' and 3' ends of respective *HAA1* constructs were used to generate compatible ends in vectors and insert. When these clones were transformed in *haa1Δ* strain, full-length *HAA1* gene cloned either under GPD promoter or TEF promoter in multicopy vector yielded only few slow growing transformants. However deletions expressed from same vectors were growing normally and transformation efficiency was comparable to respective vectors. Upon expression of *HAA1* gene from TEF promoter in a single copy vector growth was comparable to strains expressing *HAA1* deletions. Thus in further studies yeast strains overexpressing full-length *HAA1* and its deletions cloned in p416TEF were used.

The ability of *HAA1* deletions to complement acetic acid sensitivity of *haa1Δ* strain was checked by dilution spotting. It was observed that *HAA1* deletions H500, H400, N50 and N100 increased the acetic acid tolerance of *haa1Δ* strain like full-length *HAA1* gene. On the other hand, deletions H200, H50, N504 and N1004 did not rescue the acetic acid sensitivity of *haa1Δ* strain. Moreover C-terminal deletions H500 and H400 gave higher resistance to acetic acid compared to full-length *HAA1* gene. H400 shows at least three dilutions growth benefit over full-length gene on 55 mM acetic acid plate, whereas growth of H400 was comparable to that of wild type *HAA1*on 40 mM acetic acid plate, indicating that H400 can provide additional 15 mM acetic acid. Expression of N50 can provide as much resistance as *HAA1*, but for N100 the resistance conferred is moderate compared to full-length *HAA1* gene (Figure-4.5). The *haa1Δ* strain shows prolonged lag phase in acetic acid as a result of increased viability loss (Fernandes et al., 2005), the effect of reintroduction of full-length *HAA1* and its deletions on lag phase was checked. When these strains were grown in YPD broth containing 40 mM acetic acid at pH 3.0, the vector control strain (lacking functional Haa1p) showed lag phase of approximately 60 hours, the strain expressing full-length *HAA1* showed a lag phase of approx 30 hours. Interestingly expression of H400 further reduced the

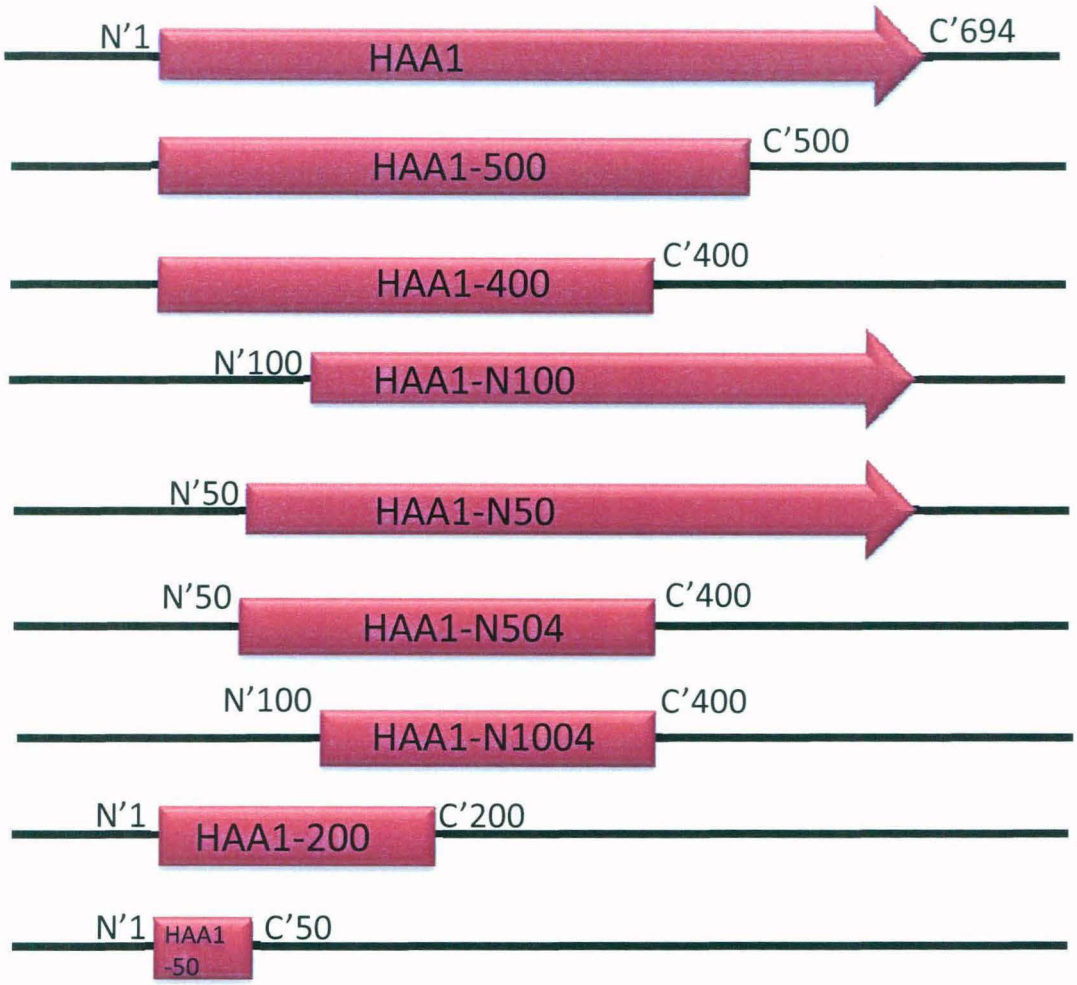


Figure 4.4: *HAA1* deletion clones. Deletions of *HAA1* constructed by PCR. the regions retained in truncated proteins are indicated by the amino acid coordinates.

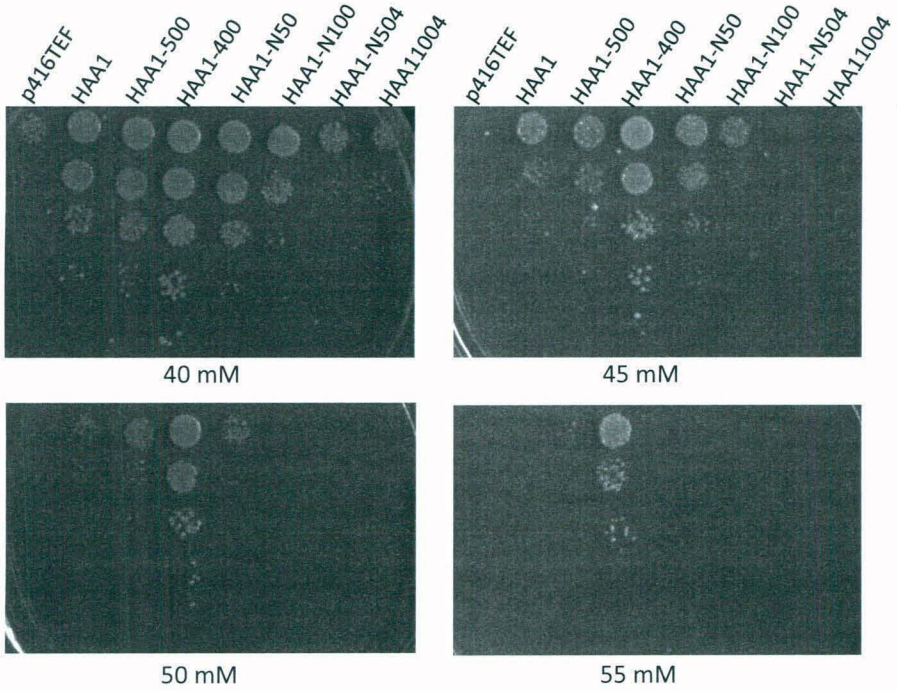


Figure 4.5: Complementation of acetic acid sensitivity of *haa1Δ* by expression of *HAA1* and its deletions under TEF promoter. Yeast strains grown up to exponential phase were serially 10-fold diluted and spotted on YPD plates with indicated conc. of acetic acid at pH 3.0.

lag phase to 15-20 hours, whereas the N100 expression showed a lag phase comparable to that of full-length *HAA1* gene. The lag phase of N504 and N1004 deletion clones was comparable to that of *haa1Δ* strain, consistent with comparable acetic acid sensitivity of these clones as seen in dilution spotting (Figure-4.6). These phenotypic studies show that presence of functional Haa1p is essential for adaptation of yeast cells to inhibitory concentrations of acetic acid. C-terminal deletions (H400 and H500) can fully complement whereas N-terminal deletions (N50 and N100) can partially complement the acetic acid sensitivity of *haa1Δ* strain. The expression level of *HAA1* and its deletions under TEF promoter were also checked by northern blotting. The size of the transcripts correlated with the length of *HAA1* gene retained, as expected. However, the transcript level of N-terminal deletions was aberrantly high compared to full-length *HAA1* or its C-terminal deletions (Figure-4.7). Moreover transcript level was found maximum in deletions lacking first 300 bp i.e. N100 and N1004, thus 5' region of *HAA1* may have some recognition sites targeting mRNA for degradation and regulating the transcript level. As overexpression of *HAA1* leads to slow growth in yeast, such a mechanism controlling through mRNA turnover could be another level of regulation of Haa1p.

4.5 Effect of *HAA1* deletions on expression of Haa1p target genes:

Since C-terminal *HAA1* deletions were showing better acetic acid resistance than full-length *HAA1* gene and N-terminal deletions were also complementing Haa1p function. The correlation between phenotype and ability to control the expression of Haa1p target genes was checked for *HAA1* deletions. Upon propionic acid and acetic acid shock, Haa1p increases the expression of genes such as *TPO2, TPO3, YGP1, PHM8* and *YLR297w* etc and deletion of *TPO2, TPO3* and *YGP1* increases the sensitivity of yeast cells to acetic acid and other weak acids (Fernandes et al., 2005). So we selected these three genes as representative of Haa1p target genes and expression of these genes was checked under the control of *HAA1* deletions by northern blot analysis.

So far we have assessed acetic acid sensitivity by dilution spotting, whereas for expression analysis yeast strains have to be grown in liquid broth. Addition of acetic acid to media at low pH results in extended lag phase of growth; moreover strains expressing different *HAA1* deletions have varying duration of lag phase, and thus do not have comparable cell density at the time of sampling. Therefore, instead of growing in acetic acid containing media, appropriately grown yeast cells were further subjected to acetic acid shock. The *haa1Δ* strains

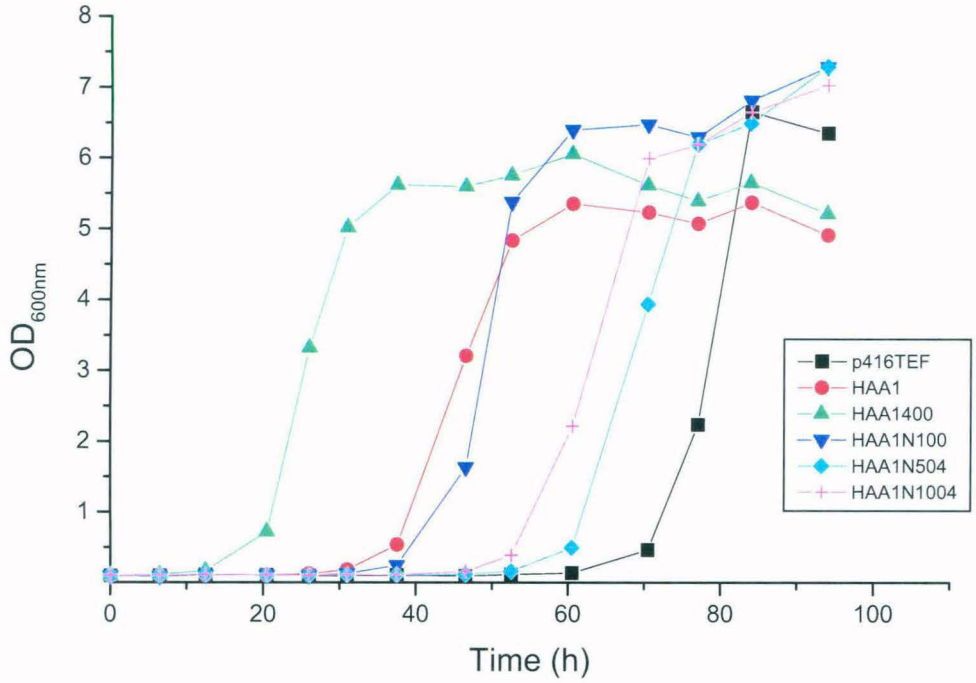


Figure 4.6: Adaptation of *HAA1* and its deletions to acetic acid. The *haa1Δ* strains expressing *HAA1* and its deletions under TEF promoter were inoculated in YPD broth containing 40 mM acetic acid at pH 3.0 at 0.05 OD_{600} and incubated at 30°C with shaking (200 rpm). Growth was monitored in term of increase in $O.D._{600}$ at intervals.

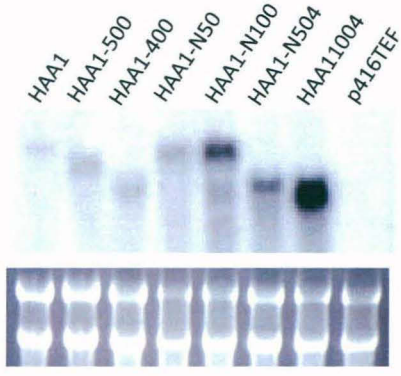


Figure 4.7: Abundance of transcripts of *HAA1* deletions expressed under TEF promoter in *haa1Δ* strain. The transcript length of *HAA1* corresponds to the size of different deletions. As loading control 25S and 18S RNA region of stained gel is shown in the lower panel.

expressing *HAA1* deletions were grown to exponential phase in SD medium supplemented with auxotrophic supplements at 30°C. Cells were harvested at 0.6-0.8 OD_{600} and incubated in YPD medium containing acetic acid (75, 100, 150 and 200 mM concentrations) at pH 3.0 at cell density corresponding to 1.0 OD_{600} at 30°C. Viability of these shocked cells was checked at 0, 2, 4 and 6 hours of incubation by dilution spotting on SD medium plates. At 200 mM acetic acid conc. all strains were unviable after 2 hours and at 150 mM acetic acid yeast strains expressing *HAA1* deletions N50, N100, N504 and N1004 were unviable after 4 hours and vector control strain was unviable after 2 hours. At 100 mM and 75 mM acetic acid conc. strains expressing *HAA1* and its deletions were viable; moreover control strain lacking functional Haa1p (*haa1Δ* strain transformed with vector alone) was significantly viable after acetic acid shock of 4 hours (Figure-4.8). Therefore acetic acid shock at 75 mM acetic acid for 4 hours was selected as sublethal condition for expression analysis of Haa1p target genes.

Yeast strains expressing different *HAA1* deletions were grown to exponential phase in minimal medium, cells were harvested for immediate RNA isolation as well as incubated in YPD medium with 75 mM acetic acid at pH 3.0 for 4 hours and RNA was isolated by hot phenol method (Mannan et al., 2009). The mRNA level of Haa1p target genes *TPO2, TPO3* and *YGP1* along with *IPP1* as loading control was checked by northern blotting. In exponentially grown cells, *HAA1* is necessary for expression of *TPO2, TPO3* and *YGP1* and upon acetic acid shock expression of *TPO2* and to some extent *YGP1* was further enhanced as earlier reported. In control strain lacking functional Haa1p, transcripts of these genes were not detectable in any condition tested. Haa1p C-terminal deletions H500 and H400 were comparable to full-length *HAA1* in regulation of target genes, whereas N-terminal deletions N50 and N100 can support the expression of *TPO3* and *YGP1* but no *TPO2* transcript was detected with or without shocked cells. Interestingly acetic acid induced increase in *TPO2* expression was maximum with H400, as faint transcript of *TPO2* was seen in untreated cells, however upon acetic acid shock expression of *TPO2* was comparable to full-length *HAA1* gene. In N50 and N100 deletions, *YGP1* transcript level was less in acetic acid treated cells compared to untreated as well as expression level in *HAA1* and C-terminal deletions (Figure-4.9). Simultaneous deletion from both N and C termini results in possibly nonfunctional Haa1p protein, since none of Haa1p target genes was expressed in strains with N504 and N1004 deletion clones (Data not shown).

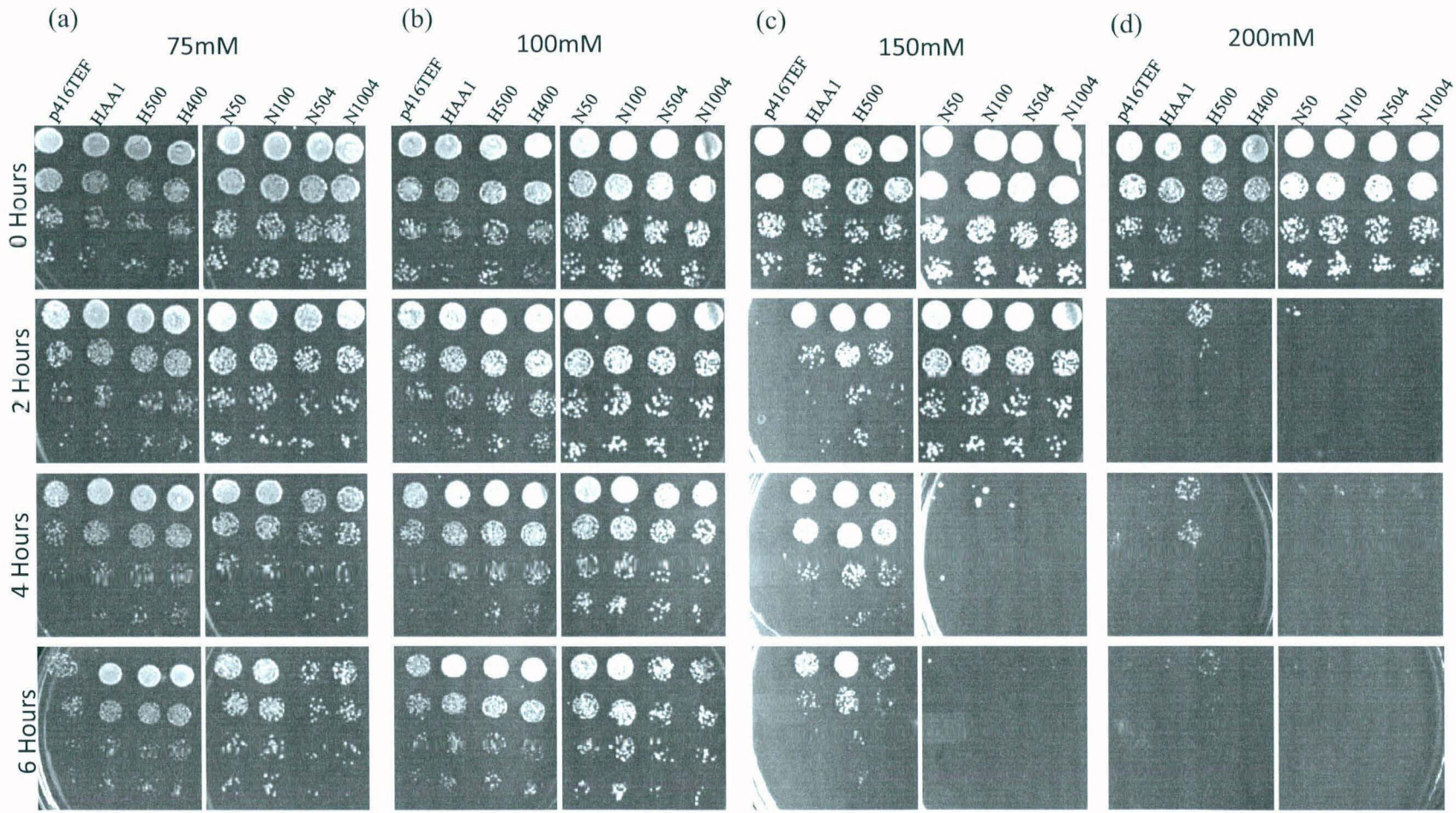


Figure 4.8: Survival time course of *HAA1* and its deletions in acetic acid at pH 3.0. Exponentially grown cells were harvested and resuspended at 1 OD_{600} into YPD broth at pH 3.0 containing (a) 75, (b) 100, (c) 150 and (d) 200 mM acetic acid. Treated cells were dilution spotted on SD plates with auxotrophic supplements, after 0, 2, 4 and 6 hours of incubation.

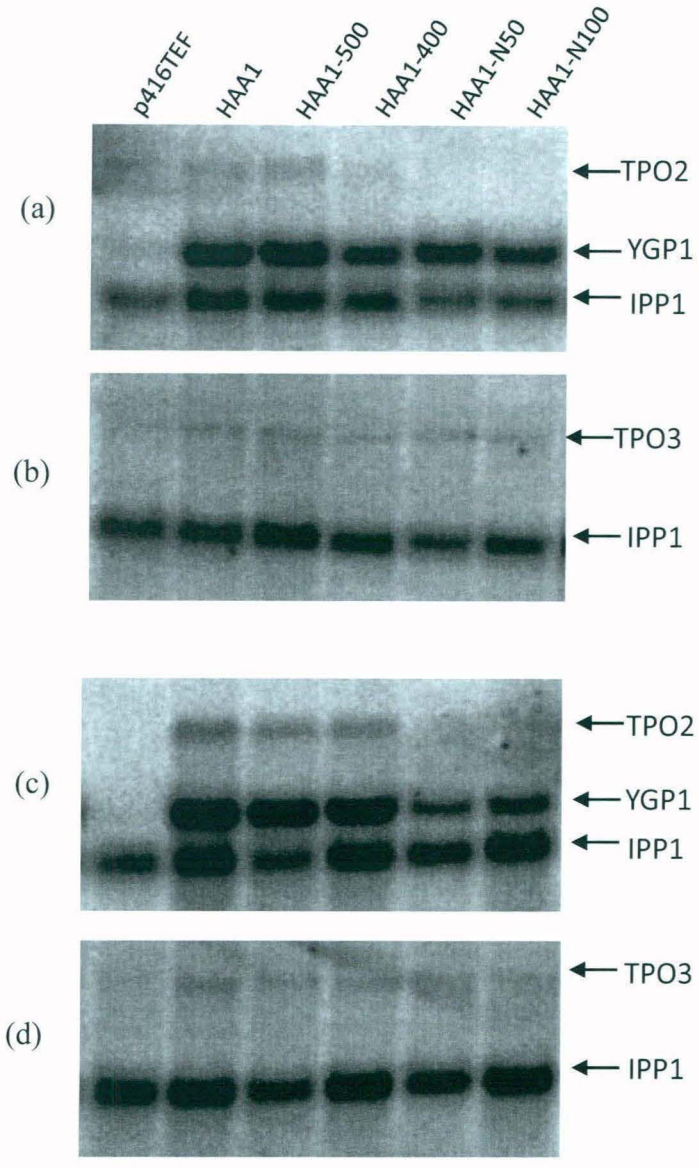


Figure 4.9: Differential regulation of Haa1p target genes by *HAA1* deletions. The *haa1Δ* strain transformed with *HAA1,* its deletions under TEF promoter (in p416TEF) were grown to exponential phase and further subjected to acetic acid shock (YPD, pH 3.0 with 75 mM acetic acid) for 4 hours. The expression of *YGP1, TPO2* and *TPO3* in untreated (a & b) or acetic acid shocked cells (c & d) was checked by northern blot. The positions of transcripts of *TPO2, TPO3* and *YGP1* are indicated. Expression of *IPP1* was used as internal loading control.

4.6 Expression and purification of Haa1p and H400p from *E. coli*:

Since *HAA1* deletions can induce expression of Haa1p target genes, the potential DNA binding ability of Haa1p and its deletions to possible promoter regions upstream of Haa1p target genes was checked. To study DNA binding ability purified protein or cell lysate having functional protein is required. Thus, *HAA1* full-length gene and H400 deletion construct were cloned in pET28c (+) at *Nde*I and *EcoR*I restriction sites, thereby adding N-terminal His tag. These clones were transformed into *E. coli* expression strain BL21-CodonPlus and protein expression was induced at 25°C with 0.25 mM IPTG at a cell density of OD_{600}=1.0 to 1.25 (Figure-4.10 (a)). Recombinant proteins thus produced were bound to Ni-NTA affinity matrix and eluted in buffer having 300 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10% glycerol and 200 mM imidazole. To check purity, eluted proteins were run on SDS-PAGE and stained with Coomassie brilliant blue R-250. Full-length Haa1p of 87 kDa and H400 protein of 47 kDa were seen, along with significant number of bands of low molecular weight that can result from poor purification or degradation of purified protein (Figure-4.10 (b)). So purity of eluted proteins were further checked by western blotting using anti His monoclonal antibody, which showed that the majority of bands were result of Haa1p degradation (Figure-4.10 (c)).

To minimize protein degradation various parameters affecting protein expression and purification were tried. IPTG induction was done at different temperatures; at 37°C induced protein mainly goes to insoluble pellet fraction after sonication, whereas at temperatures below 20°C no induction was seen. Various protease inhibitor cocktails were used in lysis buffer and elution buffer but no improvement in the quality of eluted protein was seen. Buffers at various pH ranges were also tried such as MES buffer, pH 6.0 (Figure-4.11 (b) & 4.11 (c)), carbonate buffer, Tris-HCl (pH 9.0) and phosphate buffer. No improvement was seen in the quality of protein; moreover upon dialysis significant aggregation was seen for Haa1p and H400p (Figure-4.11 (a)). In an attempt to further purify partially purified protein, we performed gel filtration chromatography of dialyzed protein on superdex S200 column. SDS-PAGE analysis of eluted fractions of gel filtration showed consistent degradation of both Haa1p and H400p (Figure-4.12). Thus Haa1p and H400p seem to highly unstable proteins as consistent pattern of degradation was seen. Purification of Haa1p and its deletion proteins from native host *S. cerevisiae* were also tried; C-terminally His tagged proteins were expressed from constitutive GPD promoter or inducible GAL1 promoter. The expressed

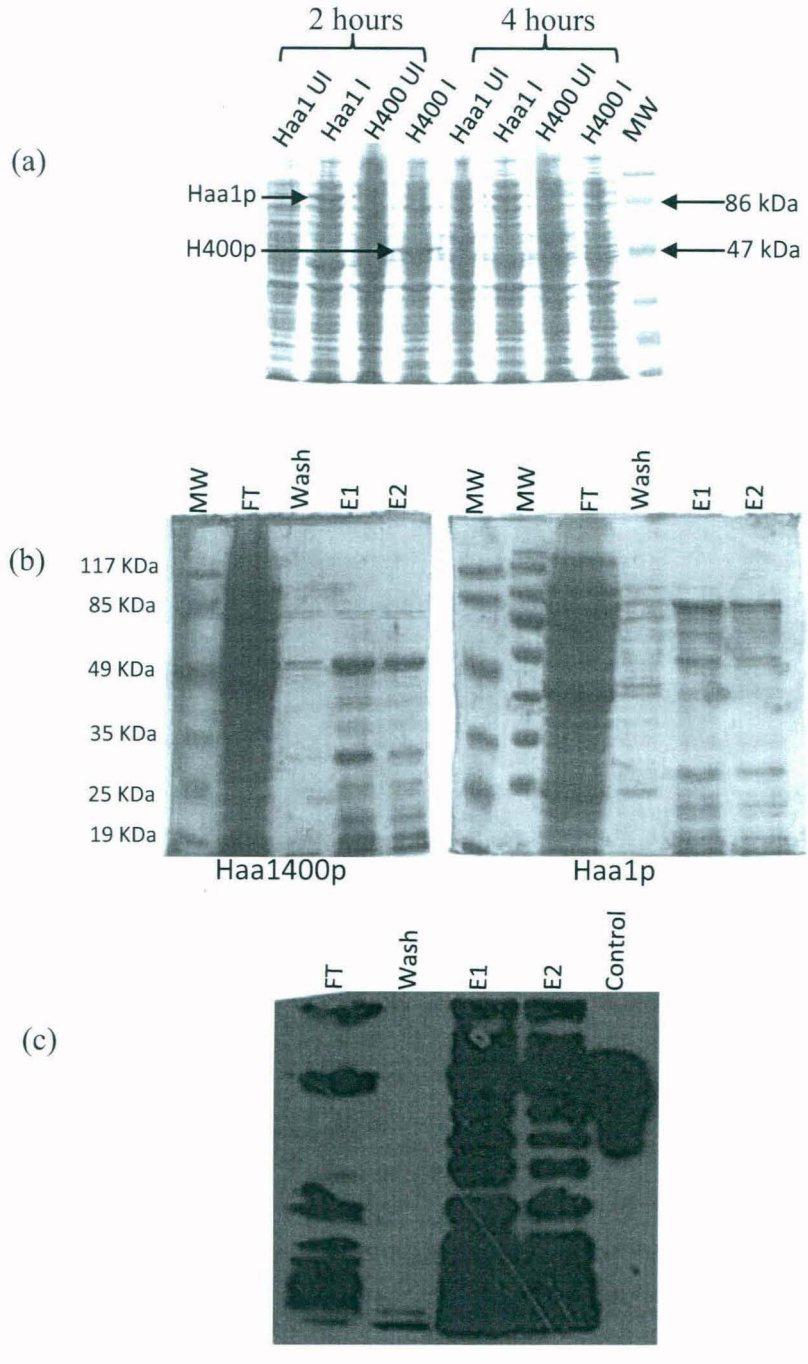


Figure 4.10: Expression and purification of Haa1p and H400p. *HAA1* and H400 were expressed from pET28c (+) in *E coli* expression strain Codon^{+}. (a) **induction of proteins**, with 0.5 mM IPTG for 2 and 4 hours at 37°C, UI (uninduced) and I (induced). (b) **Purification profile** of H400p and Haa1p eluted by Ni-NTA chromatography, MW (molecular weight marker), FT (flow through), Wash, E1 (elution 1) and E2 (elution 2) (c) **Western blot** of purified recombinant Haa1p. His tagged protein was probed with anti His mouse antibody and followed by HRP conjugated anti mouse IgG, signal was detected by Millipore chemiluminescence detection kit. FT (flow through), Wash E1 (elution 1), E2 (elution 2) and Control (Dug1p-His).

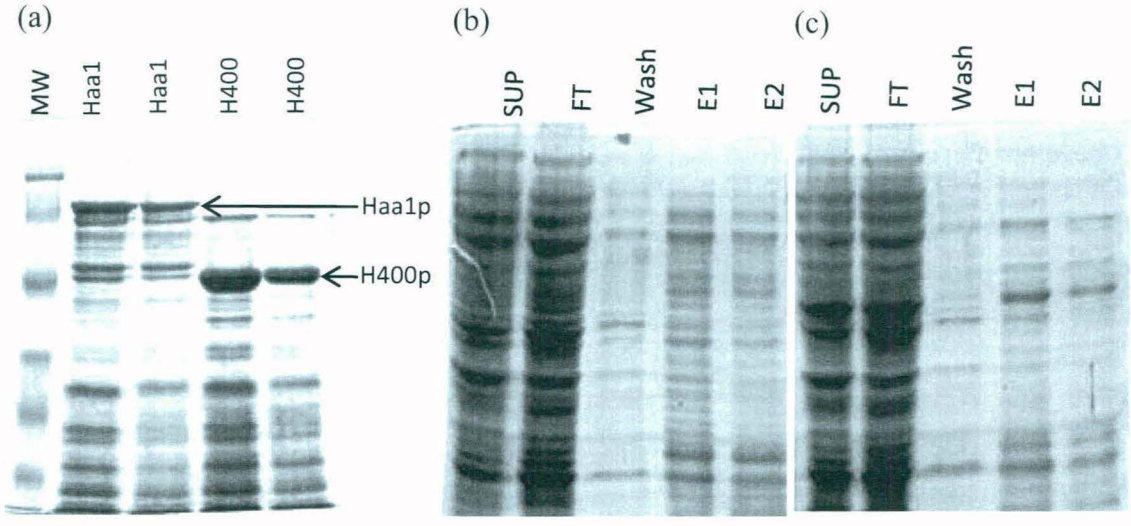


Figure 4.11: Purification of Haa1p and H400p from *E. coli*. *HAA1* and H400 were expressed in *E. coli* strain Codon$^+$ from pET28c (+) and proteins were purified by Ni-NTA affinity chromatography. (a) Protein purified from Tris-HCl pH 8.0 based buffer and dialyzed and run on SDS-PAGE. (b) Haa1p purified from MES pH 6.0 based buffer (c) H400p purified from MES pH 6.0, SUP (supernatant), FT (flow through), Wash, E1 (elution 1) and E2 (elution 2).

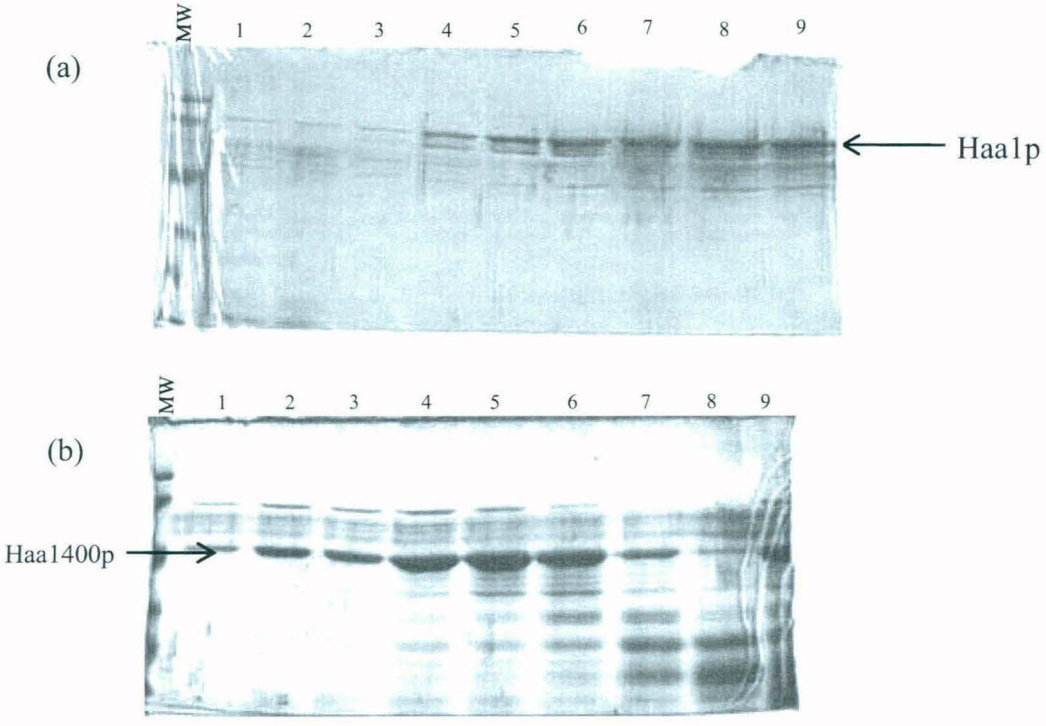


Figure 4.12: Gel filtration analysis of Haa1p and H400p, Ni-NTA purified proteins, dialyzed against 300 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10% glycerol, were, passed through superdex S200 column, Different gel filtration fractions were run on SDS-PAGE to detect homogeneity of eluted protein. (a) gel filtration fractions of Haa1p and (b) gel filtration fractions of H400p.

protein complemented the acid sensitivity of *haa1Δ* strain (Data not shown), however these proteins could not be detected by western blotting or purified.

4.7 Haa1p binds upstream DNA sequences of *YGP1* and *TPO2* in metal dependent manner:

As discussed earlier, Haa1p functions as transcription factor in yeast and has a possible N terminal DNA binding domain. Very recently Haa1p responsive element (HRE) has been identified in upstream region of *TPO3* located between nucleotides -590 and -690. Purified Haa1p DBD (N' terminal 127 amino acids) interacts with sequence 5'-(G/C)(A/C)GG(G/C)G-3' in promoter region of Haa1p target genes (Mira et al., 2011). Simultaneously we have also tried to prove DNA binding ability of Haa1p to upstream regulatory regions of *YGP1* and *TPO2* coding sequences using gel shift experiment with recombinant Haa1p and H400p. The gel shift or electophoretic mobility shift assay (EMSA) is a rapid and sensitive method to detect protein-nucleic acid interactions. It is based on the observation that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid. Generally a radioactively end labeled nucleic acid probe is incubated with protein of choice, and run on native polyacrylamide gel to study mobility of DNA-protein complexes with respect to free probe.

To study the DNA binding ability of Haa1p and H400p, upstream sequences of *TPO2* and *YGP1* coding region were PCR amplified in 3 separate overlapping fragments covering 1kb region as shown in Figure-4.13. PCR amplified DNA fragments were purified by gel extraction and used for 5' end radiolabelling using ^{32}P-γ-ATP in a reaction catalyzed by poly nucleotide kinase (PNK). After purification yield for H400p was higher than full-length Haa1p and percentage of undegraded protein was more in H400p. Therefore, initially H400p was incubated with 20 f mole probe DNA of *YGP1* upstream regions at 20°C for 30 min to study DNA binding of Haa1p. Protein DNA complexes thus formed were electrophoresed on 5% polyacrylamide native gel and analyzed for mobility shift relative to free probe molecules. With increasing concentration of H400p, mobility shift in *YGP1*-A, B and C probes were seen, similar mobility shift was also observed with *TPO2*-A, B and C probe DNA (Figure-4.14 and 4.15). DNA binding was also seen for recombinant Haa1p in mobility shift assay with *TPO2* and *YGP1* probes (Figure-4.16). Thus these preliminary observations indicated that Haa1p has direct DNA binding ability in upstream region of Haa1p target genes.

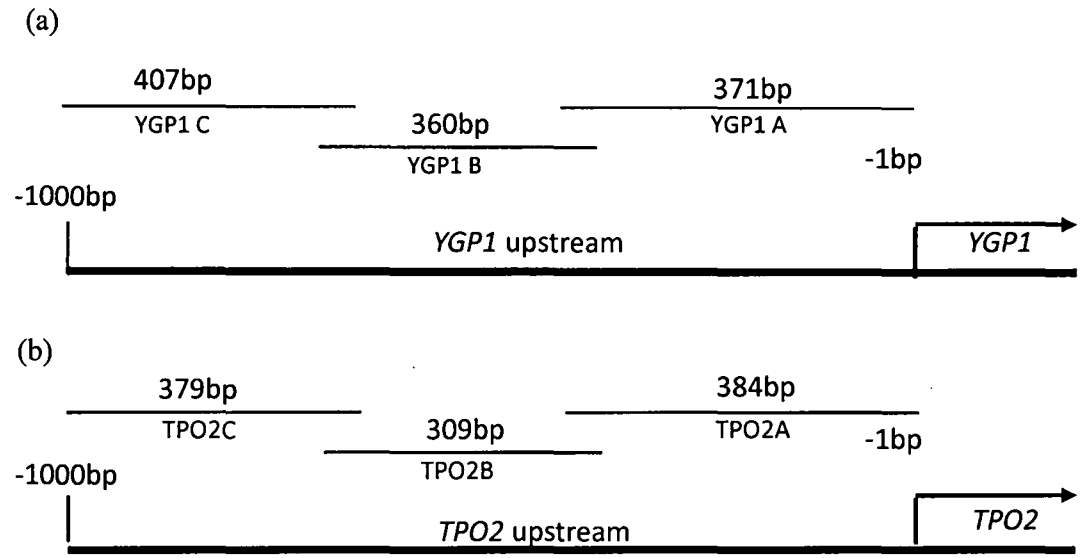


Figure 4.13: *YGP1* and *TPO2* upstream regions used as end labeled probes in DNA binding assays. 3 overlapping DNA fragments covering 1 KB upstream region were PCR amplified, end labeled with γ-^{32}P and used in DNA binding experiments with Haa1 and H400. (a) YGP1 probes, YGP1-A (-1 to -390), YGP1-B (-301 to -680) and YGP1-C (-573 to -980) and (b) TPO-2 probes, TPO2-A (-1 to -405), TPO2-B (-331 to -640) and TPO2-C (-561 to -1000).

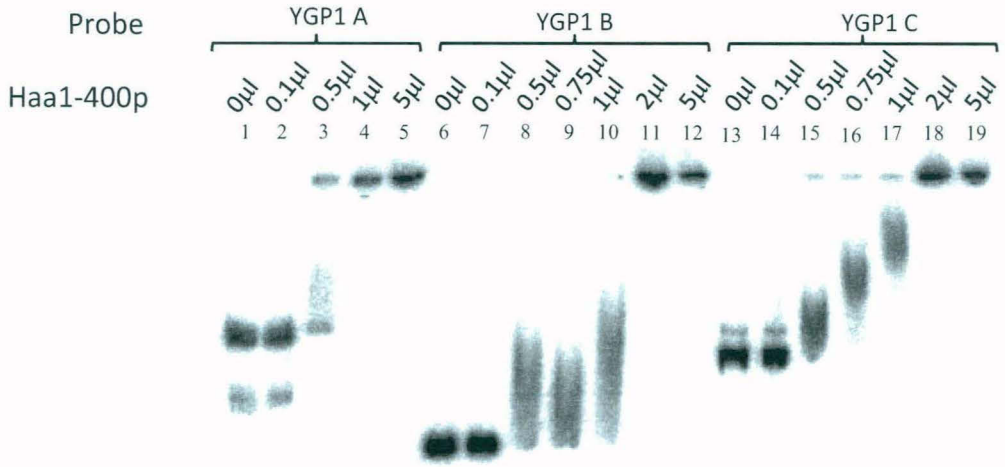


Figure 4.14: DNA binding assay of H400p with YGP1 probes. End labeled YGP1 probes were mixed with H400p proteins and incubated at 20°C for 30 minutes, as described (section 2.2.15). Samples were run on native PAGE, after which gel was dried and possible protein DNA complexes were analyzed on phosphorimager. The probe and protein amount used in reaction is indicated above the lane. One µl of protein is equivalent to 0.94 µg.

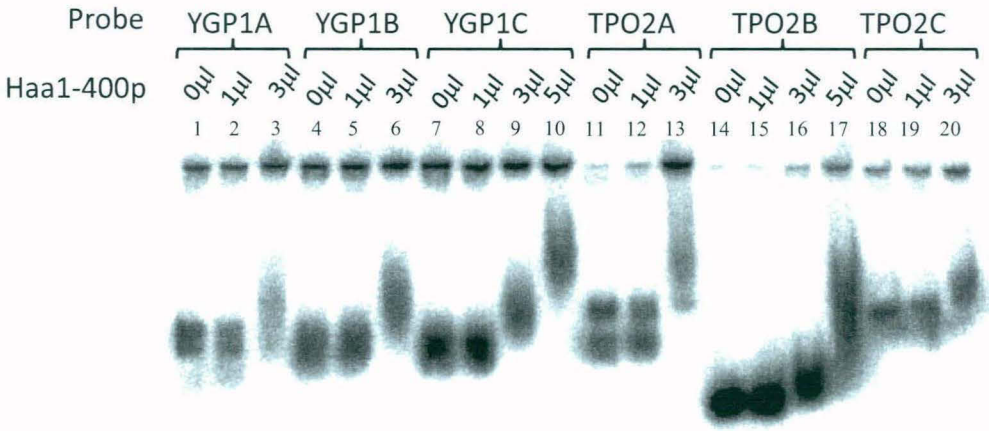


Figure 4.15: DNA binding assay of H400p with YGP1 and TPO2 probes. End labeled probes were mixed with H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. The probe and protein amount used in reaction is indicated above the lane. One µl of protein is equivalent to 0.42 µg.

Haa1p DNA binding domain has a Zn stability domain and Cu regulatory domain, and thus it is likely that these metal ions play significant role in DNA binding property of Haa1p. Although metalloregulation of Haa1p is not reported, but it has been proposed that poly copper thiolate structure may stabilize DNA binding domain of Haa1p (Keller et al., 2001). Thus, effect of chelation of metal ions present in Haa1p DNA binding domain on its DNA binding ability was checked. Recombinant Haa1p and H400p protein were either incubated with or without 1 mM EDTA at 30°C for 30 min; these protein samples were separately dialyzed and used for DNA binding experiment. Both Haa1p and H400p, once incubated with EDTA loses their ability to bind *YGP1* or *TPO2* probe molecules, as no gel shift was observed, however proteins similarly incubated without EDTA showed gel shift. These results indicate that presence of these metal ions is essential for DNA binding ability of Haa1p (Figure-4.16 and 4.17). Specificity of DNA binding ability of Haa1p with *YGP1* or *TPO2* probes were confirmed as similar set of protein did not show any binding with nonspecific Mycobacterial probe DNA used in DNA binding experiment (Figure-4.16).

Since presence of metal ions (Cu^{+} and Zn^{2+}) in DNA binding domain of Haa1p was found essential for its DNA binding ability, as EDTA inactivated proteins fail to bind target DNA probes. We tried to regenerate functional protein by incubating apoprotein (Haa1p or H400p inactivated with EDTA) with $ZnSO_4$ and $CuSO_4$. Divalent Copper salt was selected as monovalent copper salts are insoluble in aqueous medium. Moreover to find out functional significance of any other metal ion in Haa1p function, $MgSO_4$ and $FeSO_4$ were also tested. Apo H400p incubated with increasing concentration of different metal salts (1 μM, 10 μM, 100 μM and 1mM) was used for DNA binding experiment with *YGP1*-C probe. DNA binding ability of apoH400p was not regained after incubation with any of the metal ion tested (Figure-4.18). Furthermore combinations of Cu^{2+} and Zn^{2+} were also tested as possibly Haa1p DBD binds both of these metal ions. Hence apoH400p was simultaneously incubated with gradually increasing concentration of both $ZnSO_4$ and $CuSO_4$ together, and DNA binding ability was checked, but activity was not regained (Figure-4.19). These results indicate that DNA binding ability of Haa1p is metal dependent and DBD of Haa1p is stabilized by presence of metal ion.

Mac1p is a copper responsive transcription factor in *S. cerevisiae*; upon copper starvation it induces the expression *FRE1, CTR1, CTR3* and *FRE7* (Jungmann et al., 1993). Mac1p shares homology with N-terminal DBD of Haa1p and Ace1p, having Copper regulatory domain and

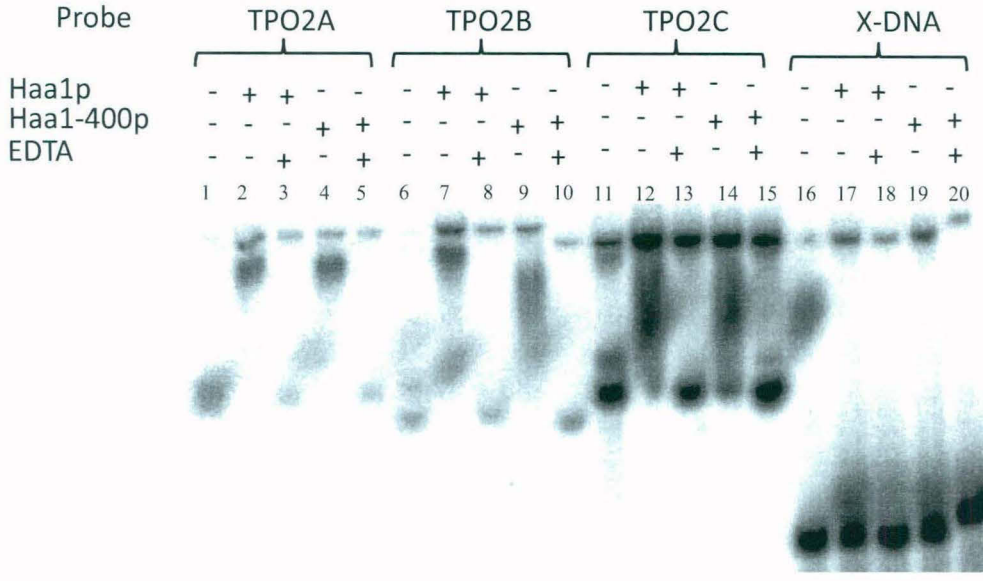


Figure 4.16: Metal ion dependent DNA binding assay of Haa1p and H400p with TPO2 and nonspecific probes. Purified Haa1p and H400p were incubated with or without 10 mM EDTA, dialyzed and further used for DNA binding assay. End labeled probes were mixed with Haa1 and H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. X-DNA is non specific Mycobacterial DNA used as probe. Proteins were used at final conc. of 1 μM.

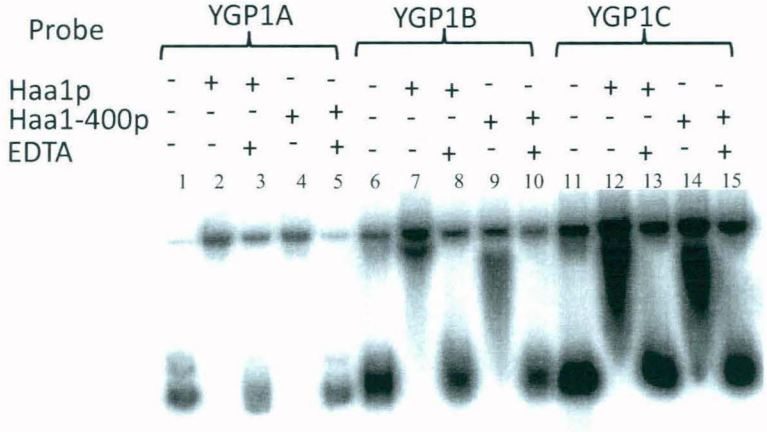


Figure 4.17: Metal ion dependent DNA binding assay of Haa1p and H400p with YGP1 probes. Purified Haa1p and H400p were incubated with or without 10 mM EDTA, proteins were dialyzed and further used for DNA binding assay. End labeled probes were mixed with these Haa1 and H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Proteins were used at final conc. of 1 μM.

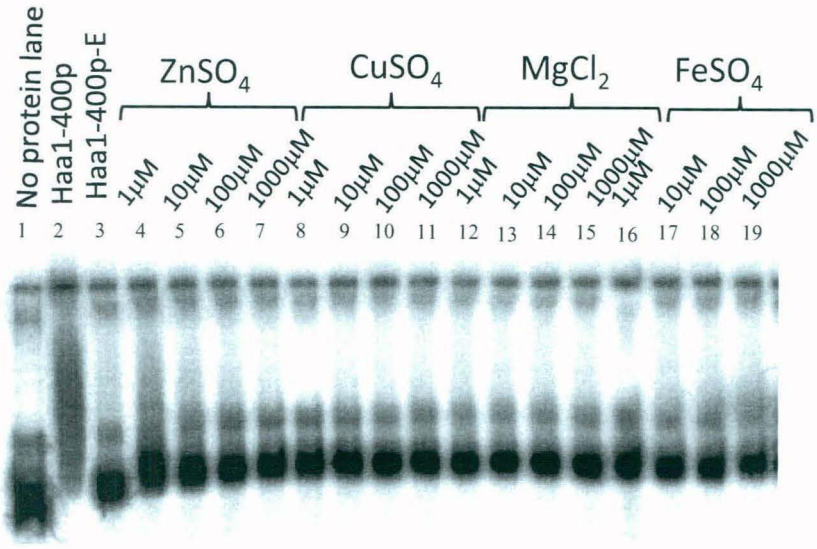


Figure 4.18: Effect of metal ions on DNA binding ability of EDTA inactivated H400p. H400p incubated with 10 mM EDTA, dialyzed and further used for DNA binding assay after incubation with $ZnSO_4$, $CuSO_4$, $MgCl_2$ or $FeSO_4$. End labeled probes were mixed with H400p, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Lane 2 is untreated and lane 3 is EDTA treated H400p. Proteins were used at final conc. of 1 μM.

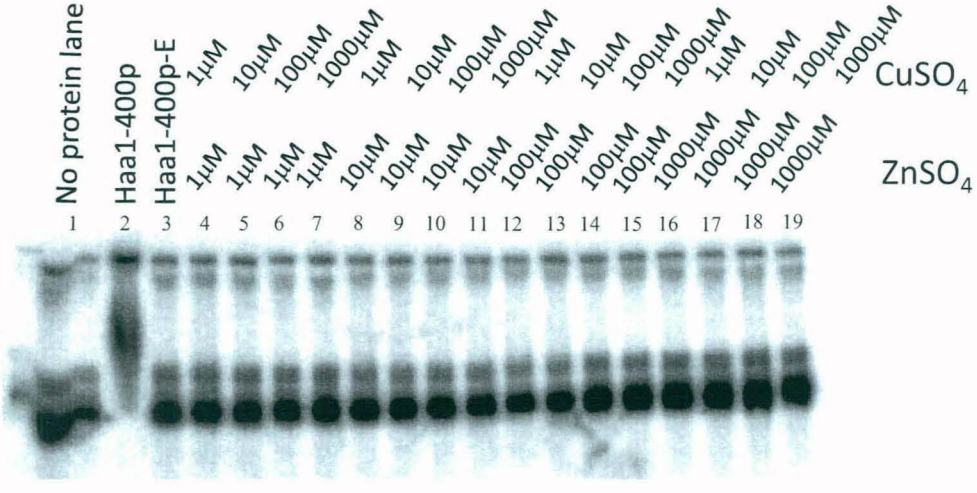


Figure 4.19: Effect of $CuSO_4$ and $ZnSO_4$ on DNA binding ability of EDTA inhibited H400p. H400p incubated with 10 mM EDTA, dialyzed and further used for DNA binding assay after incubation with $ZnSO_4$ and $CuSO_4$,. End labeled probes were mixed with H400p incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Lane 2 is untreated and lane 3 is EDTA treated H400p. Proteins were used at final conc. of 1 μM.

zinc stability domain. It have been shown that when Maclp is expressed from *E. coli* strain BL21 (DE3), addition of 1 mM $ZnSO_4$ after IPTG induction improves the protein purity (Jensen et al., 1998). Thus we checked the effect of $ZnSO_4$ on protein expression and purification of Haalp and H400p. After 30 minutes of IPTG induction of *E. coli* strain expressing Haalp and H400p, $ZnSO_4$ was added (0.25, 0.5 and 1.0 mM) and the cells were grown for additional 5 hrs. It was observed that addition of increasing concentration of $ZnSO_4$ significantly improves the purity of protein of both Haalp and H400p, but yield decreases (Figure-4.20 (a-c)). Thus proteins purified after addition of 0.5 mM $ZnSO_4$ were further used for DNA binding experiments. However, proteins expressed in the presence of additional Zn^{2+} ions lack DNA binding ability as no mobility shift in *YGP1*- probes was seen (Figure-4.20 (d)).

4.8 Promoter mapping of *YGP1* and *TPO2*:

Our previous northern blot experiments have shown that Haalp regulates the expression of *YGP1* and *TPO2* differently. Subsequent DNA binding experiments of Haalp/H400p with *YGP1* and *TPO2* upstream regions indicate the possibility of more than one binding sites. Based on DNA binding experiments, *YGP1* and *TPO2* genes were cloned with promoter having 400 bp to 1000 bp of upstream region in p315ADH replacing ADH promoter (Fig-21 (a) and 22 (a)). *YGP1* clones thus constructed were named as YS1, YS2 and YS3 (containing -390 bp, -579 bp and -1000 bp upstream region respectively) were transformed into *ygp1Δ* strain. Similarly *TPO2* clones named TS1, TS2 and TS3 (containing -405 bp, -640 bp and -1000 bp upstream region respectively) were transformed into *tpo2Δ* strain. Expression of *TPO2* was checked in respective strains with or without acetic acid shock by northern blotting. *TPO2* expression was seen at all the promoter lengths tested, in unshocked as well as acetic acid shocked cells, although *TPO2* expression increases upon acetic acid shock. Thus essential regulatory regions required for *TPO2* expression could be present within 405 nucleotides upstream of its coding sequences (Fig-21 (b)). To identify the regulatory elements important for *TPO2* expression clones having -304 bp (TS4) and -214 bp (TS5) upstream regions were also constructed and transformed in *tpo2Δ* strain. Expression of *TPO2* was checked from these clones as well as original clones. It was observed that 405 bp upstream region is required for *TPO2* expression in acetic acid shocked or unshocked cells as TS4 and TS5 shows no expression of *TPO2* (Fig-21 (c)). *TPO2* seems to have a single reglulatory element located between nucleotide -405 and -304 and it is sufficient for *TPO2*

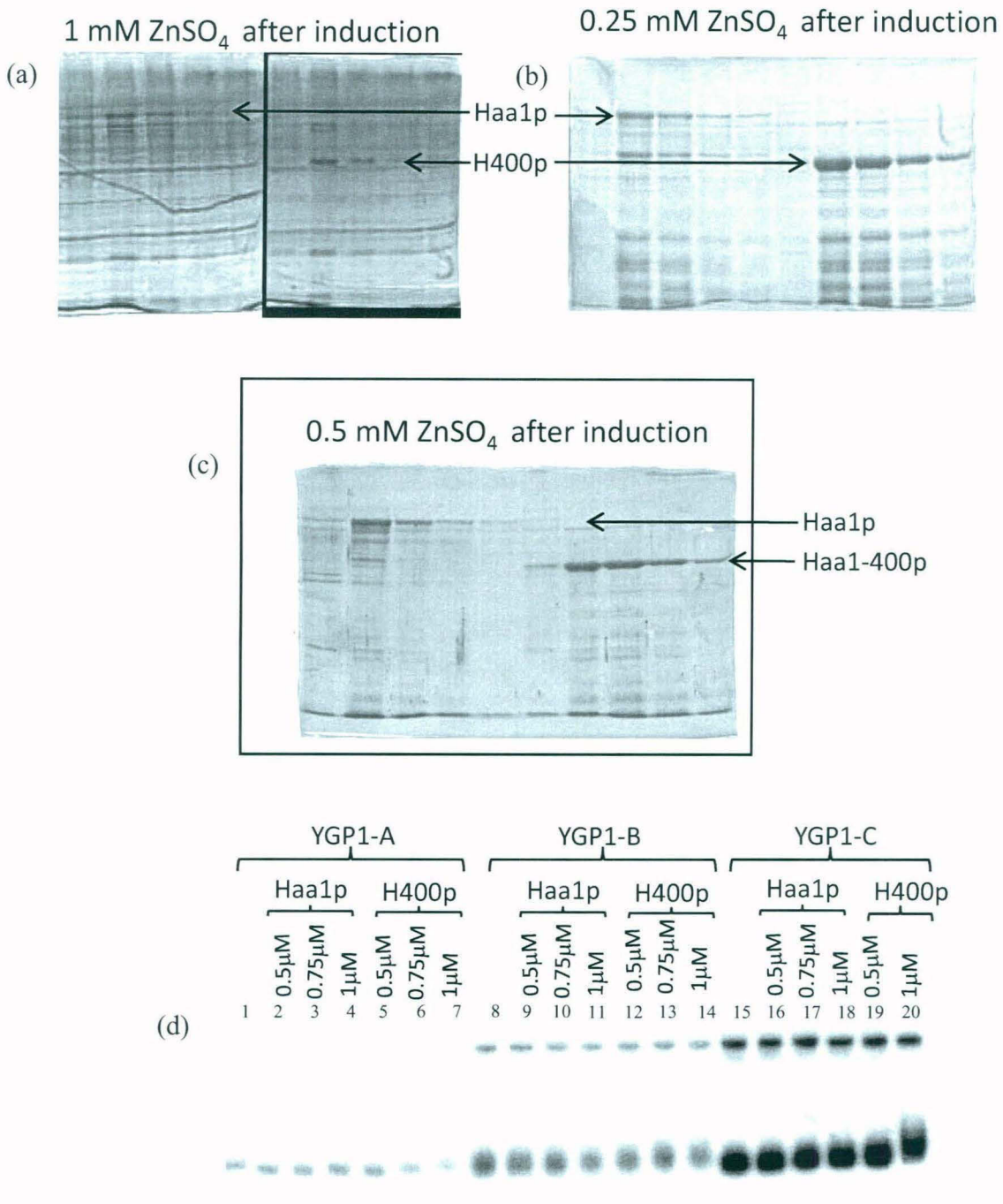


Figure 4.20: Effect of $ZnSO_4$ addition after IPTG induction on protein purification and DNA binding ability of Haa1p and H400p. After 30 min of IPTG induction of *E. coli* strain codon$^+$ carrying pET28c+*HAA1* or +H400, $ZnSO_4$ was added to induced culture and protein was purified after 5 hours of induction. Elutions of Haa1p and H400p purified from cultures added with (a) 1 mM $ZnSO_4$, (b) 0.5 mM $ZnSO_4$ and (c) 0.25 mM $ZnSO_4$. (d) DNA binding assays of Haa1p and H400p purified after addition of 0.5 mM $ZnSO_4$ with YGP1 probes. Lane 1-7 YGP1-A, 8-14 YGP1-B and YGP1-C.

expression in with or without acetic acid shock. However, based on previous reports and our own observation that *TPO2* expression increases upon acetic acid shock, we called this element as Acetic Acid Response Element (AARE) for *TPO2*.

When expression of *YGP1* was checked from YS1, YS2 and YS3 clone, it was detected at all the promoter lengths tested in unshocked cells but upon acetic acid shock YS1 clone having first 390 bps of *YGP1* upstream region was defective in *YGP1* expression (Fig-22 (b)). These observations indicated that *YGP1* expression is regulated differently in unshocked and acetic acid shocked cells, as different promoter elements seems to be required for *YGP1* expression in these conditions. To further scale down the essential regulatory elements present upstream of *YGP1* and required for acetic acid induced and constitutively induced *YGP1* expression, clones having -579 bp (YS4), -479 bp (YS5) and -290 bp (YS6) upstream region were constructed and transformed in *ygp1Δ* strain. Expression of *YGP1* was checked from these clones as well as original clones in with and without acetic acid shock. It was observed that 390 bp promoter region present in YS1 is minimum region required for *YGP1* expression in unshocked cells as in YS6 clone with 290 bp upstream region no expression is seen. In acetic acid shocked cells 579 bp promoter region present in YS4 is minimum region required for *YGP1* expression, as clones YS5 and YS1 with shorter promoter regions were defective in acetic acid induced *YGP1* expression (Fig-22 (C)). This upstream regulatory element present between nucleotide -579 and -479 is referred as Acetic Acid Response Element (AARE) for acetic acid induced *YGP1* expression, whereas upstream elements present between nucleotide -390 and -290 is referred to as Non Acetic acid Response Element (NARE) for *YGP1* expression.

4.9 Discussion:

Haa1p is a transcriptional activator involved in early adaptation to acetic acid stress in yeast. In our screening for genes conferring acetic acid resistance upon overexpression, we found multiple clones expressing *HAA1*. However, among *HAA1* clones few were expressing shortened gene product, as confirmed by sequencing and northern blot analysis. Truncated *HAA1* clones were acetic acid resistant in screening and were able to complement the acetic acid sensitivity of *haa1Δ* strain. Such a finding was interesting as possibly C-terminal part of Haa1p either has no function in Haa1p mediated acetic acid tolerance or it plays some regulatory roles which can be bypassed upon encountering acetic acid shock.

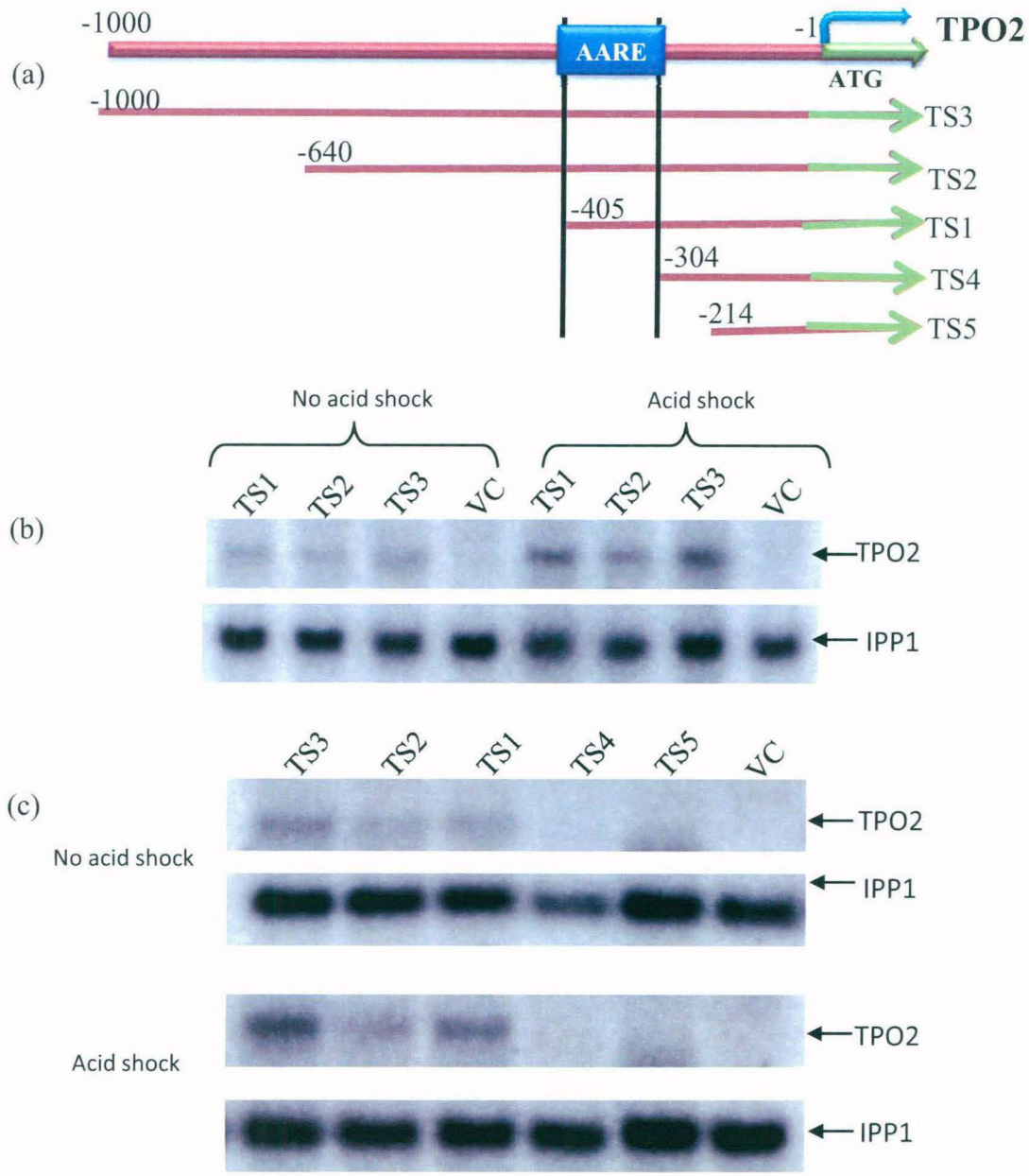


Figure 4.21: Promoter mapping of *TPO2*. (a) Schematic representation of *TPO2* promoter deletions. *TPO2* gene with 5' deletions of upstream regulatory region were cloned in pRS315, covering maximum 1000 bp and progressively reduced to 214 bp of upstream region. (b) The *TPO2* expression was initially checked in the *tpo2Δ* strain transformed with TS1, TS2, TS3 and (c) TS1 to TS5 in untreated cells and after acetic acid shock (YPD with 75 mM acetic acid at pH 3.0)., VC is vector control.

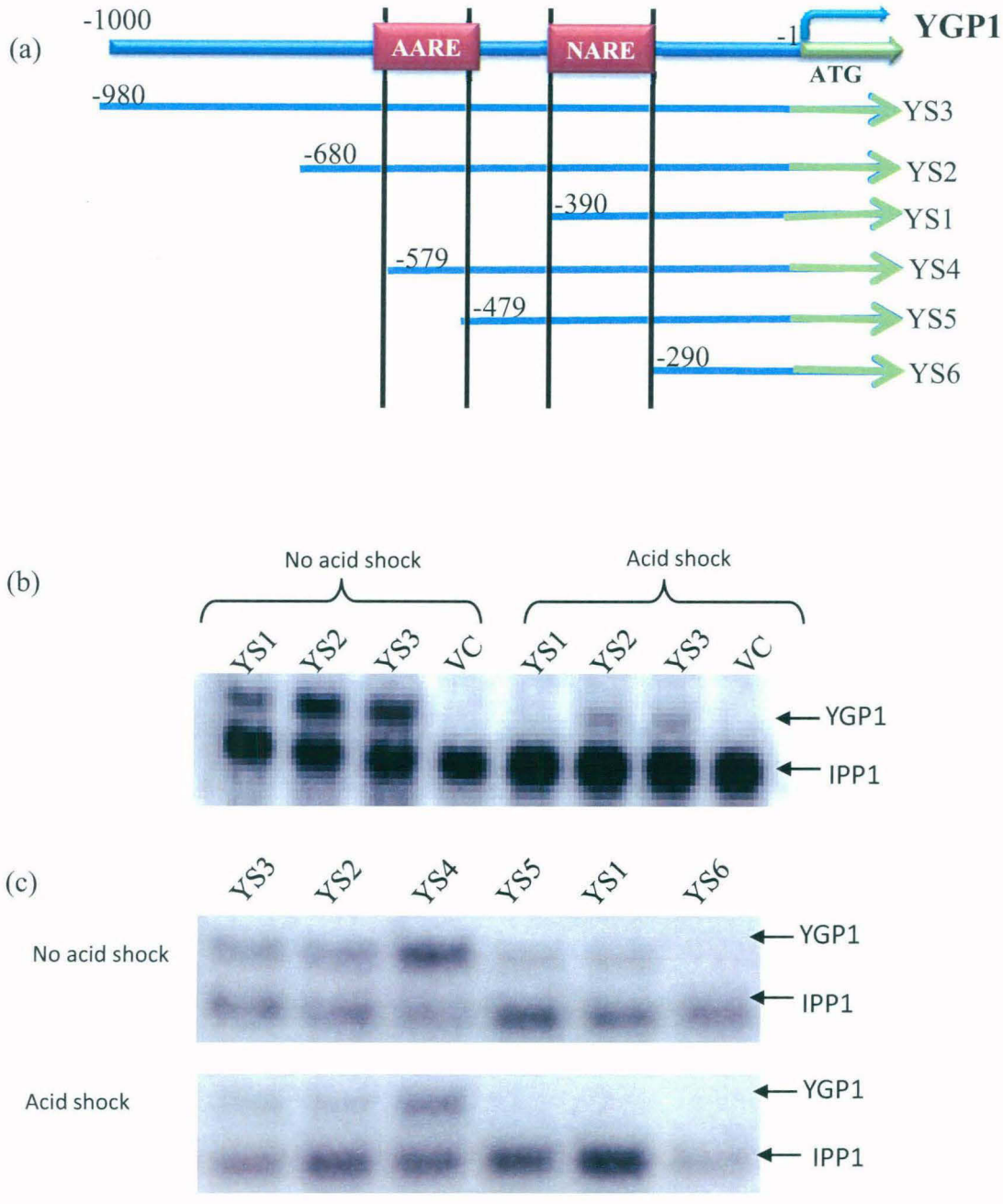


Figure 4.22: Promoter mapping of *YGP1*, (a) Schematic representation of *YGP1* promoter deletions. *YGP1* gene with 5' deletions of upstream regulatory region were cloned in pRS315, covering maximum 1000 bp and progressively reduced to 290 bp of upstream region. (b) The *YGP1* expression was initially checked in the *ygp1Δ* strain transformed with YS1, YS2, YS3 and (c) YS4, YS5, YS6 in untreated cells and after acetic acid shock (in YPD with 75 mM acetic acid at pH 3.0), VC is vector control.

To map domains or regions of Haa1p important for acetic acid adaptation in yeast, deletions of *HAA1* lacking either C-terminal or N-terminal regions or both were generated and expressed under TEF promoter. C-terminal deletions H500 and H400 can complement acetic acid sensitivity of *haa1Δ* strain and provide tolerance to acetic acid stress better than full-length *HAA1*. Like full-length *HAA1*, expression of H400 and H500 can induce expression of Haa1p target genes *TPO2, TPO3* and *YGP1*. Haa1p mediated regulation of *YGP1* and *TPO3* was relatively similar in yeast cells treated or untreated with acetic acid, whereas expression of *TPO2* increases upon acetic acid shock. Relative upregulation of *TPO2* upon acetic acid shock was highest under H400, although *TPO2* transcript level is not higher than that seen in full-length *HAA1* or H500. Moreover H400 provide higher resistance than full-length or any other deletions tested on acetic acid containing plates and causes least hours of lag phase in acetic acid containing broth. Thus there is a possibility that C-terminal region of Haa1p beyond first 400 or 500 amino acids has some regulatory region that represses Haa1p activity. Here an intriguing possibility is that C-terminal region of Haa1p is subject to repression via other repressor proteins in normal growth conditions and upon acetic acid shock Haa1p is released from repression to perform its function. Thus C-terminal deletion clones H400 and H500 lacking Haa1p domains or regions subjected to repression are more active in providing acetic acid resistance in yeast cell. Expression of full-length *HAA1* from GPD promoter results into slow growth phenotype, whereas slow growth is not seen upon expression of C-terminal deletions from GPD promoter. Thus it is possible that full-length Haa1p regulates the expression of additional genes, not regulated by H400 and H500, resulting in growth defect. Thus, C-terminal deletions can be preferably used in strain improvement as they provide more acetic acid tolerance without causing growth defect upon overexpression.

Deletions lacking N-terminal 50 (N50) or 100 (N100) amino acids of Haa1p can partially complement the *haa1Δ* strain for acetic acid sensitivity and induction of Haa1p target genes. N-terminal deletions can regulate the expression of *YGP1* and *TPO3*, but they seem unable to activate expression of *TPO2*. N-terminal deletions lack possible DNA binding region of Haa1p transcription factor, consisting of Zn module responsible for stability and Cu regulatory domain. Such DNA binding regions are present in copper regulated transcription factors Ace1p and Mac1p, responding to copper status of the cell. But it is known that expression of Haa1p target genes is not affected by copper status of the yeast cell and DNA binding of Haa1p is copper independent (Keller et al., 2001). A recent report shows that Haa1p DBD (1-127 aa) can interact with Haa1p responsive elements present in *TPO3*

upstream region (Mira et al., 2011). Our results show that N-terminal 50 or 100 amino acids are essential for upregulation of *TPO2* expression. Earlier it is shown that N-terminal minimal DNA binding region of Haa1p (1-124 amino acids) fused with VP16 activation domain can induce expression of *TPO2*. Thus, these results indicate that N-terminal region of *HAA1* has DNA binding region for *TPO2*. On the other hand N-terminal deletions are able to induce expression of *TPO3* and *YGP1*, thus N-terminal region is not essential for *TPO3* and *YGP1* expression. Moreover clones having simultaneous deletion at N and at C termini of *HAA1*, N504 and N1004 are unable to activate expression of any Haa1p target gene tested and did not complement the *haa1Δ*. These results indicate that Haa1p regulates the expression of *TPO3* and *YGP1* differently than it does for *TPO2*. A Haa1p region beyond first 100 amino acids may have a DNA binding region that in association with C-terminal region beyond 400 amino acids can alternatively activate the expression of *TPO3* and *YGP1* in N-terminal deletions. Haa1p mediated upregulation of *TPO3* and *YGP1* may represent general response of Haa1p, whereas *TPO2* expression is induced mainly upon acetic acid shock. Haa1p may have dual regulatory regions; one responding to generalized and other responding to acetic acid induced expression of Haa1p target genes. N-terminal region of Haa1p may function as acetic acid induced DNA binding domain, as it is essential for *TPO2* expression. There could also be proteins that differentially bind to Haa1p and H400, thereby modulating their function at different promoters.

The histidine tagged Haa1p and its truncated proteins purified from heterologous *E. coli* host were consistently degraded in similar manner. Possibly Haa1p has intrinsic instability associated with its structure in heterologous host, moreover we were unable to purify Haa1p or its deletion proteins from native host. DNA binding of partially pure Haa1p and H400 with upstream sequences of *YGP1* and *TPO2* coding region were seen. Haa1p protein showed DNA binding with three separate overlapping regions within 1 kb upstream region of *TPO2* and *YGP1* coding sequences. On the other hand Haa1p does not show any DNA binding with non specific Mycobacterial probe DNA used. These results show that Haa1p specifically binds to multiple binding sites in *TPO2* and *YGP1* regulatory regions. Exact Haa1p binding sites were not mapped as protein was partially purified and possibility of multiple binding sites is still to be validated fully. N terminal DNA binding domain in Haa1p similarly have Cu and Zn ions associated with it, as shown by atomic force spectroscopy data (Data not shown). Haa1p chelated with EDTA loses the metal ions associated with it and also DNA binding ability. Moreover loss of DNA binding ability of Haa1p is not restored upon

incubation of EDTA inactivated Haa1p with copper or zinc salt either alone or together. These results show that Haa1p DNA binding domain is stabilized by Zn and Cu ions and presence of Zn^{2+} and Cu^{+} ion is essential for DNA binding ability of Haa1p. Like Haa1p, H400p also shows similar metal dependent DNA binding with *YGP1* and *TPO2* upstream probes. Thus H400p has all DNA binding regions present in Haa1p that are required for activation of *TPO2* and *YGP1* expression.

Promoter mapping of *YGP1* and *TPO2* to identify regulatory regions required for Haa1p mediated expression, revealed that both genes are regulated quite differently. *YGP1* has two regulatory elements in upstream region, one responding to acetic acid called, Acetic Acid Response Element (AARE) and another responding in untreated cells which is not required for acetic acid induced expression, referred to as Non Acetic acid Response Element (NARE). On the other hand *TPO2* has single regulatory element in upstream region, mainly responding to acetic acid shock, thus referred as AARE. Since Haa1p response element has been identified in upstream region in one of the Haa1p target gene *TPO3* and DNA binding has been established (Mira et al., 2011), we propose that Haa1p has two possible binding sites in upstream region of *YGP1* in AARE and NARE respectively, whereas *TPO2* has single Haa1p binding in its AARE. Since *HAA1* C-terminal deletions H400 and H500 as well as N-terminal deletions N100 and N50 can induce expression of *YGP1* independently. It is possible that Haa1p has two DNA binding domain and earlier shown N-terminal DBD preferably recognizes to AARE like in *TPO3* and as it can be proposed for *TPO2*, whereas NARE can be recognized by additionally or alternatively by separate Haa1p domain.

Chapter 5
Regulation of Haa1p mediated acetic acid resistance

5.1 Introduction:

Stress conditions induce expression of stress responsive proteins and repress the expression of housekeeping proteins including events of protein synthesis in yeast. Thus, stress induced transcriptional responses, while favoring survival of yeast cells retard growth (Lelandais and Devaux, 2010). Activation of transcription by stress induced transcription factors is highly regulated process, which can be imparted at the level of activity or expression of transcription factor itself. As discussed in previous chapter, *HAA1* overexpression from constitutive and strong GPD promoter was toxic for yeast growth. On the other hand, overexpression of *HAA1* deletion clones from same plasmid and promoter was not toxic. Thus toxicity of *HAA1* overexpression cannot be simply attributed to extra cost of overproducing a protein. It is likely that Haa1p mediated adaptation to acetic acid involves heavy metabolic cost as yeast cells select between survival and growth. Thus, Haa1p function must be tightly regulated in yeast cells to avoid unwanted growth inhibition. Here we have studied the regulation of Haa1p function at various levels to understand how cellular machinery controls the Haa1p function.

5.2 Haa1p interacting proteins:

Global genomic and proteomic approaches have yielded information on possible genetic and physical interactions of Haa1p with other proteins. The Biogrid, an online interaction database (Stark et al., 2006) presents various genetic and physical interaction data for Haa1p. Haa1p presumably interacts with several proteins involved in various cellular processes, though proteins involved chromatin remodeling and transcriptional regulation are enriched (Table5.1). Brief descriptions of these proteins are given below, based on information available in Sachharomyces Genome Database (SGD).

Haa1p presumably interacts with proteins involved in chromatin remodeling such as Hta2p, Hos2p, Ngg1p, Yta7p and Nto1p. Hta2p is histone H2A, a core histone protein required for chromatin assembly and chromosome function. Hos2p is a histone deacetylase required for gene activation via specific deacetylation of lysines in H3 and H4 histone tails, a subunit of the Set3p complex and a meiotic specific repressor of sporulation specific genes. Ngg1p is a component of transcriptional adaptor and histone acetyltransferase (HAT) complexes, such as the ADA, the SAGA and SLIK complexes. Nto1p is subunit of the NuA3p HAT complex that acetylates histone H3 and it contains PHD finger domain that interacts with methylated

Gene name	Experimental Evidence code	Role	Publication
Genes involved in chromatin remodelling function			
HTA2	Mass spectrometry	Hit	Krogan NJ (2006)
HOS2	Phenotypic Suppression	Bait	Beltrao P (2009)
NGG1	Mass spectrometry	Hit	Krogan NJ (2006)
NTO1	Negative Genetic	Bait	Zheng J (2010)
	Phenotypic Enhancement	Bait	Beltrao P (2009)
YTA7	Phenotypic Suppression	Hit	Beltrao P (2009)
Transcription activators			
GLN3	Negative Genetic	Hit	Zheng J (2010)
NGG1	Mass spectrometry	Hit	Krogan NJ (2006)
SEF1	Negative Genetic	Hit	Zheng J (2010)
URE2	Negative Genetic	Hit	Zheng J (2010)
Genes involved in transcription			
DST1	Mass spectrometry	Hit	Krogan NJ (2006)
HRR25	Mass spectrometry	Bait	Breitkreutz A (2010)
SIN4	Positive Genetic	Bait	Beltrao P (2009)
RPA135	Mass spectrometry	Bait	Schneider DA (2006)
Translation and mRNA transport			
NAB2	Affinity capture RNA	Bait	Batisse J (2009)
ERB1	Mass spectrometry	Hit	Krogan NJ (2006)
HEK2	Affinity capture RNA	Bait	Hasegawa Y (2008)
RIA1	Negative Genetic	Bait	Costanzo M (2010)
Signalling and nuclear localization			
MSN5	Yeast Two-Hybrid Data	Bait	Uetz P (2000)
FUS1	Yeast Two-Hybrid Data	Bait	Tong AH (2002)
			Tonikian R (2009)
YAK1	Dosage Resue	Bait	Malcher M (2010)

Table 5.1: List of Haa1p interacting proteins. Haa1p interacting proteins listed in Biogrid (Stark et al, 2006, thebiogrid.org) protein interaction dataset are categorized based on functional classes.

histone H3. Yta7p localizes to chromatin and plays role in regulation of expression of histone gene; it has a bromodomain-like region that interacts with the N-terminal tail of histone H3, and an ATPase domain potentially phosphorylated by Cdc28p.

Haa1p shows possible interactions with other yeast transcription activators such as Gln3p, Sef1p, Ure2p and Ngg1p. Gln3p is transcriptional activator of genes regulated by nitrogen catabolite repression (NCR), its localization and activity are regulated by quality of nitrogen source in media. Ure2p also functions as NCR transcriptional regulator that acts by inhibition of Gln3p transcription in good nitrogen source. Ure2p also has glutathione peroxidase activity and its altered form creates [URE3] prion. Sef1p is putative transcription factor in *S. cerevisiae* and has homolog in *Kluyveromyces lactis*. Sef1p contains a Zn(2)-Cys(6) binuclear cluster motif and it compensates for the essential function of *RPM2*. Besides being component of HAT complexes, Ngg1p functions as a transcription regulator involved in glucose repression of Gal4p-regulated genes and also represses the Pdr1p mediated transcription.

Haa1p interacts with several components of yeast transcription machinery, such as Dst1p, Hrr25p, Sin4p and Rpa135p. Dst1p is a general transcription elongation factor TFIIS that enables RNA polymerase II to read through blocks to elongation by stimulating cleavage of nascent transcripts stalled at transcription arrest sites. Hrr25p is protein kinase involved in regulating diverse events including vesicular trafficking, DNA repair and chromosome segregation. Hrr25p binds with C-terminal repeat domain (CTD) of RNA pol II thus function as CTD kinase and its activity is required for normal phosphorylation of the CTD on elongating RNA polymerase II. Sin4p is subunit of RNA polymerase II mediator complex; it associates with core polymerase subunits to form the RNA pol II holoenzyme and contributes to both positive and negative transcriptional regulation. Rpa135p is second largest subunit of RNA polymerase I called A135 and similar subunits encoded by other genes are also present in RNA polymerase II and III.

Haa1p also has possible interactions with protein involved in translation and mRNA transport from nucleus to cytosol such as Nab2p, Erb1p and Hek2p. Nab2p is a nuclear polyadenylated RNA binding protein required for nuclear mRNA export and poly (A) tail length control; it also helps in auto regulation of mRNA level. Erb1p is constituent of 66S pre-ribosomal particles; it forms a complex with Nop7p and Ytm1p that is required for maturation of the large ribosomal subunit. Haa1p also interacts with Msn5p, a karyopherin involved in nuclear

import and export of proteins, including import of replication protein A and export of Swi6p, Farlp and pho4p. Several proteins involved in signal transduction in yeast, such as Fuslp, Ste20p and Yaklp are also shown to interact with Haalp. Fuslp localizes to shmoo tip and is proposed to coordinate signaling, fusion and polarization events required for cell fusion. Ste20p is Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family; it is involved in pheromone response, pseudohyphal/invasive growth, vacuole inheritance and down-regulation of sterol uptake. Yaklp is serine-threonine protein kinase that is part of a glucose-sensing system and it regulates growth in response to glucose availability.

Diverse interaction of Haalp suggests that activity of Haalp must be tightly regulated in expression of Haalp target genes. Haalp shows interactions with proteins involved in chromatin remodeling and components of transcription machinery, suggesting that Haalp is directly involved in transcription of its target genes and their induction upon acid shock involve interplay of multiple regulatory proteins. Possible interaction of Haalp with Msn5p a karyopherin shows that, Msn5p may play important role in determining localization of Haalp. Similarly proteins involved in signal transduction like Yaklp, Fuslp and Ste20p may play important role in activation of Haalp upon acetic acid shock and therefore induction of its target genes. The information in Biogrid Database may not be comprehensive for Haalp interacting proteins, but still many important aspects of regulation of Haalp in yeast cells can be understood by studying the Haalp function in strains deleted for genes encoding the interacting proteins.

5.3 Haalp function in strains deleted for Haalp interacting or target genes:

Haalp interacting proteins may also play a role in acetic acid adaptation of yeast cells by modulating the activity of Haalp. Thus acetic acid sensitivity of strains deleted in Haalp interacting proteins was checked by dilution spotting on acetic acid containing YPD agar plates at pH 3.0. It was observed that *ngg1Δ, ste20Δ, fus1Δ, msn5Δ* and *tda6Δ* deletion strains have similar acetic acid sensitivity or resistance as wildtype strain *BY4741,* whereas *haa1Δ* strain was sensitive. Simultaneously acetic acid sensitivity of strains deleted in Haalp target genes such as *com2Δ, ygp1Δ, tpo2Δ, phm8Δ, spi1Δ, yro2Δ* and genes encoding components of vacuolar ATPases such as *vph1Δ* and *vma1Δ* were also checked. As previously reported, *tpo2Δ, vph1Δ* and *vma1Δ* strains were found to be sensitive to acetic acid whereas *com2Δ,*

ygp1Δ, *spi1Δ* and *yro2Δ* were marginally sensitive or comparable to wildtype (Figure 5.1) (Fernandes et al., 2005; Mira et al., 2010b)

Since strains deleted in genes encoding Haa1p interacting proteins were not much affected in acetic acid sensitivity, the effect of overexpression of *HAA1* or its H400 deletion in these strains and in strains deleted for Haa1p target genes was studied. The *ngg1Δ*, *ste20Δ*, *fus1Δ*, *msn5Δ*, *ygp1Δ*, *tpo2Δ*, *phm8Δ*, *spi1Δ*, *tpo3Δ* and *BY4741* strains were transformed with p416TEF-HAA1-HA and p416TEF-H400-HA plasmids and the acetic acid sensitivity/resistance was further checked. Overexpression of *HAA1* and H400 enhance acetic acid resistance in wildtype strain *BY4741* and in *haa1Δ* strain. Similarly in *ngg1Δ*, *ste20Δ*, *fus1Δ*, *ygp1Δ*, *tpo2Δ*, *phm8Δ*, *spi1Δ* and *tpo3Δ* strains overexpression of *HAA1* and H400 enhances acetic acid resistance; moreover strains expressing H400 were slightly more resistant compared to those expressing full-length *HAA1*. However, in *msn5Δ* strain, overexpression of *HAA1* decreased acetic acid resistance, whereas overexpression of H400 enhanced acetic acid resistance as in other strains tested (Figure-5.2).

We also observed that few deletion strains overexpressing *HAA1* were severely retarded for growth in SD medium with auxotrophic supplements. Thus some of the initial experiments had to be abandoned as sufficient comparable growth was not obtained for these strains. When similar set of strains were grown in SC media without uracil a decent growth was observed, thus the growth rates of these strains were checked in SD medium with auxotrophic supplements. Deletion strains overexpressing *HAA1*, H400 and vector control were grown overnight in SC medium to achieve sufficient growth and further inoculated in SD medium in multiwell plates and growth was monitored at intervals by $O.D._{600}$ using a plate reader. Overexpression of *HAA1* under constitutive TEF promoter in *ngg1Δ*, *ste20Δ*, *fus1Δ*, *msn5Δ*, and *spi1Δ* strains caused slow growth with prolonged lag phase, whereas overexpression of H400 or vector control did not cause such slow growth in the same set of deletion strains (Figure-5.3). Growth retardation caused by *HAA1* overexpression was maximum in *msn5Δ* strain, where almost no growth was observed. The *ngg1Δ* strain was as such slow growing in minimal medium and upon *HAA1* overexpression its growth was further retarded. However overexpression of H400 did not reduce the growth as much as *HAA1*, but it was slower than vector control. In wildtype strain *BY4741* and in *ygp1Δ*, *tpo2Δ*, *phm8Δ*, and *tpo3Δ* strains overexpressing *HAA1* or H400 do not cause any growth defect compared to vector control. *HAA1* overexpression associated growth defect is seen only in deletion strains

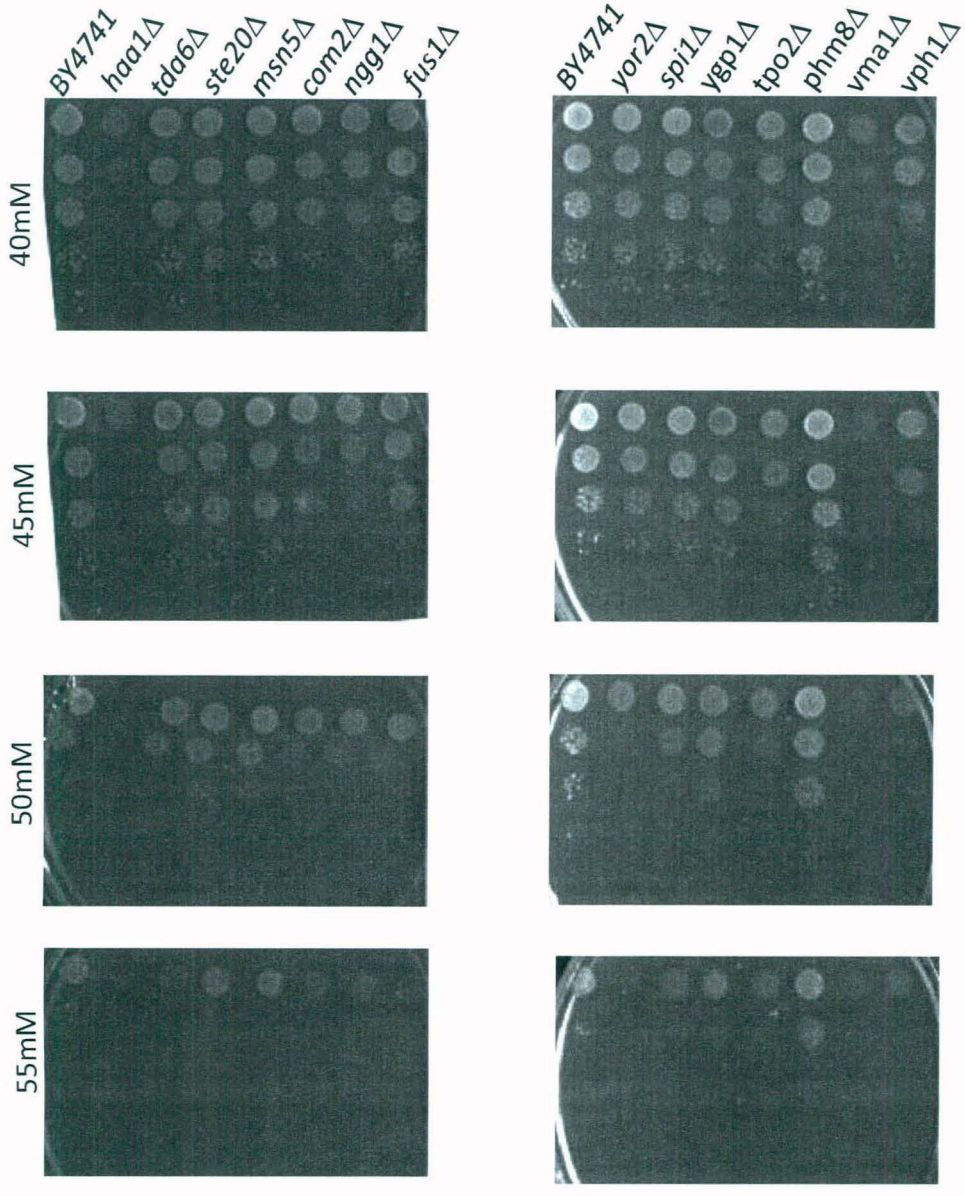


Figure 5.1: Acetic acid sensitivity of yeast deletion strains. *S. cerevisiae* deletion strains of selected Haa1p interacting proteins and Haa1p regulated genes were checked for acetic acid sensitivity by dilution spotting on YPD plates at pH 3.0 with indicated concentration of acetic acid, as described in materials and methods (Section 2.2.7).

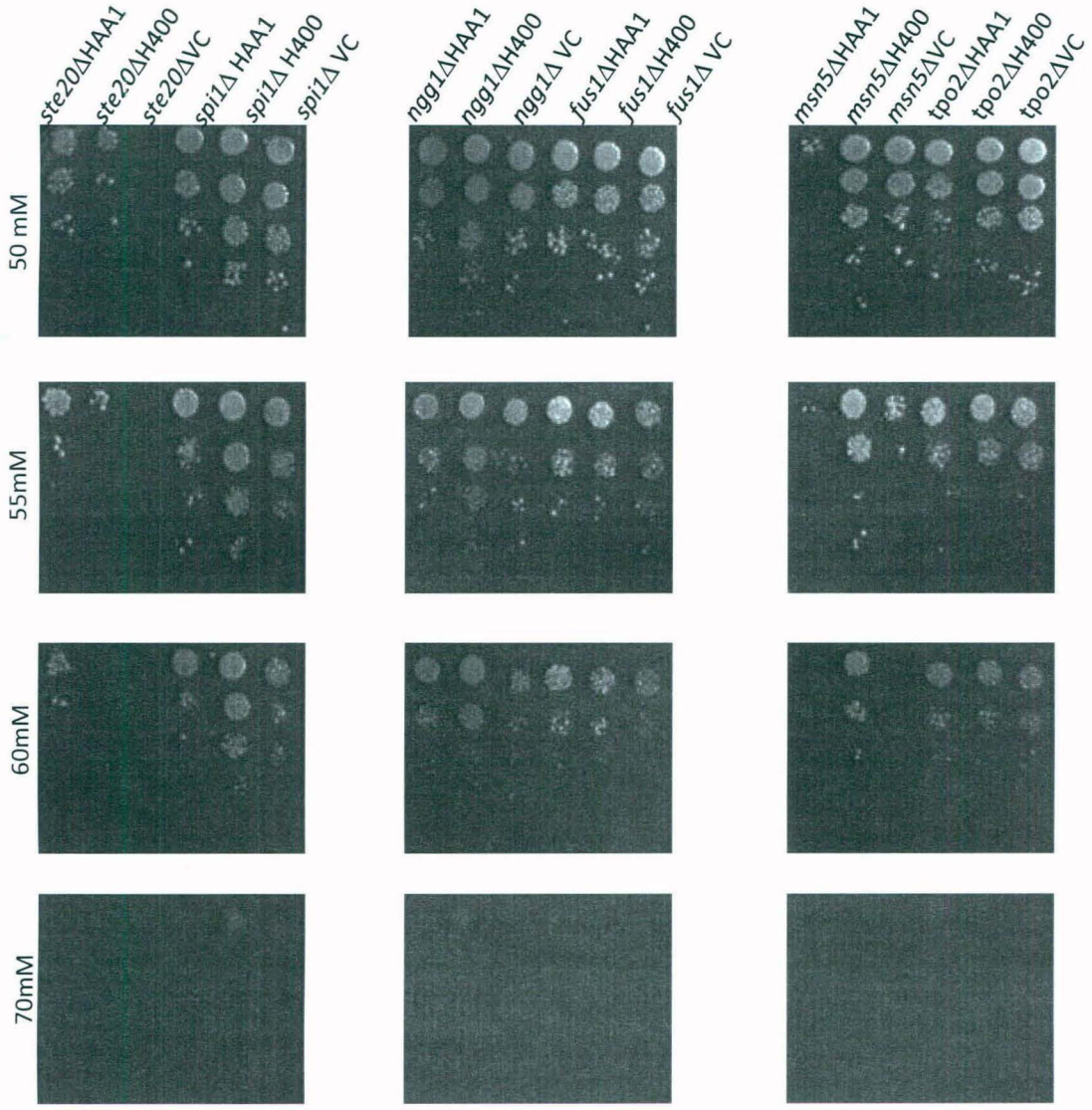


Figure 5.2: Acetic acid sensitivity of yeast deletion strains upon expression of *HAA1* or H400. *S. cerevisiae* strains deleted in selected Haa1p interacting proteins and Haa1p regulated genes were transformed with p416TEF-*HAA1*, -H400 and vector p416TEF. Acetic acid sensitivity of transformants was checked by dilution spotting on YPD agar plates at pH 3.0 with indicated conc. of acetic acid.

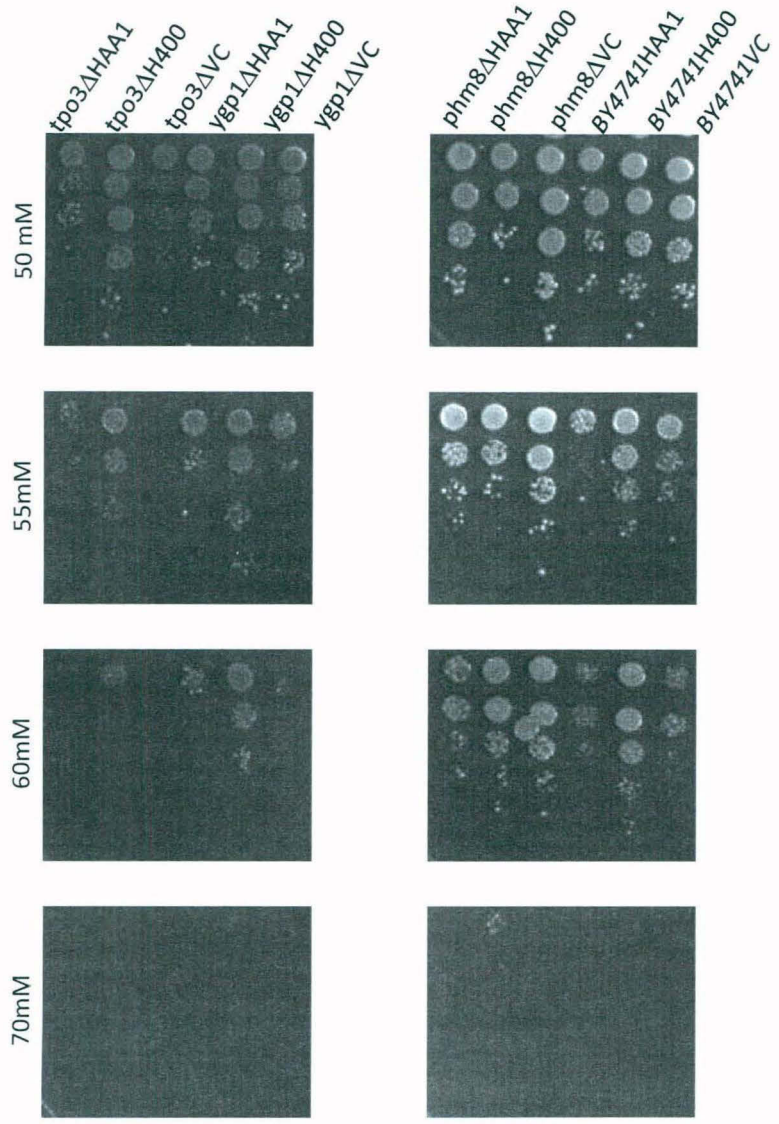


Figure 5.2 (continued): Acetic acid sensitivity of yeast deletion strain upon overexpression of *HAA1* or H400.

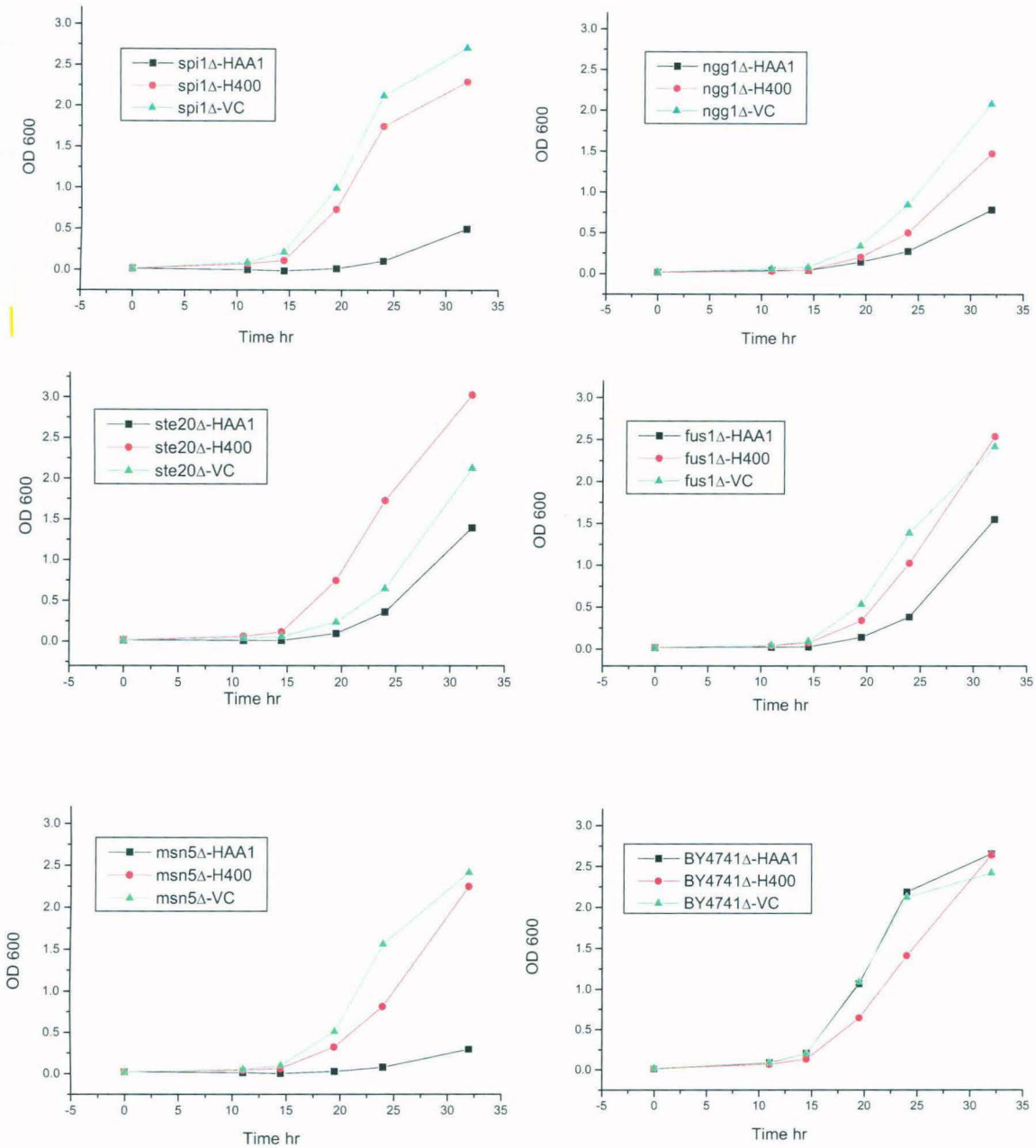


Figure 5.3: Growth of yeast deletion strains upon overexpression of *HAA1* and H400. *S. cerevisiae* deletion strains of selected Haa1p interacting proteins and Haa1p regulated genes were transformed with p416TEF-*HAA1*, -H400 and vector p416TEF. Transformants grown overnight in SC (synthetic complete) - Ura medium were reinoculated in SD (synthetic dextrose) medium with auxotrophic supplements at 0.05 $O.D_{600}$ in 96 well plates and incubated at 30°C without shacking. At indicated time points cells were resuspended by shacking on thermomixer at 1000rpm for 1 min and $O.D_{600}$ was measured in a multiwell plate reader after shacking for 5 seconds.

of Haa1p interacting proteins and in *SPI1* (an *HAA1* target gene) but not in wildtype strain. Such phenotypes seem to be related to regulation of Haa1p function during acetic acid adaptation, as in strains lacking interacting proteins mechanisms regulating the Haa1p function are likely to be absent.

5.4 Msn5p affects the localization of HA tagged Haa1p and H400p:

Keller et al (2001), have shown that Haa1p-GFP fusion expressed under *GAL1* promoter localizes to nucleus. We have found that full-length *HAA1* overexpression in *msn5Δ* strain results in growth defect in minimal media and acetic acid sensitivity, whereas overexpression of H400 in *msn5Δ* neither causes growth defect nor acetic acid sensitivity. Msn5p is a soluble transport receptor called karyopherin that mediates the recognition of specific nuclear import and export signals. Msn5p is involved in diverse cellular processes, like phosphate and carbon source metabolism, stress response, calcium signaling, mating response, pseudohyphal differentiation and cell cycle control. Msn5p acts as a nuclear export receptor for several proteins, including the transcription factors Pho4p, Mig1p, Msn2/4p, Crz1p, Rtg1/3p, Swi6p and other proteins like Cln-Cdc28p inhibitor Far1p and MAPK cascade scaffold protein Ste5p (Queralt and Igual, 2003). Moreover Msn5p is able to function bidirectionally since it also acts as importin of replication protein A (Yoshida and Blobel, 2001). Recently it was shown that Msn5p is required for re-export of mature tRNAs after their retrograde import from the cytoplasm (Murthi et al., 2010).

Given the fact that Haa1p possibly interact with Msn5p and Msn5p is involved in nuclear export and import of many other transcription activators, we checked the effect of Msn5p on localization of Haa1p. The p416TEF-HAA1-HA and p416TEF-H400p-HA plasmids were transformed into *haa1Δ* and *msn5Δ* strains and the localization of constitutively overexpressed Haa1p-HA and H400-HA was visualized by immunefluorescence as described (Materials and Methods, Sec 2.2.14). In h*aa1Δ* strain expressing Haa1p-HA or H400p-HA significant immunofluorescence signal as a result of immunostaining of HA tagged protein, colocalized with DAPI staining of nucleus but signal is also seen from cytosol. On the other hand, in *msn5Δ* strain immunofluorescence signal of Haa1p-HA and H400p-HA distinctly colocalized with DAPI staining of nucleus and no cytoplasmic signal is seen (Figure-5.4). These results suggest that possibly Msn5p exports the Haa1p and also H400p from nucleus to cytoplasm in normal growth conditions. Upon acetic acid shock, Haa1p is rescued from

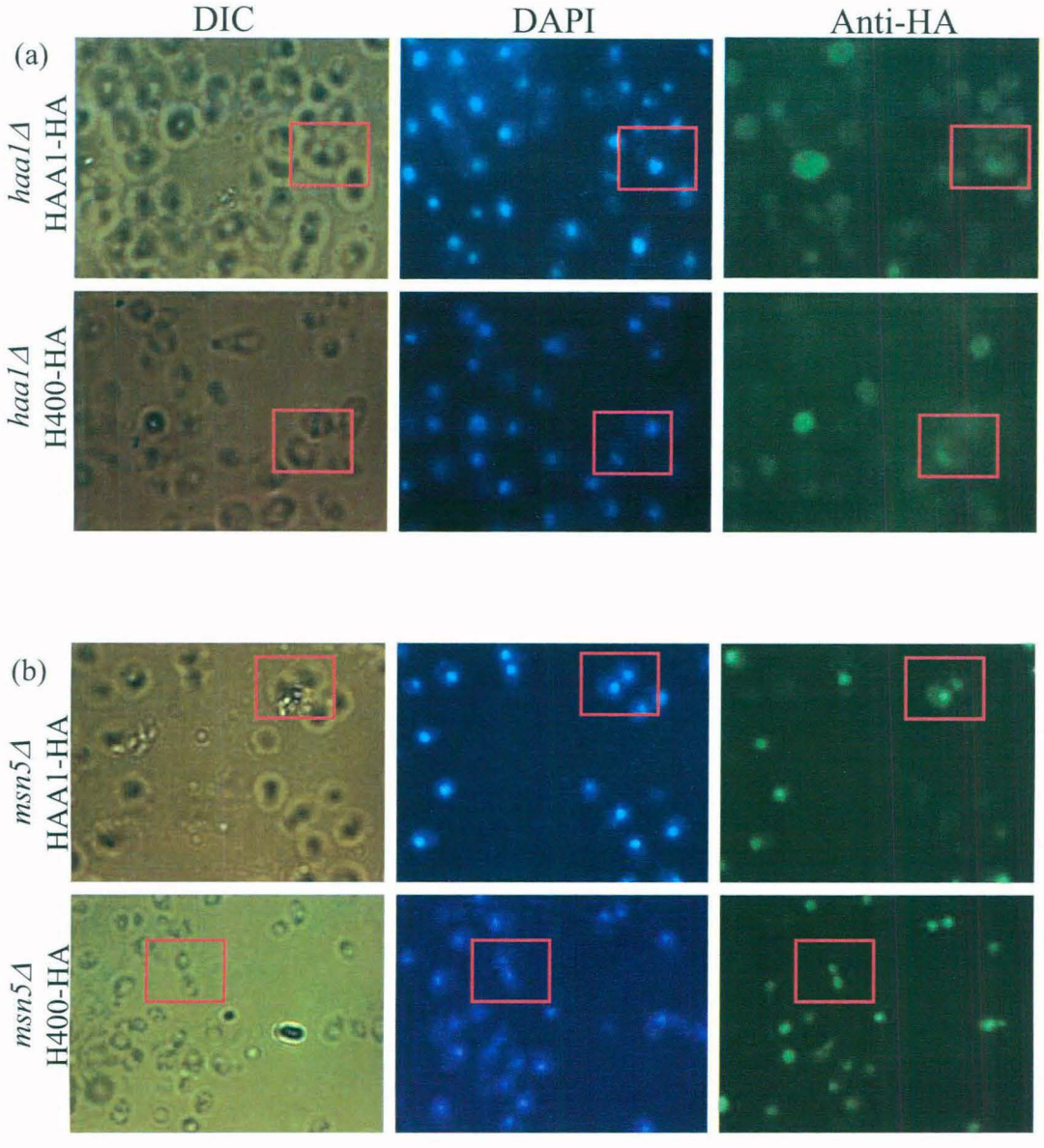


Figure 5.4: Localization of Haa1p-HA and H400-HA, HA tagged proteins were detected by immunofluorescence (a) in *haa1Δ* strain and (b) *msn5Δ* strain transformed with p416TEF-*HAA1*-HA and –H400-HA. Yeast strains were stained and visualized as described in materials and methods (Section 2.2.14).

Msn5p mediated export from nucleus and Haa1p localizes to nucleus as in *msn5Δ* strain. Moreover nuclear localization signal and Msn5p interacting domain of Haa1p must be present within the N-terminal 400 amino acids, since H400p also localizes to the nucleus in Msn5p dependent manner.

Since deletion of *MSN5* results in complete nuclear localization of Haa1p and H400p, the expression of Haa1p target genes *TPO2*, *YGP1* and *HAA1* itself was checked in *msn5Δ* strain by northern blot. While overexpression of Haa1p in *msn5Δ* strain did not significantly increase the expression of *TPO2* and *YGP1* form vector control having genomic copy of *HAA1*, although H400p increased the expression of these genes. (Figure-5.5). Since, constitutive nuclear localization of Haa1p in *msn5Δ* strain only marginally increases the expression of its target genes, tight regulation of Haa1p activity inside the nucleus is likely.

5.5 Expression analysis of deletion strains of Haa1p interacting proteins:

Since overexpression of *HAA1* in deletion strains of Haa1p interacting proteins confer slow growth in minimal media, these proteins must be affecting the functional activity of Haa1p in yeast cell. Haa1p interacting proteins may directly or indirectly control gene expression in yeast as they are involved in signal transduction, nuclear export and import and activation or repression of yeast transcriptional responses. Therefore we checked the expression of Haa1p target genes in these deletion strains. Yeast strains *BY4741*, *ngg1Δ*, *ste20Δ*, *fus1Δ*, *msn5Δ*, and *spi1Δ* were grown to exponential phase in minimal media and further subjected to acetic acid shock in YPD, 75 mM acetic acid at pH 3.0 for 4 hours. Total RNA was isolated from these strains and expression was checked by northern blot.

In wildtype stain *BY4741*, in the presence of functional Haa1p the expression of *YGP1* and *TPO2* is seen in with or without acetic acid shocked cells. However upon acid shock, expression of *TPO2* enhances, whereas *YGP1* expression remains comparable to unshocked cells. Similarly in *ste20Δ*, *fus1Δ*, *msn5Δ*, and *spi1Δ* deletion strains expression of *YGP1* or *TPO2* remains comparable to *BY4741* strain. However, in *ngg1Δ* strain expression of *YGP1* was less than that in *BY4741* strain without acid shock and expression of *TPO2* remains as in *BY4741*strain. Upon acetic acid shock in *ngg1Δ* strain expression of *YGP1* completely diminishes and expression of *TPO2* exponentially increases. Moreover *ngg1Δ* strain also shows increased expression of *HAA1* upon acetic acid shock (Figure-5.6). These gene

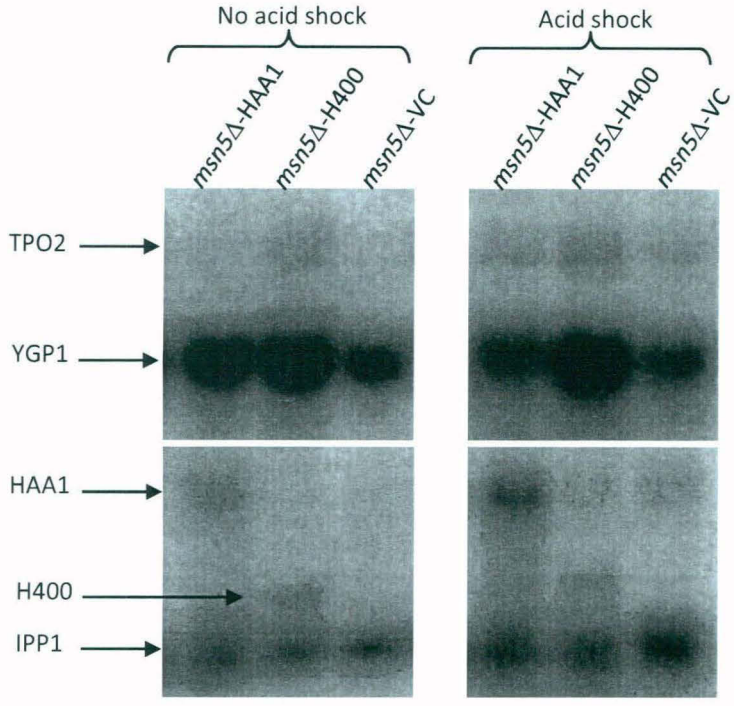


Figure 5.5: Expression of Haa1p target genes in *msn5Δ* strain with p416TEF-*HAA1*, -H400 and vector p416TEF. Yeast strains were grown to exponential phase and subjected to acetic acid shock (75 mM acetic acid at pH 3.0) for 4 hrs. The expression of *YGP1, TPO2* and *HAA1* was checked by northern blot. Levels of *IPP1* transcripts were detected and used as internal control.

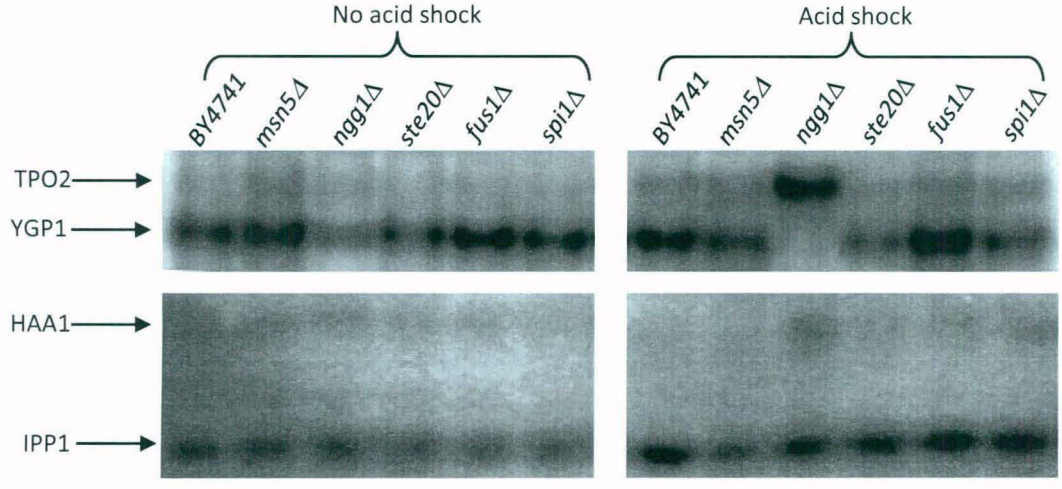


Figure 5.6: Expression of Haa1p target genes in yeast strains deleted in genes encoding Haa1p interacting proteins. The deletion strains were grown to exponential phase and subjected to acetic acid shock (75 mM acetic acid at pH 3.0) for 4 hrs. The expression of *YGP1, TPO2* and *HAA1* was checked by northern blot .

expression analyses were repeated for at least 3 times and similar results were observed. Therefore these observations indicate that Ngg1p is possibly involved in differential regulation of Haa1p activity upon acid shock. As in *ngg1Δ* strain expression of *YGP1* is reduced whereas expression of *TPO2* is exponentially increased upon acetic acid shock. Deletion of *MSN5,* which affects the localization of Haa1p, does not affect the expression of *YGP1* and *TPO2,* although a marginal increase in *TPO2* expression can be seen in without acetic acid shock compared to wildtype. This increase in *TPO2* expression may result from increased access of Haa1p to target regions in yeast genome as a result of constitutive nuclear localization, yet difference in *TPO2* expression was not very high as multiple regulatory mechanisms must be controlling the Haa1p activity inside the nucleus.

5.6 Ngg1p regulates Haa1p activity in acetic acid adaptation:

In previous section we have shown that deletion of *NGG1* results in differential expression of Haa1p target genes. *NGG1* or *ADA3* was initially identified as gene required for glucose repression of the GAL10-related his3-G25 promoter and its function was attributed to inhibition of Gal4p activity (Brandl et al., 1993). Deletion of *NGG1* confers growth defect in minimal medium and no growth in any media at 37°C (Pina et al., 1993). Ngg1p has two separate and non overlapping domains (amino and carboxyl terminal domains), which separately cannot complement the slow growth phenotype in minimal medium but simultaneous expression of both domains rescue the growth defect. Moreover Ngg1p forms heterodimeric complex with Ada2p and Gcn5p, where C-terminal domain of Ngg1 interacts with Ada2p (Horiuchi et al., 1995). Ngg1p is also shown to be a component of chromatin modifying Histone acetyltransferase (HAT) complexes, such as ADA (Horiuchi et al., 1995), SAGA (Eberharter et al., 1999) and SLIK (Pray-Grant et al., 2002). Amino terminal domain of Ngg1p is involved in transcriptional repression of the GAL genes by glucose. In Ngg1p a region essential for glucose repression lies within residues 274-373 and more precisely to a segment rich in Phenyl alanine residues, which is possibly part of an amphipathic α helix. Ngg1p also has an amino terminal transcriptional activation domain and the function of this domain in activation requires the presence of Ada2p (Brandl et al., 1996). The amino terminal domain of Ngg1p also interacts with the C-terminal activation domain of Pdr1p and Pdr3p. Interaction of Ngg1p with Pdr1p partially requires presence of Ada2p and results in inhibition of transcriptional activation by Pdr1p (Martens et al., 1996). In humans, Ngg1p

homolog hAda3p is shown to interact with p53 tumor suppressor protein and it is essential for DNA damage induced acetylation and stabilization of p53 as well as p53 target gene induction (Nag et al., 2007).

Based on the structure function and domain analysis of Ngg1p by Horiuchi et al, 1995 and Brandl et al, 1996 different deletions of *NGG1* were constructed. Horiuchi et al, 1995 have shown that slow growth phenotype of *ngg1Δ* strain can be complemented with expression of wildtype or even truncated allele lacking initial 214 amino acids. Moreover co-expression of carboxyl terminal domain of Ngg1p (364-702) with amino terminal domain of Ngg1p (1-346) or truncated domain (214-346) can fully complement the slow growth phenotype of *ngg1Δ* strain. Thus Ngg1p is supposed to have two separate domains which fold independently into functional units and interact noncovalently in the same functional pathway. Similar domain structure of Ngg1p also complements the defect in transcriptional regulation of Gcn4p in *ngg1Δ* strain (Horiuchi et al., 1995). In a separate study the Ngg1p regions from amino acids 274 to 373 was shown to be critical for glucose repression by Ngg1p, as in frame deletions $NGG1_{\Delta 274\text{-}307}$ and $NGG1_{\Delta 308\text{-}373}$ were defective in this function (Brandl et al., 1996).

To map the domains of Ngg1p controlling Haa1p function, deletions of *NGG1* were constructed encoding 1-702, 1-346, 214-702, 214-346, 1-671, 1-273/308-702 and 1-307/374-702 amino acids of Ngg1p (Figure-5.7) and cloned in p416ADH. The full-length *NGG1* and its deletions constitutively expressed under ADH promoter were transformed into *ngg1Δ* strain. Initially complementation of slow growth defect of *ngg1Δ* strain in minimal medium by *NGG1* deletions was checked by dilution spotting on SD medium with appropriate auxotrophic supplements. As previously shown, full-length *NGG1*, and its deletion clones 214-702 and 1-671 were only able to complement slow growth defect on minimal media (Figure-5.8). Further, expression of Haa1p target genes was checked in *ngg1Δ* strain transformed with *NGG1* deletion clones. These strains were grown to exponential phase in SD medium, subjected to acetic acid shock and expression was checked. The expression of functional *NGG1* clones that complement the slow growth defect in minimal media can also reverse the differential regulation of *YGP1* and *TPO2* expression to wildtype pattern. In presence of full-length *NGG1* or its deletion clone 214-702 or 1-671 expression of *YGP1* is seen to wildtype level in growing cells; moreover upon acetic acid shock *YGP1* expression is restored compared to no expression in *ngg1Δ* strain. In the same set of strains, *TPO2*

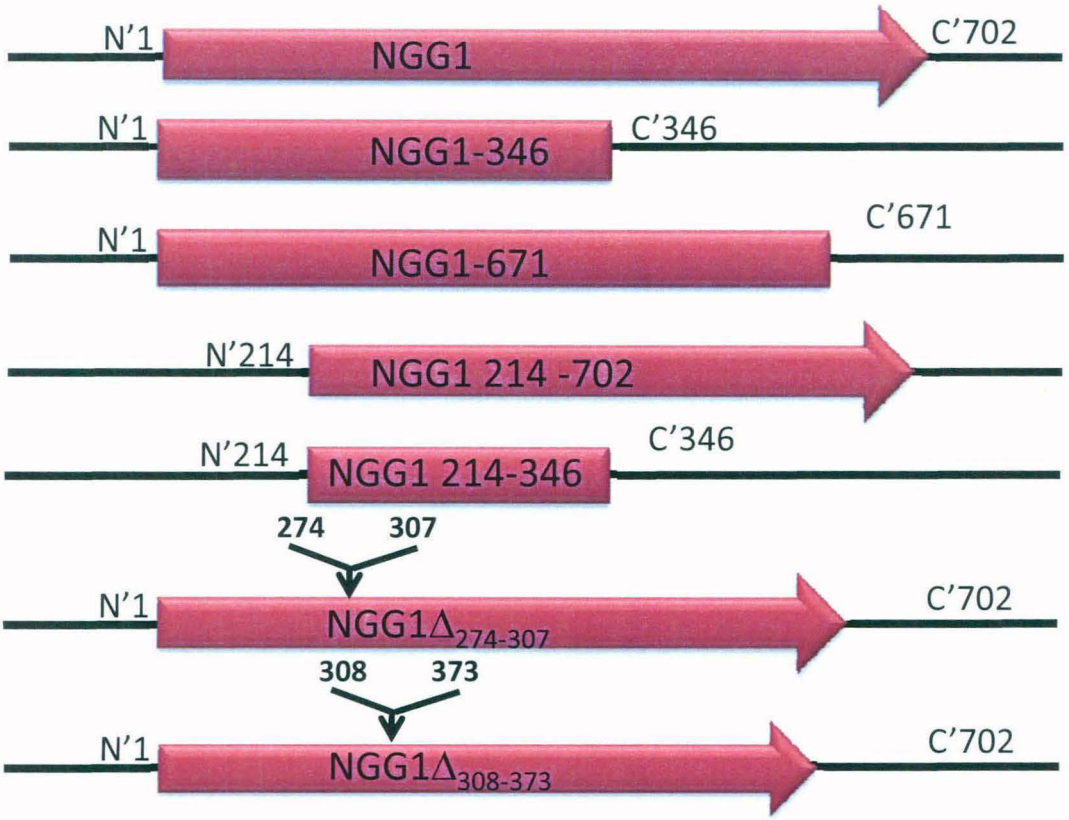


Figure 5.7: Schematic representation of *NGG1* deletions. Deletion clones of *S. cerevisiae* transcription regulator *NGG1* were constructed by PCR amplification of different regions, designated by amino acid position. Internal deletions were made by overlap extension PCR. All deletion clones were cloned in p416ADH yeast expression vector at *BamH*I and *Eco*RI restriction sites.

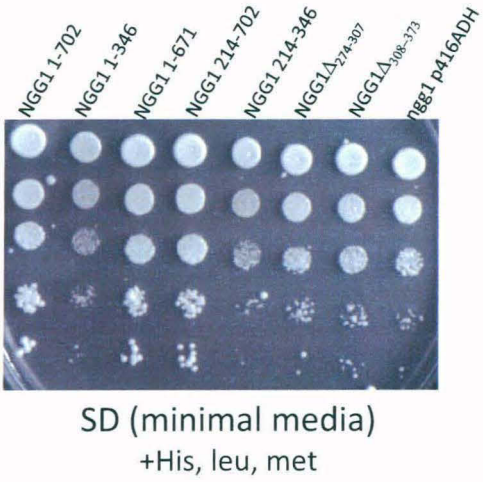


Figure 5.8: Complementation of slow growth phenotype of *ngg1Δ* strain. The *ngg1Δ* strains transformed with *NGG1* deletions and vector p416ADH were grown to exponential phase and rescue of slow growth phenotype in *ngg1Δ* strain was checked by dilution spotting on SD agar plates.

expression level is less than that in *ngg1Δ* strain, but comparable to wildtype. Rest of the *NGG1* deletions does not significantly affect the expression of Haa1p target genes in *ngg1Δ* strain (Figure-5.9). These results indicate that Ngg1p effect on the expression Haa1p target genes is likely to be through a mechanism similar to that of glucose repression of *GAL* genes and transcriptional regulation of Gcn4.

To further establish that role of Ngg1p in regulation of *TPO2* and *YGP1* expression is through modulation of Haa1p activity. We deleted the genomic copy of *HAA1* in *ngg1Δ* strain by *HIS3* auxotrophic selection marker as follows. *HIS3* gene from pRS303 plasmid was PCR amplified using primers having 40 base pair homology to region upstream and downstream of *HAA1*coding sequence. PCR amplified deletion cassette was gel purified and transformed into *ngg1Δ* strain. Transformants selected for histidine prototrophy were further confirmed by PCR based strategy to ensure correct integration at HAA1 locus, followed by dilution spotting to show increased acetic acid sensitivity as a result of *HAA1* deletion. Expression of Haa1p target genes was checked in *ngg1Δhaa1Δ* and *ngg1Δ* strains without and with acetic acid shock. Upon deletion of *HAA1* in *ngg1Δ* strains expression of *TPO2* and *YGP1* was lost at both conditions (Figure-5.10) indicating that Ngg1p regulates expression of *TPO2* and *YGP1* possibly by modulating the Haa1p activity. It also shows the genetic interaction of Ngg1p with Haa1p and supports the possible interaction shown between these by Krogan et al., 2006. Since constitutive over-expression of *HAA1* or H400 in *ngg1Δ* strain reduces growth rate, we checked the expression of *TPO2* and *YGP1* in these strains. *YGP1* expression is repressed in *ngg1Δ* strain, but upon overexpression of *HAA1* or H400 from TEF promoter in *ngg1Δ* strain, very high expression of *YGP1* is seen in cells with or without acetic acid shock. Expression of *TPO2* slightly increases upon acetic acid shock in *ngg1Δ* strain overexpressing *HAA1* or H400. (Figure-5.11). These observations indicate that constitutive overexpression of *HAA1* results in suppression of effects caused by *NGG1* deletion on Haa1p mediated expression of *YGP1*. This further supports the hypothesis that Ngg1p regulates the activity of Haa1p in acetic acid induced expression of Haa1p target genes. These results supports the hypothesis that Haa1p may have different preferences for target genes in cells with and without acid shock, *TPO2* expression seems to induced mainly upon acetic acid shock whereas *YGP1* seems to be constitutively expressed and Ngg1p may acts as a modulator of Haa1p activity.

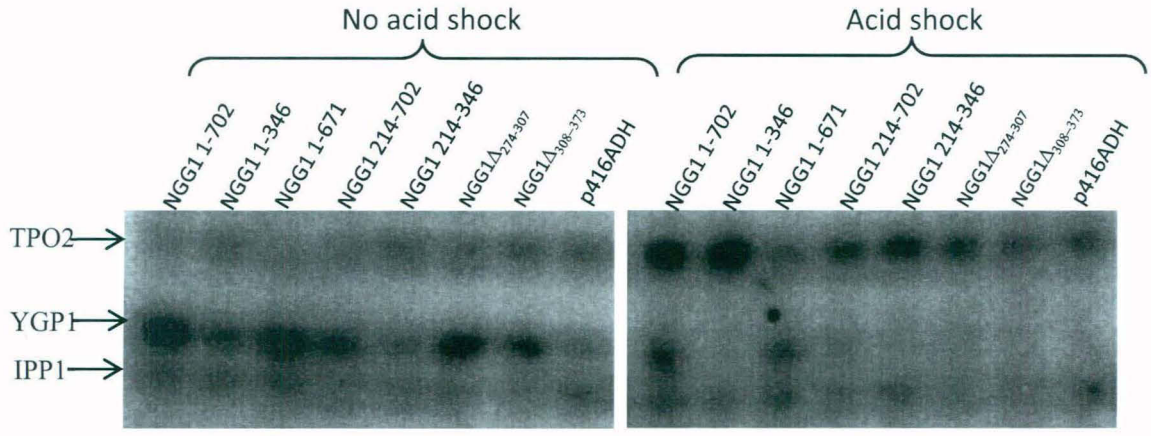


Figure 5.9: Mapping *NGG1* regions modulating differential expression of Haa1p target genes. The *ngg1Δ* strain transformed with *NGG1* deletion clones and vector p416ADH were grown to exponential phase and subjected to acetic acid shock (75 mM acetic acid and pH 3.0). The expression of *YGP1* and *TPO2* in cells with and without acetic acid shock was checked by northern blot as previously described.

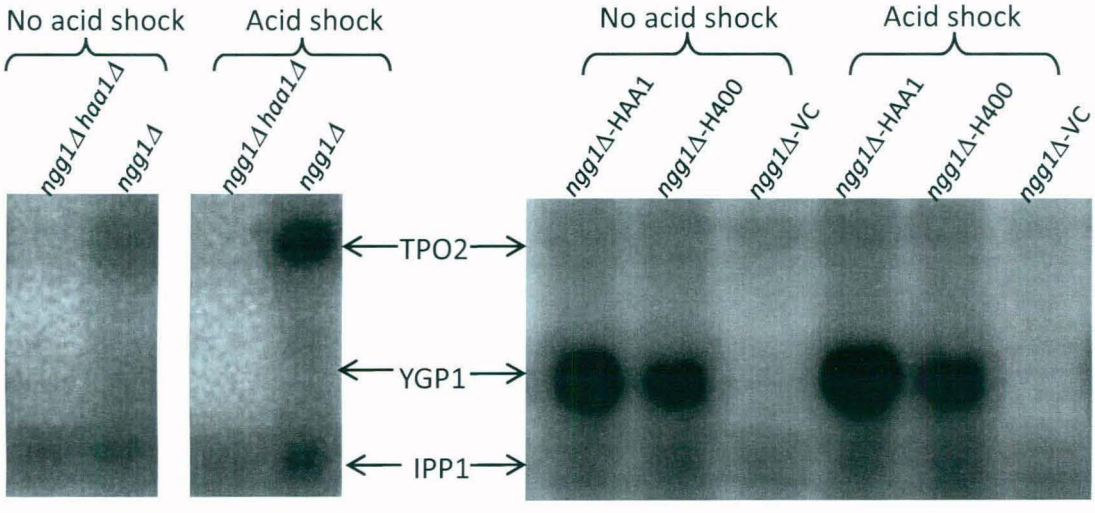


Figure 5.10: Expression of Haa1p target genes in *ngg1Δ* and *ngg1Δhaa1Δ* strains. The expression of *YGP1* and *TPO2* was checked in *ngg1Δ* and *ngg1Δ haa1Δ* strains with and without acetic acid shock.

Figure 5.11: Expression of Haa1p target genes in *ngg1Δ* strain overexpressing *HAA1* or H400. The expression of *YGP1* and *TPO2* was checked by northern blot in *ngg1Δ* strain transformed with p416TEF-*HAA1*, -H400 and vector p416TEF with and without acetic acid shock.

5.7 Effect of *HAA1* and its deletion clones expression in *ngg1Δ* strain:

In previous section, we have shown that deletion of *NGG1* results in differential regulation *TPO2* and *YGP1* upon acetic acid shock; similarly upon expression of Haa1p deletions differential regulation of *TPO2* and *YGP1* was seen. Overexpression of full-length Haa1p or C-terminal deletion clones H400 and H500 can activate expression of *TPO2* as well as *YGP1*, whereas N-terminal deletion clones N50 and N100 are able to activate *YGP1* but not *TPO2* (Section 4.5). Thus, any possible correlation between role of Ngg1p and Haa1p deletions in regulating expression of Haa1p target genes was examined.

Overexpression of *HAA1* or H400 under constitutive TEF promoter in *ngg1Δ* strain suppressed the effect of *NGG1* deletion on differential expression of *TPO2* and *YGP1*. Thus, it was decided to express Haa1p and Haa1p deletions under *HAA1* promoter. Since upstream regulatory regions of *HAA1* are not mapped, we cloned the entire 1640 bp region upstream of *HAA1* (*YPR008w*) up to *REC8* (*YPR007c*) coding region as *HAA1* promoter in p416TEF, replacing TEF promoter. The new vector with the *HAA1* promoter is designated as p416HAA1 (S1A1) and all Haa1p deletions were then cloned under *HAA1* promoter in this vector. These constructs were transformed in *haa1Δ* and *ngg1Δhaa1Δ* stains and acetic acid tolerance was checked by dilutions spotting. The relative pattern of acetic acid tolerance provided by Haa1p deletions in *haa1Δ* and *ngg1Δhaa1Δ* strains were found to be comparable. C-terminal deletions were providing better acetic acid resistance than full length *HAA1* whereas expression of N-terminal deletions contributed to only partial complementation of *HAA1* deletion. Interestingly, expression of Haa1p deletions in *ngg1Δhaa1Δ* provides better acetic acid tolerance compared to their expression in h*aa1Δ* strain. In *ngg1Δhaa1Δ* strain expressing full-length *HAA1* or its C-terminal deletions growth can be seen up to 60 mM acetic acid containing plates whereas in *haa1Δ* strain relative growth can be seen only up to 50 mM acetic acid containing plates (Figure-5.12). Thus possibly Ngg1p functions as repressor of Haa1p activity and absence of Ngg1p results in better Haa1p mediated adaptation to acetic acid.

Expression of Haa1p target genes were also checked in *haa1Δ* and *ngg1Δhaa1Δ* strains expressing *HAA1* deletions under *HAA1* promoter in exponentially growing cells and after acetic acid shock. In *haa1Δ* strain, expression of full-length *HAA1* gene and C-terminal deletions H400 and H500 facilitates high level expression of *YGP1* in with or without acetic

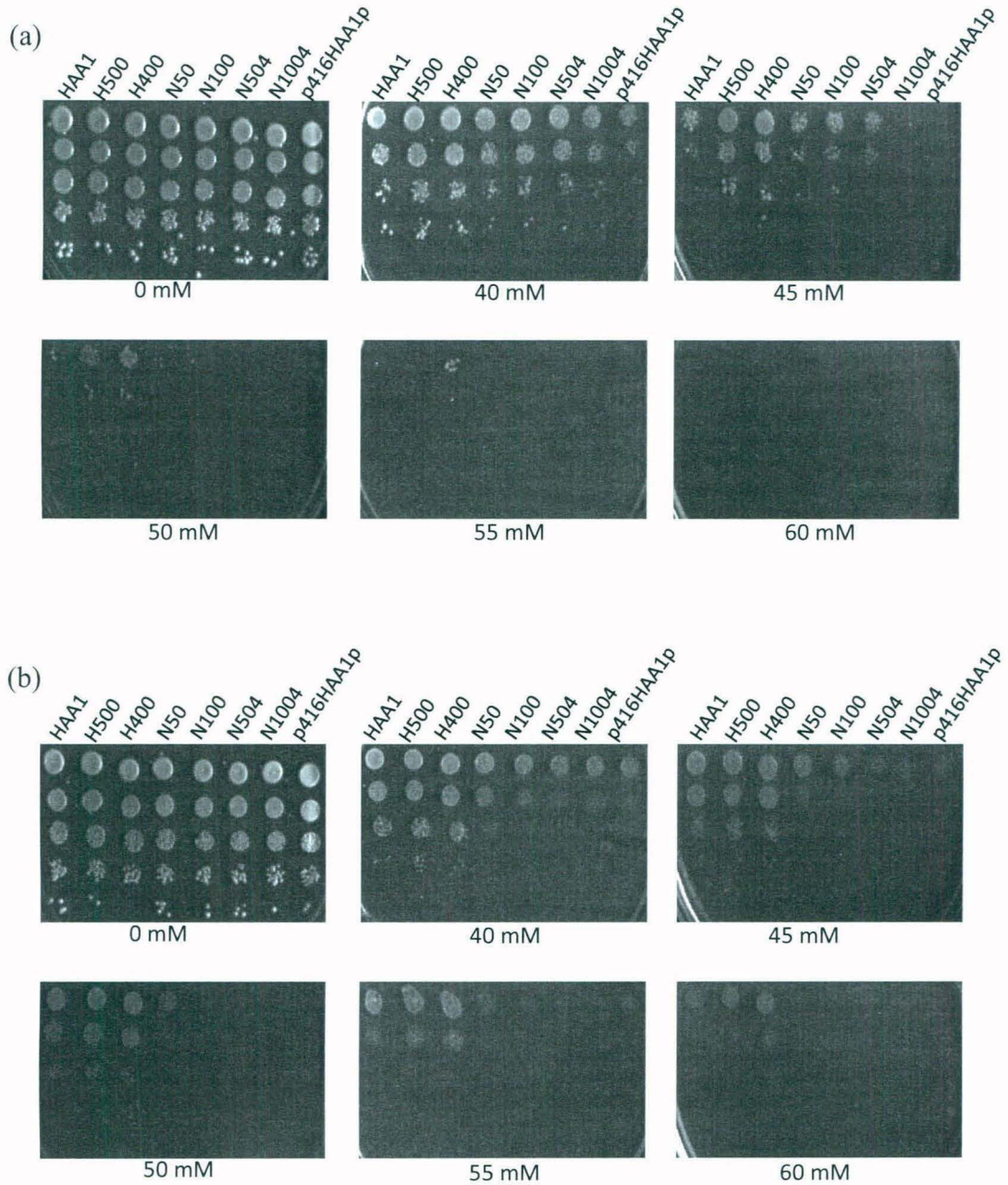


Figure 5.12: Acetic acid tolerance of *haa1Δ* and *ngg1Δ haa1Δ* strains expressing *HAA1* deletions. (a) *haa1Δ* and (b) *ngg1Δ haa1Δ* strains expressing *HAA1* deletions under *HAA1* promoter were grown to exponential phase and acetic acid tolerance was checked by dilution spotting on YPD plates containing indicated conc. of acetic acid at pH 3.0.

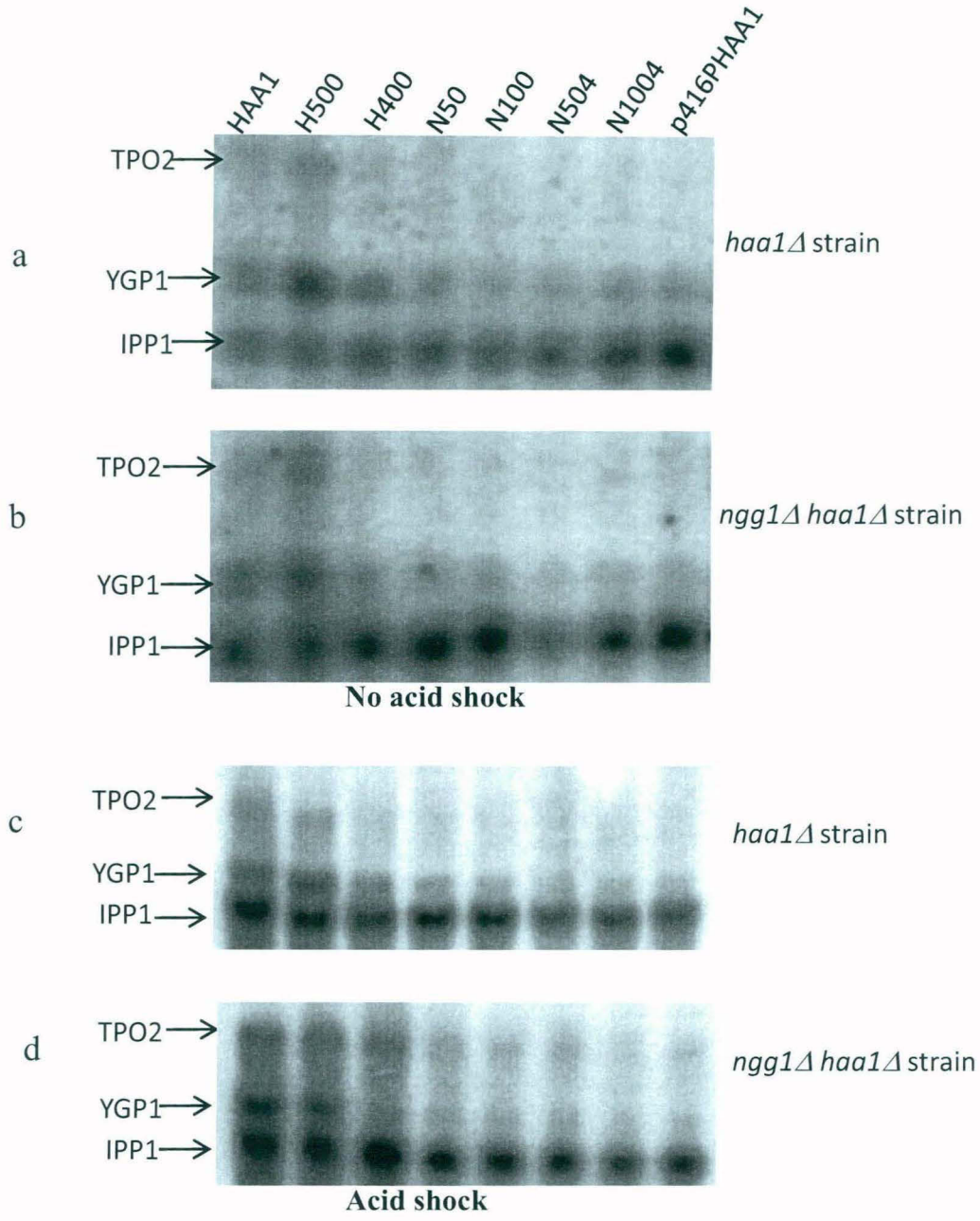


Figure 5.13: Regulation of Haa1p target genes by *HAA1* deletions in *haa1Δ* and *ngg1Δ haa1Δ* strains. The *haa1Δ* (a & c) and *ngg1Δ haa1Δ* strains (b & d) expressing *HAA1* deletions under *HAA1* promoter were grown to exponential phase and subjected to acetic acid shock (75 mM acetic acid and pH 3.0). The expression of *YGP1* and *TPO2* in acetic acid treated (a & b)and untreated cells (c & d) was checked by northern blot. The names of *HAA1* deletions are marked above the lanes and p416HAA1 is vector control.

acid shocked cells. The expression of *TPO2* was seen only upon acetic acid shock under full-length Haa1p or H500 deletion. The N-terminal deletions N50 and N100 can induce expression *YGP1* but *TPO2* expression is not seen in any condition tested. In *ngg1Δhaa1Δ* strain expressing of full-length Haa1p or C-terminal deletions, *TPO2* expression is induced upon acetic acid shock and *YGP1* expression is either decreased or remains comparable to without shock conditions (Figure-5.13). These results support the hypothesis that Ngg1p functions as a repressor of Haa1p activity and divert Haa1p activity towards generalized function, *YGP1* expression. Whereas, upon acetic acid shock Haa1p function more specifically to activate expression of genes required for acetic acid adaptation in yeast, such as *TPO2*.

5.8 Pho85p and Pho80p regulate Haa1p activity upstream of Ngg1p:

Our studies have identified that Msn5p and Ngg1p regulate the Haa1p activity by affecting its nuclear localization and transcriptional response upon acetic acid shock, respectively (Section 5.4 and 5.5). However, the mechanism of activation of Haa1p activity upon acetic acid shock is still unknown. Therefore to identify regulatory proteins involved in activation of Haa1p, we checked the expression of Haa1p target genes *YGP1* and *TPO2* in strains deleted in genes for possible Haa1p regulatory proteins, namely *nto1Δ*, *gln3Δ*, *yak1Δ*, *sap30Δ*, *pho85Δ*, *pho80Δ* and *pcl10Δ* strains. Interactions of Nto1p, Gln3p and Yak1p with Haa1p are described in Biogrid Dataset and their functions have been described in section 5.2. Haa1p regulates the expression of *SAP30*, which encodes a subunit of histone deacetylase complex and presumably modulates yeast transcriptional responses to acetic acid (Mira et al., 2010a). In a genome level study to identify the interactions of protein kinases and phosphatases with their regulatory subunits and substrates, Haa1p was shown to interact with Pho80p and Pcl10p cyclins (Breitkreutz et al., 2010). Both these in turn interact with cyclin dependent kinase Pho85p, to phosphorylate the target proteins and such phosphorylation is shown to regulate cellular responses to nutrient levels (Huang et al., 2007). Thus, possible role of *sap30Δ*, Pho85p, Pho80p and Pcl10p in regulation of Haa1p activity was checked.

Expression of *TPO2* and *YGP1* was checked in these strains with or without acetic acid shock and compared with wildtype strain BY4741. Upon acetic acid shock transcripts of *YGP1* and *TPO2* were detected in wildtype stain as well as in *nto1Δ*, *gln3Δ*, *yak1Δ*, *sap30Δ* and *pcl10Δ* strains. However, transcript level of both *YGP1* and *TPO2* was reduced in *yak1Δ* and *sap30Δ* strains, consistent with their proposed role in Haa1p regulation (Malcher et al., 2011; Mira et

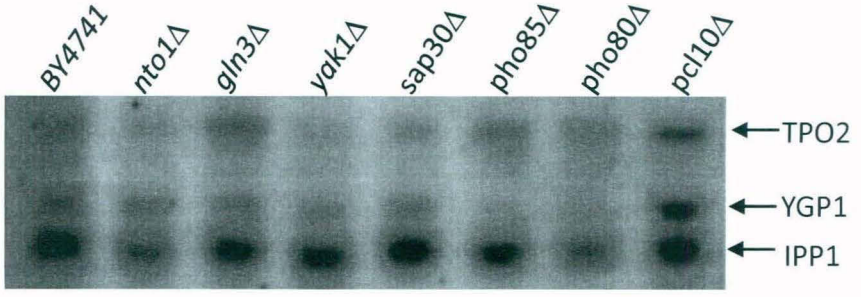


Figure 5.14: Expression of Haa1p target genes in deletion strains of genes encoding proteins possibly regulating Haa1p activity. The deletion strains were grown to exponential phase and subjected to acetic acid shock (75 mM acetic acid at pH 3.0) for 4 hrs. The expression of *YGP1* and *TPO2* was checked by northern blot. Transcript level of *IPP1* was used as internal loading control.

al., 2010a). Interestingly, in *pho85*Δ and *pho80*Δ strains *YGP1* transcript was not detected, whereas *TPO2* transcript was detected as in wildtype strain (Figure-14). This is similar to the results obtained with *ngg1*Δ strain (Section 5.5 and Figure-5.6). Thus it is very much possible that Pho80p and Pho85p function upstream of Ngg1p in regulating Haa1p upon acetic acid shock. Since Pho80p and Pho85p mediated phosphorylation is shown to regulate transcriptional activity in yeast, similar role in Haa1p regulation can be proposed.

5.9 *HAA1* promoter mapping:

As mentioned in section 5.7, regulatory regions present upstream of *HAA1* gene are not yet mapped. Between *HAA1* (*YPR008w*) coding region and upstream *REC8* (*YPR007c*) coding region lies 1640 bp non coding region, with small sequences coding for tRNA-gly and Ty1-LTR. Initially this entire region was considered as regulatory region required for *HAA1* expression and thus functioning as *HAA1* promoter. In our screening of *FY3* strain transformed with overexpression library for acetic acid resistance, *HAA1* clones with different lengths of upstream regions were identified. Among these clones, 3606-3 was especially interesting as it starts just downstream of Ty1-LTR sequence and thus lacks the *HAA1* upstream region beyond tRNA-gly coding sequence including *REC8* coding sequence. On the other hand, clone 3606-1 begins from *REC8* coding sequence thus has all possible regulatory regions present between *HAA1* and *REC8* coding sequences. The *haa1Δ* strain transformed with clone 3606-3 showed very high level of *HAA1* expression and two transcripts of nearly similar size were observed. On the other hand, *HAA1* expression from clone 3606-1 showed single transcript and expression was much less compared to 3606-3. Thus it appeared that the presence of Ty element or upstream region beyond Ty element may play important role in the regulation of *HAA1* expression.

To test the above, different lengths of *HAA1* upstream regions between the *HAA1* and *REC8* coding sequence were cloned in p416TEF vector replacing TEF1 promoter; these plasmids were named as p416S1A1, p416S2A1, p416S3A1, and p416-Ty. *HAA1* promoter sequence begins from upstream of *HAA1* coding sequence and ends just before *REC8* coding sequence in p416S1A1, uptoTy1-LTR sequence in p416S2A1, ends before Ty1-LTR sequence in p416S3A1, and simply lacks Ty element but has all upstream sequences between *HAA1* and *REC8* in p416-Ty (Figure-5.15). *HAA1* coding region was cloned downstream of different *HAA1* promoters, transformed into *haa1Δ* strain and acetic acid tolerance was checked. *HAA1* expressed from all *HAA1* promoters was able to complement loss of *HAA1* function in terms

of acetic acid tolerance (Figure-5.16). Expression of *HAA1* under different *HAA1* promoters was checked by northern blot analysis with *IPP1* expression as loading control. *HAA1* expression from original library clones 3606-1 and 3606-3 were used as control for expression under full regulatory region between *HAA1* and *REC8* coding region (3606-1) or one lacking Ty1-LTR and upstream region (3606-3). Deletion of Ty1-LTR from *HAA1* promoter region in p416S3A1 and p416-Ty does not affect the expression of *HAA1* gene, although control strain 3606-3 lacking Ty1-LTR and upstream region beyond it was still showing very high expression of *HAA1* (Figure-5.17). Thus, we could not identify the reason for the aberrantly high *HAA1* expression from clone 3606-3 and possible role of Ty elements in this could not be established. Nevertheless, it can be concluded that *HAA1* upstream region from start codon to until Ty1-LTR can function as *HAA1* promoter.

5.10 Discussion:

Our earlier studies indicated that function of Haa1p must be highly regulated, since constitutive expression of full-length *HAA1* under GPD promoter is toxic to yeast cells. Response of yeast cells to weak acids has huge metabolic cost, as it activates plasma membrane ATPases to pump out proton in ATP dependent manner to avoid acidification of cytosol, as well as transporters for effluxing weak acid anions and other cellular metabolites (Mira et al., 2010c; Piper et al., 2001). Possibly constitutive overexpression of *HAA1* leads to activation of these processes, resulting in wastage of metabolic energy and growth defect. Thus it is likely that Haa1p function is negatively regulated, and it is activated only under acetic acid stress.

Haa1p is predicted to interact with many proteins involved in regulation of transcription, chromatin remodeling, nuclear translocation and signaling. These interactions may play crucial role in regulating the activity of Haa1p in acetic acid stress. Expression of full-length *HAA1* under TEF promoter in deletion strains of Haa1p interacting proteins (*ngg1Δ, ste20Δ, fus1Δ,* and *msn5Δ)* and *spi1Δ* result into growth defect. Possibly it results from toxicity of *HAA1* overexpression in yeast, although *HAA1* expression from TEF promoter in centromeric vector in wildtype strain does not cause such effect. Perhaps these interacting proteins negatively regulate Haa1p function and their deletion results in growth defect associated with activation of Haa1p function. Expression of H400 deletion in these strains does not cause growth defect, possibly because H400 lacks regulation imparted by these interacting proteins

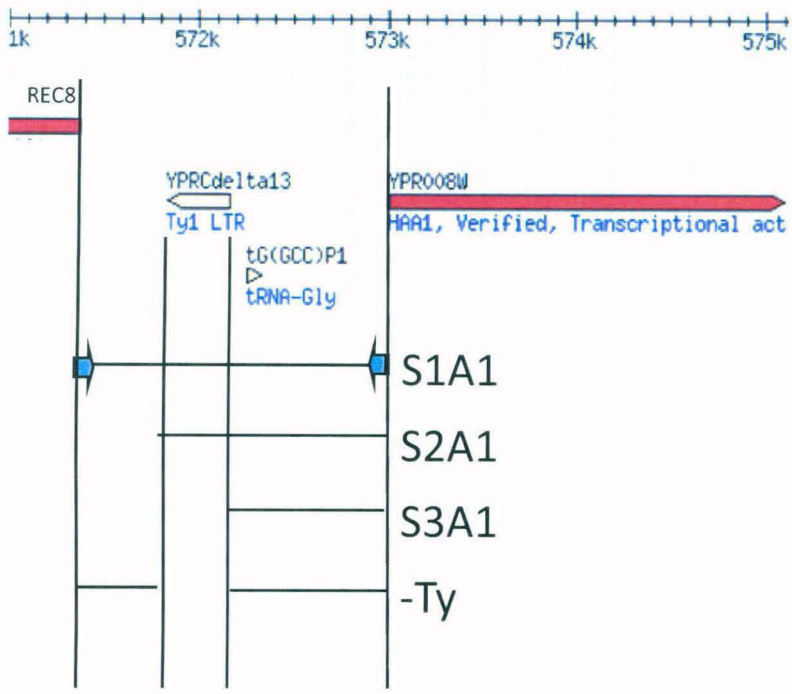


Figure 5.15: Promoter mapping of *HAA1*. Indicated genomic regions upstream of *HAA1* coding sequence were cloned in p416TEF vector replacing TEF promoter. Figure adopted and modified from SGD.

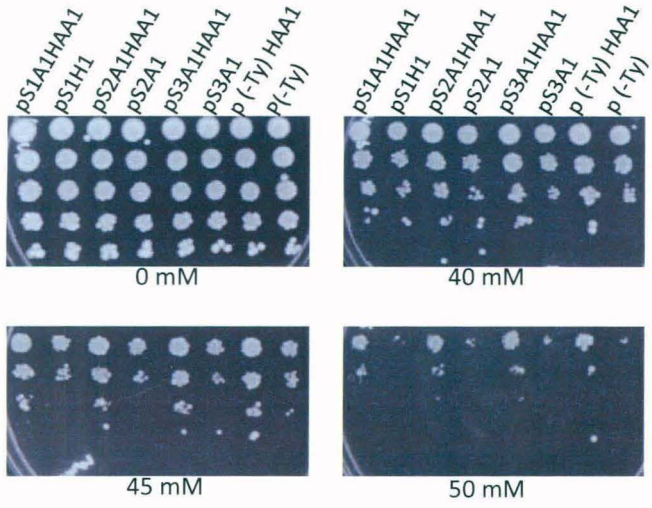


Figure 5.16: Complementation of acetic acid sensitivity of *haa1Δ* strain upon expression of *HAA1* from varying regions of *HAA1* promoter. The *haa1Δ* strains expressing *HAA1* gene under varying length of *HAA1* promoter were checked for acetic acid tolerance by dilution spotting on YPD plates containing indicated conc. of acetic acid at pH 3.0.

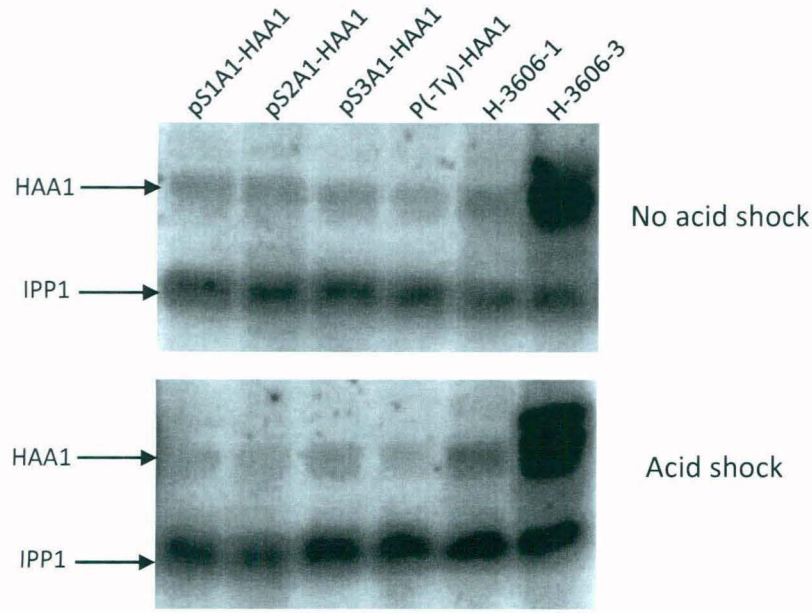


Figure 5.17: Expression of *HAA1* under varying *HAA1* promoter length . The *haa1Δ* strain transformed *HAA1* gene under varying length of *HAA1* promoter were checked for *HAA1* expression in growing cells and upon acetic acid shock. The expression of *HAA1* clones 3606-1 and 3606-3 having different length of *HAA1* upstream region and obtained in initial acetic acid screening (Section 3.4 and 4.5) were used as controls. Expression of *IPP1* were used as internal loading control.

or it lacks the ability to activate expression of some Haa1p target genes which upon activation results in suppression of growth.

Msn5p was earlier shown to be involved in import and export of proteins from nucleus, such as Pho4p, Swi6p and Far1p (Queralt and Igual, 2003). Constitutive expression of full-length *HAA1* in *msn5Δ* strain results in growth defect and increased acetic acid sensitivity, but expression of H400 did not result in any growth defect, moreover tolerance to acetic acid increases. Thus Msn5p must be playing important role in regulation of Haa1p activity and may regulate activity of full-length Haa1p and H400p differently. Haa1p and H400p completely localize to nucleus in *msn5Δ* strain, but Haa1p and H400p partially localizes to nucleus and rest lies in cytosol in the strain with *MSN5*. Thus as earlier proposed for other transcription factors, Msn5p may be involved in exporting Haa1p protein into cytosol from nucleus and loss of Msn5p results in accumulation of Haa1p and its deletion H400p within nucleus. These results suggest that when yeast cells are not stressed with acetic acid Msn5p exports Haa1p from the nucleus, thereby minimizing its activity in the nucleus. Upon acetic acid shock Haa1p somehow avoids Msn5p dependent export from the nucleus and remains within nucleus. Haa1p also possibly interacts with Pho80p and Pcl10p cyclins (Breitkreutz et al., 2010), both of which interact with cyclin dependent kinase Pho85p. Pho80p-Pho85p complex is known to phosphorylate Pho4p transcription factor in high phosphorus growth conditions, thus promoting its Msn5p dependent export from nucleus (Kaffman et al., 1998). Thus, there is a possibility that like in Pho4p, localization of Haa1p is also regulated by Pho80p or Pcl10p dependent phosphorylation by Pho85p. Since full-length Haa1p and H400p localize in the nucleus, possible nuclear localization signal must be present within first 400 amino acids of Haa1p. Expression of *TPO2* and *YGP1* is higher in *msn5Δ* strain expressing H400 compared to full-length *HAA1,* although no drastic changes were observed. Therefore acetic acid sensitivity and growth defect caused by constitutive expression of *HAA1* in *msn5Δ* strain is not caused by its ability to activate target genes tested or any alteration in the localization of protein.

When expression of Haa1p regulated genes was checked in strains deleted for Haa1p interacting proteins, we found that in *ngg1Δ* strain, expression of *YGP1* decreases in growing cells and upon acetic acid shock *TPO2* is highly induced and *YGP1* is completely repressed. Moreover reintroduction of functional *NGG1* in *ngg1Δ* strain can restore the wildtype like expression of *TPO2* and *YGP1*, whereas deletion of *HAA1* in *ngg1Δ* strain results in loss of

expression of *YGP1* and *TPO2*. Therefore Ngg1p must be functioning in regulation of Haa1p activity in adaptation of yeast cells to acetic acid stress. We have shown that Haa1p mediated expression of *YGP1* is generalized response as it is equally expressed in unshocked and acetic acid shocked cells whereas *TPO2* expression is more specifically induced upon acetic acid stress. Ngg1p may function as repressor of acetic acid induced response of Haa1p (*TPO2* expression), but on the other hand activates the generalized response of Haa1p (*YGP1* expression). Thus in normal growth conditions when yeast cells do not face acetic acid stress Ngg1p represses *TPO2* expression, upon acetic acid stress Haa1p is released from Ngg1p repression and it preferably induces the expression of *TPO2*. Domains of Ngg1p important for glucose repression and transcriptional control are also found essential for regulation of Haa1p activity in our study. Thus possible mechanism of modulation of Haa1p activity by Ngg1p should be similar to its previously described function. Ngg1p in a trimeric HAT complex with Ada2p and Gcn5p is shown interact with transcription factors Pdr1p and Gal4p to inhibit their activity. Similarly Ngg1p can interact with Haa1p to regulate its activity, but how *YGP1* and *TPO2* are regulated in not yet clear.

Like in *ngg1Δ* strain *YGP1* transcript is not seen in *pho85Δ* and *pho80Δ* strains, whereas *TPO2* expression is seen at wild type level. Thus role of Pho80p-Pho85p cyclin-CDK complex in regulating the Haa1p activity is proposed. Pho80p-Pho85p mediated phosphorylation of Pho4p transcription factor in phosphate rich medium results in its nuclear export and inhibition of its transcriptional response (Kaffman et al., 1998). Similarly Pho80p-Pho85p mediated phophorylation or any other possible modification of Haa1p may regulate its activity upon acetic acid stress. Moreover, upon acetic acid shock increased ATP consumption to pump out protons and acetate ions may lead to a condition similar to growth in low phosphate media; Pho85p may not phosphorylate Haa1p under these conditions, resulting in nuclear accumulation of Haa1p and its transcriptional response. Haa1p is likely to recognize different upstream elements of *YGP1* depending on presence or absence of acetic acid, in *TPO2* has single upstream element that is mainly acetic acid responsive. Thus it can be further proposed that Pho80p-Pho85p mediated modification of Haa1p, target it to Ngg1p mediated repression resulting in suppression of target gene expression from acetic acid responsive elements, whereas expression of genes with non acetic acid response elements is not affected. On the other hand, upon acetic acid shock, Haa1p is released from Pho80p-Pho85p-Ngg1p mediated repression, allowing it to activate transcription of genes required under acetic acid stress (Figure-5.18).

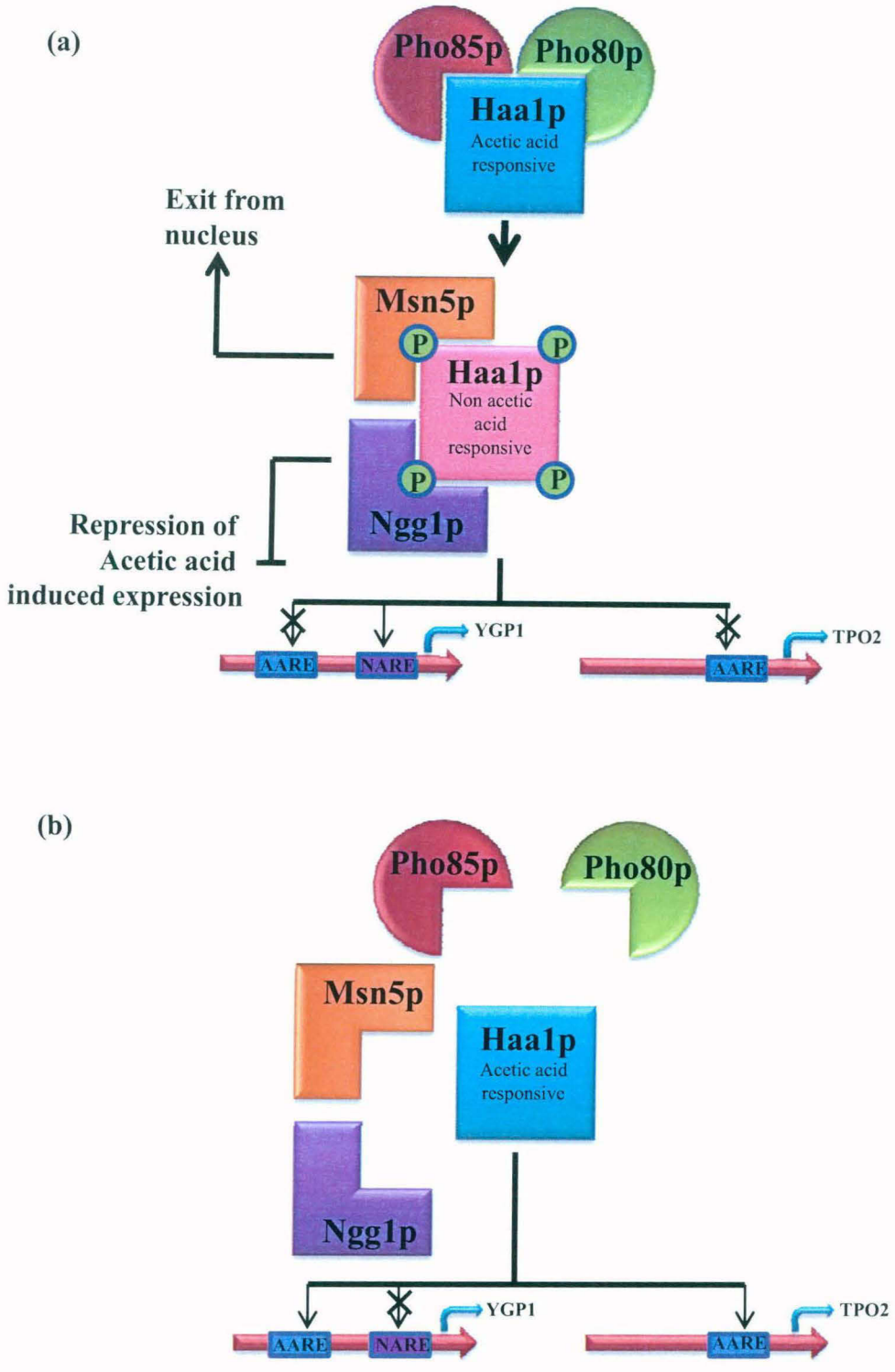


Figure 5.18: Model for regulation of Haa1p activity by Pho85p-Pho80p Cdk-cyclin complex, and Msn5p nucleopherin and Ngg1p transcriptional regulator in (a) untreated and (b) acetic acid treated cells.

Summary

Acetic acid is generated as a byproduct of ethanolic fermentation by yeast. Acid hydrolysis of lignocellulosic substrates to be used for ethanolic fermentation also generates acetic acid. Acetic acid inhibits growth and fermentating efficiency of yeast strains and this inhibition is synergistic with ethanol. Acetic acid is known to effect yeast viability through apoptosis and chronological aging of yeast population. Thus, identifying and characterizing genes involved in acetic acid tolerance is essential for a better understanding of the mechanisms of adaptation of yeast to acetic acid stress. The genes identified might be useful for improving yeast strains for higher acetic acid tolerance and ethanol production, particularly from biomass hydrolyasates.

Screening for genes conferring resistance to acetic acid in yeast *S. cerevisiae*

We screened yeast transformants of multicopy yeast genomic library to identify genes that upon overexpression increase the tolerance to acetic acid at pH 3.0 or 4.5. Plasmids from resistant yeast clones were isolated and retransformed in yeast to confirm the acetic acid tolerance, and further sequenced to identify the effector genes. Our screening yielded *HAA1* gene in multiple acetic acid resistant clones, which is already known to be involved in acetic acid adaptation in yeast, thereby validating our approach. The other genes identified provided only marginal improvement in acetic acid tolerance of yeast.

From one such clone we identified *YLR297w*, an uncharacterized gene in yeast, which upon overexpression provided acetic acid tolerance only in late growth phase (24 hours growth in synthetic dextrose media). Moreover, even constitutive overexpression of *YLR297w* under strong GPD promoter did not increase the acetic acid resistance of exponentially growing yeast cells, but only that of cells in late growth phase. *YLR297w* overexpression (unlike *HAA1*) also resulted in resistance to lipophilic acids like octanoic acid and benzoic acid, but only in late growth phase. It is likely that the growth phase specific effect of *YLR297w* depends on the expression of some other protein(s) in late growth phase or stationary phase. Deletion of *YLR297w* rendered the yeast cells only marginally sensitive to acetic acid and other weak acids tested. Thus, *YLR297w* does not appear to be critical for adaptation of yeast cells to weak acids and its function is either redundant or can be bypassed.

We found that *YLR297w* mediated acetic acid tolerance in late growth phase requires acidification of pregrowth media as in SD medium. Pregrowth in YPD medium, media with reduced glucose or buffered to near neutral pH, acidification is not observed, and under these

conditions *YLR297w* did not increase acetic acid tolerance. Burtner et al., 2009, have shown that acidification of pregrowth media in stationary phase is associated with release of organic acids in growth media, and release of acetic acid among them causes chronological aging. Thus we checked the role of acetic acid in pregrowth media in activation of *YLR297w* function. When exponentially grown yeast strains overexpressing *YLR297w* were subjected to acetic acid containing low pH media resembling spent media in late growth phase, *YLR297w* mediated acetic acid resistance was increased. Thus Ylr297wp may increase survival of yeast cells by modulating acetic acid induced apoptosis and chronological aging.

Insights into molecular mechanism of Haa1p mediated acetic acid resistance

Among the multiple clones with *HAA1* gene conferring acetic acid resistance upon overexpression, few had *HAA1* truncated at 3' termini, thus encoding Haa1p as short as 450 aa out of 695 aa sequence of Haa1p These truncated clones were complementing *HAA1* deletion for acetic acid sensitivity and producing shorter transcripts thus further confirming their size. Based on this observation we wished to identify the functional domains of Haa1p important in regulation of acetic acid tolerance. Various deletions of Haa1p missing either C-terminal or N-terminal region were made and expressed under TEF promoter in *haa1*Δ strain. The C-terminal deletions encoding initial 400 or 500 amino acid residues (H400 and H500 respectively) were found to fully complement the *HAA1* deletion; moreover these provided better acetic acid resistance than full-length protein. Like full-length Haa1p, C-terminal deletions can also activate expression of tested Haa1p target genes, namely *TPO2, TPO3* and *YGP1*. N-terminal deletions lacking initial 50 or 100 amino acids (N50 and N100 respectively) which constitute the DNA binding region were partially complementing the *HAA1* deletion and could support expression of *TPO3* and *YGP1* but not *TPO2*. Thus, presence of N-terminal DBD is essential for *TPO2* expression, whereas possibly it is not essential for *YGP1* and *TPO3* expression. Only simultaneous deletion at N-terminus (first 50 or 100 aa) and C-terminus beyond 400 amino acid rendered the protein non functional in complementing acetic acid sensitivity of *haa1Δ* strain, and also in induction of Haa1p target genes. Here it was also seen that expression of *TPO2* is induced upon acetic acid shock, whereas *YGP1* and *TPO3* expression was comparable in with or without acetic acid shocked cells.

We also tried to purify the Haa1p and its deletions from yeast as well as *E. coli* hosts. Protein purification from yeast was not fruitful whereas protein purified from *E coli* host was

consistently degraded. Such partially purified Haa1p and H400 deletion proteins were used to study their DNA binding ability with *TPO2* and *YGP1* upstream regions. Haa1p showed metal dependent and specific DNA binding ability with *TPO2* and *YGP1* upstream regions, since EDTA inactivated protein was unable to show any DNA binding. Moreover, DNA binding of Haa1p was seen with multiple and overlapping probes from *YGP1* and *TPO2* upstream regions. Thus possibility of existence of multiple Haa1p binding sites in upstream region of target genes is likely. Promoter regions of *YGP1* and *TPO2* were mapped by progressive deletions in 1000 bp upstream region of respective genes to identify essential regulatory elements. We identified that *YGP1* has two upstream regulatory elements, one responding to acetic acid shock and another in untreated cells only, and these were named as Acetic Acid Response Element (AARE) and Non Acetic acid Response Element (NARE), respectively. In *TPO2,* single regulatory element mainly responding to acetic acid shock was identified, thus named as AARE. Thus differential regulation of *YGP1* and *TPO2* by Haa1p can be explained by regulatory elements present upstream of these genes. Moreover, presence of dual DNA binding domain in Haa1p cannot be ruled out as N50 and N100 deletions of Haa1p lacking N-terminal DBD is functional in activating *YGP1* and furthermore two different DBD recognizing different *YGP1* promoter elements cannot be ruled out.

Regulation of Haa1 mediated acetic acid tolerance

We have also studied the regulation of Haa1p activity in adaptation to acetic acid stress. Possible Haa1p interacting proteins were shortlisted from interaction datasets, and acetic acid sensitivity of strains deleted for genes encoding these proteins, were checked. Expression of full-length *HAA1* under constitutive TEF promoter in *ngg1Δ, msn5Δ, fus1Δ* and *ste20Δ* strains resulted in very long lag phase of growth in minimal media, whereas expression of H400 deletion did not cause any growth defect. Growth defect was also seen when full-length *HAA1* was expressed under strong and constitutive GPD promoter, perhaps resulting from excessive Haa1p activity in the absence of regulatory proteins. Based on the preliminary results and known functions of individual Haa1p interacting proteins, we tested their role in regulation of Haa1p function. Since Msn5p is known to function as nucleopherin in yeast, we checked the localization of Haa1p and H400 deletion protein in *msn5Δ.* strain. HA tagged Haa1p and H400 expressed under TEF promoter localize to nucleus only in *msn5Δ* whereas in *haa1Δ* it partially localize to nucleus as significant cytosolic signal is seen. Thus role of Msn5p can be implicated in restricting Haa1p out of nucleus when Haa1p

function is not required. Haa1p is also shown to interact with Pho80p cyclin, which is known to be involved in phosphorylation of Pho4p transcription factor in association with Pho85p CDK. Phosphorylation of Pho4p leads to its Msn5p dependent exit from nucleus. Similarly, Pho85p-Pho80p complex might be involved in Msn5p mediated export of Haa1p from nucleus.

Expression of *HAA1* and its target genes *TPO2* and *YGP1* was checked in strains deleted in genes for Haa1p interacting proteins. In *ngg1Δ* strain expression of *YGP1* is reduced; upon acetic acid shock expression of *TPO2* is substantially increased, but no expression of *YGP1* is seen. Ngg1p is a component of HAT complexes which is known to regulate the activity of many transcription factors in yeast. When *NGG1* or its functional deletions which complement the slow growth defect of *ngg1Δ* strain are expressed in *ngg1Δ* strain, the wildtype like expression of *YGP1* and *TPO2* is seen. Deletion of *HAA1* in *ngg1Δ* strain results in loss of expression of *TPO2* and *YGP1*, indicating that the Ngg1p regulation of *TPO2* and *YGP1* expression is by modulating the activity of Haa1p. Haa1p and its deletions expressed under its own promoter in *ngg1Δhaa1Δ* strain provide better acetic acid tolerance than its expression in *haa1Δ* strain, indicating that Ngg1p may function as repressor of Haa1p activity in yeast growing under normal conditions, but upon acetic acid shock Ngg1p repression is released and Haa1p functions to regulate the adaptation of yeast to acetic acid stress. Like in *ngg1Δ* strain *YGP1* expression is not seen upon acetic acid shock in *pho85Δ* and *pho80Δ* strains, whereas *TPO2* expression is seen. Thus, it is likely that Pho85p-Pho80p complex function upstream of Ngg1p in regulating activity of Haa1p.

Thus, a working hypothesis for Haa1p regulation, which needs to be experimentally proved, is as follows. Pho85p-Pho80p complex may phosphorylate Haa1p in untreated cells leading to its export from nucleus in Msn5p dependent manner, and repression of acetic acid induced transcriptional response Haa1p via Ngg1p. Upon acetic acid shock, increased metabolic cost and ATP consumption may lead to cellular conditions similar to phosphate starvation. Thus, Pho85p-Pho80p complex cannot phosphorylate Haa1p, and unphosphorylated Haa1p is released from Msn5p and Ngg1p mediated repression, leading to acetic acid induced transcriptional response by Haa1p.

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