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MINI-REVIEW

Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*

Menggen Ma · Z. Lewis Liu

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Abstract *Saccharomyces cerevisiae* is a superb ethanol producer, yet is also sensitive to higher ethanol concentrations especially under high gravity or very high gravity fermentation conditions. Ethanol tolerance is associated with interplay of complex networks at the genome level. Although significant efforts have been made to study ethanol stress response in past decades, mechanisms of ethanol tolerance are not well known. With developments of genome sequencing and genomic technologies, our understanding of yeast biology has been revolutionarily advanced. More evidence of mechanisms of ethanol tolerance have been discovered involving multiple loci, multi-stress, and complex interactions as well as signal transduction pathways and regulatory networks. Transcription dynamics and profiling studies of key gene sets including heat shock proteins provided insight into tolerance mechanisms. A transient gene expression response or a stress response to ethanol does not necessarily lead to ethanol tolerance in yeast. Reprogrammed pathways and interactions of cofactor regeneration and redox balance observed from studies of tolerant yeast demonstrated the significant importance of a time-course study for ethanol tolerance. In this review, we focus on current advances of our understanding for ethanol-tolerance mechanisms of *S. cerevisiae* including gene expression responses, pathway-based analysis, signal transduction and regulatory networks. A prototype of global system model for mechanisms of ethanol tolerance is presented.

M. Ma · Z. L. Liu (✉)
U.S. Department of Agriculture, ARS, Bioenergy Research,
National Center for Agricultural Utilization Research,
1815 N University St.,
Peoria, IL 61604, USA
e-mail: ZLewis.Liu@ars.usda.gov

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Introduction

Yeasts have been widely used for alcohol related brewing and fermentation in all of human civilization history. With renewed interest in ethanol as a sustainable and clean transportation fuel, economic cellulosic ethanol production has recently become a focal point of considerable research and development effort worldwide (Outlaw et al. 2005; Sanchez and Cardona 2008; Wall et al. 2008; Vertes et al. 2010). Low-cost and high-titer of ethanol production are significant challenges in developing a bio-based economy. *Saccharomyces cerevisiae* is a superb ethanol producer among numerous fermentative microorganisms (Lin and Tanaka 2006; Liu et al. 2008). Although it is a traditional ethanol-producing microbe, yeast is also sensitive to higher concentrations of ethanol, especially for high gravity or very high gravity fermentation conditions. Accumulation of ethanol in a medium inhibits cell growth and viability, affects different transport systems, and reduces ethanol titer in the culture (Casey and Ingledew 1986; D'Amore and Stewart 1987; D'Amore et al. 1990; Bai et al. 2004; Pina et al. 2004). High levels of ethanol affect the integrity of cell membrane, damage permeability to numerous ionic species, decrease fluidity of plasma membrane leading to dissipation of transmembrane electrochemical potential, and subsequently acidify intracellular and vacuolar conditions (Van Uden 1985; Salgueiro et al. 1988; Rosa and Sá-Correia 1996; Teixeira et al. 2009). At high concentrations, ethanol has also been shown to perturb protein conformation causing protein denaturation and dysfunction, for example, key glycolytic enzymes pyruvate kinase and hexokinase

(Millar et al. 1982; Pascual et al. 1988). High ethanol affects uptake of glucose, maltose, ammonium and amino acid and causes cell leakage of nucleotides, amino acids, and potassium (Piper 1995).

Several hundred genes were identified associated with ethanol tolerance involving a broad range of functional categories including protein biosynthesis, amino acid metabolism, nucleotide metabolism, transport, cell cycle and growth, lipid metabolism, fatty acid and ergosterol metabolism, membrane and cell wall organization, proline biosynthesis, and tryptophan biosynthesis (Gasch et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Kubota et al. 2004; Fujita et al. 2006; van Voorst et al. 2006; Gibson et al. 2007; Auesukaree et al. 2009; Dinh et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009; Ma and Liu (submitted)). As classified by different functional categories, every single gene induced by ethanol has more than one function (Table 1). This greatly complicates delineating interactions of gene relationships. Although extensive efforts have been made, mechanisms of ethanol tolerance are not fully understood. The large number of genes involved, multiple layers of functions and interactions, complexity of yeast network response, and frequent reprogrammed pathways made the task difficult regardless of how the tolerance was controversially defined. Compositions and functions of plasma membrane related to ethanol tolerance in yeast have been recently summarized, as well as stress responses of some genes (Ding et al. 2009). This review focuses on the most up-to-date knowledge in ethanol-tolerance mechanisms based on comprehensive gene expression analysis, pathway-based and integrated resistance, and regulatory networks. In addition, our improved understanding in mechanisms of ethanol tolerance in yeast uncovered new aspects in dynamic studies of the tolerance over time. Snap shots of transient expression responses are not necessarily meaningful and may not lead to a tolerance response in yeast. A prototype of the global system model of mechanisms for ethanol tolerance in yeast is presented.

Membrane and cell wall

Many genes identified in association with ethanol tolerance are involved in membrane composition. For example, *ETR1*, *OAR1*, *SUR4*, *FEN1*, and *HTD2* related to saturated fatty acid metabolism, *COQ1*, *CDS1*, *KCS1*, *ARG82*, *AYR1*, *LCB5*, *LIP5*, *PDX3*, *LDB19*, and *OPI3* for lipid and phospholipid metabolism, and *DEP1*, *FEN2*, *UME6*, *HAC1*, and *PEX15* involved in regulation of lipid, fatty acid and isoprenoid metabolism are commonly observed (Kubota et al. 2004; Fujita et al. 2006; Van Voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009). In addition, *ETR1*, *GPD1*, *DAK1*, *PCT1*, *OPI3*, *MCR1*, *FAA1*, and *GRE2* involved in fatty acid, lipid, and isoprenoid metabolism were also reported to be up-regulated under ethanol stress (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)). Up-regulated genes related to cell wall structure for ethanol tolerance include *TIP1* for mannoprotein metabolism, *SED1* for glycoprotein metabolism, *SPI1* for weak acid resistance, and *HSP150* for cell wall organization (Ogawa et al. 2000; Chandler et al. 2004; Ma and Liu (submitted)). Numerous cell wall genes were reported to be associated with ethanol tolerance by gene deletion mutation studies including *SMI1* in the regulation of cell wall synthesis, *ANP1*, *MNN10*, *MNN11*, and *HOC1* encoding four of five subunits of mannosyltransferase complex, *LDB7* and *VMA9* involved in mannoprotein biosynthesis, *KRE6* encoding β-glucan synthase for β-1, 6-glucan biosynthesis, *WSC3*, *SLG1* and *SLT2* encoding sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway involved in maintenance of cell wall integrity, and *MID2*, *ROM2*, and *SIT4* related to cell wall organization (Kubota et al. 2004; Fujita et al. 2006; Van Voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009).

Although many genes involved in membrane and cell wall composition are important as suggested by deletion mutation assays, most of them are down-regulated under ethanol stress. It is possible that membrane and cell wall structures may undergo significant remodeling processes in response to ethanol stress. Changes of membrane and ergosterol composition in response to ethanol-induced stress have been observed including unsaturated fatty acids (UFAs) index and phospholipids (Alexandre et al. 1994; Chi and Arneborg 1999; You et al. 2003; Aguilera et al. 2006). Remodeling of yeast cell wall during ethanol shock was suggested by 1,3-β-glucanase sensitivity assay (Teixeira et al. 2009). Yeast cells grown on a culture medium in the absence of ethanol were more susceptible to lyticase activity than cells pre-exposed to ethanol. Ethanol-tolerant mutants K11 and SR4-3, tolerant to ~20% ethanol, showed strong resistance to K1 killer toxin and a cell wall lysis enzyme zymolyase (Ogawa et al. 2000).

The mono-unsaturated fatty acids palmitoleic acid and oleic acid are key plasma membrane components in *S. cerevisiae* to compensate deficits caused by ethanol stress through increased fluidity of plasma membrane. The amount of these two UFAs in cellular lipids was higher in ethanol-tolerant strains (Sajbidor et al. 1995; You et al. 2003). Oleic acid is considered as the main determinant of ethanol tolerance in *S. cerevisiae*. However, both palmitoleic acid and oleic acid were formed by the same catabolic membrane desaturase encoded by *OLE1* through oxygen- and NADH-dependant desaturation of palmitic acid and

Table 1 Gene ontology (GO) categories and terms for induced genes by ethanol challenge from *Saccharomyces cerevisiae*

GO ID	Term	Gene[a]
Cellular component		
GO:0005737	Cytoplasm	*SSA1*, *CDC19*, *PRX1*, *SSA3*, *ATG8*, *NTH2*, *ETR1*, *YRO2*, *HSP26*, *TPS1*, *YBR139W*, *ADH5*, *RTC2*, *TOS1*, *SSE2*, *SDS24*, *YBR287W*, *GRX1*, *GLK1*, *YCL042W*, *PDI1*, *CIT2*, *PGK1*, *GPM2*, *GPD1*, *YDL124W*, *STF1*, *SFA1*, *COS7*, *NTH1*, *TPS2*, *SED1*, *HSP42*, *SDH4*, *YDR248C*, *HSP78*, *CCC2*, *HXT7*, *HXT6*, *GRX2*, *EMI2*, *EUG1*, *GLC3*, *UBC8*, *SPF1*, *CYC7*, *YEL047C*, *PRB1*, *YAT2*, *PIC2*, *GIP2*, *HOR2*, *RGI1*, *SER3*, *SSA4*, *COX15*, *HSP12*, *GSY1*, *HXK1*, *PNC1*, *PKP2*, *PYC1*, *STF2*, *CTT1*, *RTS3*, *TDH3*, *PDX1*, *PCT1*, *SOL4*, *ENO1*, *GND2*, *COS6*, *AIM17*, *SOD2*, *ARG4*, *GRE3*, *ENO2*, *CTR2*, *OYE2*, *PFK26*, *YJL016W*, *TDH1*, *MPM1*, *KHA1*, *OPI3*, *UGP1*, *LHS1*, *LAP4*, *MCR1*, *YKL151C*, *SSA2*, *HSP104*, *TPO1*, *UBI4*, *AHP1*, *PUT1*, *CPR6*, *GSY2*, *ATP14*, *DAK1*, *ATP18*, *TSL1*, *ERO1*, *ADH3*, *PGM2*, *ALD2*, *ICY1*, *HOR7*, *ADH2*, *PBI2*, *APJ1*, *YNL134C*, *CIT1*, *DDR2*, *ATP19*, *ADH1*, *MCH4*, *GRE2*, *GCY1*, *SRL1*, *RDL1*, *FAA1*, *ALD4*, *IRC15*, *SSE1*, *HSP82*, *ATH1*, *GPH1*, *GDB1*
GO:0005739	Mitochondrion	*PRX1*, *NTH2*, *ETR1*, *YRO2*, *RTC2*, *CIT2*, *PGK1*, *STF1*, *SFA1*, *COS7*, *TPS2*, *SED1*, *SDH4*, *HSP78*, *HXT7*, *HXT6*, *GRX2*, *SPF1*, *CYC7*, *YEL047C*, *PIC2*, *COX15*, *GSY1*, *HXK1*, *PKP2*, *STF2*, *TDH3*, *PDX1*, *ENO1*, *AIM17*, *SOD2*, *ENO2*, *OYE2*, *TDH1*, *MPM1*, *KHA1*, *OPI3*, *MCR1*, *SSA2*, *PUT1*, *ATP14*, *ATP18*, *ADH3*, *APJ1*, *CIT1*, *ATP19*, *RDL1*, *FAA1*, *ALD4*, *GDB1*
GO:0016020	Membrane	*SSA1*, *ATG8*, *AGP1*, *HSP30*, *PMP1*, *STF1*, *SDH4*, *CCC2*, *HXT7*, *HXT6*, *PDR15*, *SPF1*, *COX15*, *DDI1*, *HSP12*, *STF2*, *ENO2*, *CTR2*, *KHA1*, *MCR1*, *PTR2*, *SSA2*, *TPO1*, *YPS3*, *ATP14*, *ATP18*, *ICY1*, *HOR7*, *MEP2*, *ATP19*, *MCH4*, *RDL1*, *FAA1*, *SSU1*, *DIP5*
GO:0005634	Nucleus	*SSA1*, *APN2*, *HSP26*, *ADH5*, *GRX1*, *RPN4*, *YDL124W*, *HOR2*, *RGI1*, *SSA4*, *GRX4*, *HSP12*, *PNC1*, *NQM1*, *RTS3*, *PCT1*, *SOL4*, *COS8*, *PCL5*, *GRE3*, *OYE2*, *PHD1*, *HSP104*, *HHT2*, *APJ1*, *YNL134C*, *GRE2*, *GCY1*, *GSP2*
GO:0005624	Membrane fraction	*SSA1*, *CDC19*, *GLK1*, *PGK1*, *HSP30*, *YDL124W*, *HXT7*, *HXT6*, *YEL047C*, *HSP12*, *TDH3*, *ENO1*, *GND2*, *ENO2*, *TDH1*, *MPM1*, *UGP1*, *PTR2*, *SSA2*, *AHP1*, *HOR7*, *ADH1*, *FAA1*
GO:0005773	Vacuole	*SSA1*, *ATG8*, *YBR139W*, *TOS1*, *PRB1*, *ENO1*, *COS6*, *ENO2*, *CTR2*, *LAP4*, *SSA2*, *TPO1*, *ICY1*, *PBI2*, *DDR2*, *MCH4*, *SRL1*, *ATH1*
GO:0005886	Plasma membrane	*AGP1*, *HSP30*, *PMP1*, *HXT7*, *HXT6*, *DDI1*, *HSP12*, *ENO2*, *PTR2*, *TPO1*, *YPS3*, *HOR7*, *MEP2*, *SSU1*, *DIP5*
GO:0005618	Cell wall	*SSA1*, *TIP1*, *TOS1*, *SED1*, *SPI1*, *TDH3*, *FLO5*, *TDH1*, *HSP150*, *SSA2*, *YPS1*, *HOR7*, *SRL1*, *ATH1*
GO:0005740	Mitochondrial envelope	*STF1*, *SDH4*, *CYC7*, *COX15*, *STF2*, *MCR1*, *ATP14*, *ATP18*, *ATP19*, *RDL1*, *FAA1*
GO:0005783	Endoplasmic reticulum	*YBR287W*, *PDI1*, *EUG1*, *SPF1*, *OPI3*, *LHS1*, *ERO1*, *HOR7*, *RDL1*
GO:0012505	Endomembrane system	*CCC2*, *SPF1*, *PCT1*, *COS8*
GO:0005777	Peroxisome	*CIT2*, *GPD1*, *PNC1*
GO:0005794	Golgi apparatus	*CCC2*, *PCT1*, *KHA1*
GO:0005933	Cellular bud	*YRO2*, *TPO1*, *SRL1*
GO:0005840	Ribosome	*SED1*, *YEL047C*
GO:0005856	Cytoskeleton	*HSP42*, *IRC15*
GO:0005576	Extracellular region	*HSP150*, *YGP1*
GO:0016023	Cytoplasmic membrane-bounded vesicle	*CCC2*
GO:0030427	Site of polarized growth	*SRL1*
GO:0005694	Chromosome	*HHT2*
GO:0005575	Cellular component unknown	*YCR013C*, *YDR133C*, *YFL066C*, *YGL117W*, *YGR146C*, *PAU13*, *YHL050C*, *YKL044W*, *GLG1*, *YMR018W*, *YOL157C*, *SIA1*, *OYE3*, *HSP32*
Other	Other	*ADH7*, *HSP31*, *UGA1*
Biological process		
GO:0006950	Response to stress	*SSA1*, *APN2*, *PRX1*, *SSA3*, *ATG8*, *HSP26*, *TPS1*, *GRX1*, *HSP30*, *RPN4*, *GPD1*, *YDL124W*, *NTH1*, *TPS2*, *HSP42*, *HSP78*, *GRX2*, *PRB1*, *HOR2*, *SSA4*, *GRX4*, *HSP12*, *STF2*, *CTT1*, *SOD2*, *GRE3*, *LHS1*, *MCR1*, *SSA2*, *HSP104*, *UBI4*, *AHP1*, *DAK1*, *TSL1*, *HOR7*, *DDR2*, *GRE2*, *GCY1*, *HSP82*, *ATH1*

Table 1 (continued)

GO ID	Term	Gene[a]
GO:0044262	Cellular carbohydrate metabolic process	*CDC19*, *NTH2*, *TPS1*, *GLK1*, *CIT2*, *PGK1*, *NTH1*, *TPS2*, *YDR248C*, *GLC3*, *UBC8*, *SPF1*, *GIP2*, *HOR2*, *GSY1*, *HXK1*, *PYC1*, *TDH3*, *SOL4*, *ENO1*, *GND2*, *ENO2*, *PFK26*, *TDH1*, *UGP1*, *GLG1*, *HSP104*, *GSY2*, *DAK1*, *TSL1*, *PGM2*, *ATH1*, *GPH1*, *GDB1*
GO:0006810	Transport	*SSA1*, *SSA3*, *ATG8*, *SDS24*, *AGP1*, *GLK1*, *PMP1*, *HSP78*, *CCC2*, *HXT7*, *HXT6*, *PDR15*, *SPF1*, *PIC2*, *SSA4*, *DDI1*, *HXK1*, *CTR2*, *KHA1*, *LHS1*, *PTR2*, *SSA2*, *TPO1*, *ATP14*, *ATP18*, *MEP2*, *ATP19*, *MCH4*, *SIA1*, *FAA1*, *SSU1*, *HSP82*, *DIP5*
GO:0006091	Generation of precursor metabolites and energy	*CDC19*, *ETR1*, *ADH5*, *GLK1*, *PGK1*, *SDH4*, *GLC3*, *CYC7*, *GIP2*, *HOR2*, *GSY1*, *HXK1*, *TDH3*, *ENO1*, *ENO2*, *PFK26*, *TDH1*, *UGP1*, *GLG1*, *GSY2*, *ATP14*, *ATP18*, *ADH3*, *PGM2*, *ADH2*, *CIT1*, *ATP19*, *ADH1*, *GPH1*, *GDB1*
GO:0042221	Response to chemical stimulus	*PRX1*, *TPS1*, *TOS1*, *GRX1*, *RPN4*, *YDL124W*, *PDR15*, *GRX2*, *EMI2*, *SPI1*, *GRX4*, *HSP12*, *CTT1*, *SOD2*, *GRE3*, *LHS1*, *MCR1*, *HSP104*, *AHP1*, *GCY1*
GO:0006519	Cellular amino acid and derivative metabolic process	*ADH5*, *CIT2*, *SFA1*, *YAT2*, *SER3*, *UGA1*, *PCT1*, *ARG4*, *OPI3*, *PUT1*, *ADH3*, *ALD2*, *ADH2*, *CIT1*, *ADH1*
GO:0006457	Protein folding	*SSA1*, *SSA3*, *HSP26*, *SSE2*, *PDI1*, *HSP78*, *EUG1*, *SSA4*, *SSA2*, *HSP104*, *CPR6*, *ERO1*, *SSE1*, *HSP82*
GO:0051186	Cofactor metabolic process	*ADH5*, *GPD1*, *SDH4*, *COX15*, *PNC1*, *PYC1*, *PDX1*, *SOL4*, *GND2*, *ADH3*, *ADH2*, *CIT1*, *ADH1*, *ALD4*
GO:0006766	Vitamin metabolic process	*ADH5*, *GPD1*, *YAT2*, *PNC1*, *PYC1*, *SOL4*, *GND2*, *ADH3*, *ADH2*, *ADH1*, *ALD4*
GO:0019725	Cellular homeostasis	*PRX1*, *GRX1*, *GPD1*, *CCC2*, *GRX2*, *SPF1*, *GRX4*, *AHP1*, *PGM2*
GO:0006464	Protein modification process	*UBC8*, *SPF1*, *GIP2*, *PKP2*, *UGP1*, *UBI4*, *ERO1*, *FAA1*
GO:004255	Cellular lipid metabolic process	*ETR1*, *PCT1*, *OPI3*, *MCR1*, *GRE2*, *FAA1*
GO:0046483	Heterocycle metabolic process	*SFA1*, *COX15*, *PUT1*, *ATP14*, *ATP18*, *ATP19*
GO:0006350	Transcription	*RPN4*, *EMI2*, *PNC1*, *PCL5*, *PHD1*
GO:0016044	Membrane organization	*ATG8*, *SDS24*, *ENO1*, *ENO2*, *PBI2*
GO:0045333	Cellular respiration	*ETR1*, *SDH4*, *CYC7*, *CIT1*
GO:0007005	Mitochondrion organization	*SSA1*, *SED1*, *HSP78*, *HSP82*
GO:0007047	Cell wall organization	*TIP1*, *SED1*, *HSP150*, *YPS3*
GO:0006259	DNA metabolic process	*APN2*, *RPN4*, *IRC15*, *HSP82*
GO:0044257	Cellular protein catabolic process	*UBC8*, *PRB1*, *DDI1*, *LAP4*
GO:0007033	Vacuole organization	*ENO1*, *ENO2*, *PBI2*
GO:0051276	Chromosome organization	*HHT2*, *IRC15*, *HSP82*
GO:0030435	Sporulation resulting in formation of a cellular spore	*EMI2*, *PRB1*, *UBI4*
GO:0016070	RNA metabolic process	*EMI2*, *PNC1*, *PHD1*
GO:0016192	Vesicle-mediated transport	*ATG8*, *SDS24*, *DDI1*
GO:0007010	Cytoskeleton organization	*HSP42*, *IRC15*
GO:0007049	Cell cycle	*RPN4*, *IRC15*
GO:0007124	Pseudohyphal growth	*PHD1*, *MEP2*
GO:0006412	Translation	*SSA1*, *HSP78*
GO:0000910	Cytokinesis	*SDS24*
GO:0007059	Chromosome segregation	*IRC15*
GO:0007126	Meiosis	*IRC15*
GO:0016050	Vesicle organization	*ATG8*
GO:0006997	Nucleus organization	*GSP2*
GO:0070271	Protein complex biogenesis	*HSP82*
GO:0006725	Cellular aromatic compound metabolic process	*YDL124W*
GO:0008150	Biological process unknown	*YRO2*, *RTC2*, *YBR287W*, *YCL042W*, *YCR013C*, *GPM2*, *COS7*, *YDR133C*, *HSP31*, *RGI1*, *YFL066C*, *YGL117W*, *NQM1*, *YGR146C*, *RTS3*, *COS6*, *AIM17*, *PAU13*, *COS8*, *YHL050C*, *OYE2*, *YJL016W*, *MPM1*, *YKL044W*, *YKL151C*, *YMR018W*, *ICY1*, *APJ1*, *YNL134C*, *YOL157C*, *RDL1*, *OYE3*, *HSP32*
Other	Other	*YBR139W*, *ADH7*, *STF1*, *YEL047C*, *FLO5*, *YPS1*, *YGP1*, *SRL1*

Table 1 (continued)

GO ID	Term	Gene[a]
Molecular function		
GO:0016491	Oxidoreductase activity	*PRX1*, *ETR1*, *ADH5*, *GRX1*, *PDI1*, *ADH7*, *GPD1*, *YDL124W*, *SFA1*, *SDH4*, *GRX2*, *EUG1*, *YEL047C*, *SER3*, *COX15*, *GRX4*, *CTT1*, *TDH3*, *GND2*, *SOD2*, *GRE3*, *OYE2*, *TDH1*, *MCR1*, *AHP1*, *PUT1*, *ERO1*, *ADH3*, *ALD2*, *ADH2*, *YNL134C*, *ADH1*, *GRE2*, *GCY1*, *ALD4*, *OYE3*
GO:0016787	Hydrolase activity	*SSA1*, *APN2*, *SSA3*, *NTH2*, *TIP1*, *YBR139W*, *NTH1*, *TPS2*, *HSP78*, *CCC2*, *PDR15*, *HSP31*, *SPF1*, *PRB1*, *HOR2*, *SSA4*, *PNC1*, *SOL4*, *YHL050C*, *LAP4*, *SSA2*, *HSP104*, *YPS1*, *YPS3*, *ATP14*, *TSL1*, *GSP2*, *HSP82*, *HSP32*, *ATH1*, *GDB1*
GO:0016740	Transferase activity	*CDC19*, *TPS1*, *GRX1*, *GLK1*, *CIT2*, *PGK1*, *YDR248C*, *GRX2*, *GLC3*, *YAT2*, *GSY1*, *HXK1*, *PKP2*, *UGA1*, *NQM1*, *PCT1*, *PFK26*, *OPI3*, *UGP1*, *GLG1*, *GSY2*, *DAK1*, *TSL1*, *CIT1*, *IRC15*, *GPH1*, *GDB1*
GO:0005515	Protein binding	*SSA1*, *SSA3*, *HSP26*, *HSP42*, *HSP78*, *HSP31*, *SSA4*, *DDI1*, *PDX1*, *LHS1*, *SSA2*, *HSP104*, *UBI4*, *CPR6*, *APJ1*, *IRC15*, *HSP82*, *HSP32*
GO:0005215	Transporter activity	*AGP1*, *CCC2*, *HXT7*, *HXT6*, *PDR15*, *SPF1*, *PIC2*, *CTR2*, *KHA1*, *PTR2*, *TPO1*, *ATP14*, *ATP18*, *MEP2*, *ATP19*, *MCH4*, *SSU1*, *DIP5*
GO:0030234	Enzyme regulator activity	*SSE2*, *PMP1*, *GIP2*, *PCL5*, *LHS1*, *TSL1*, *PBI2*, *SSE1*
GO:0008233	Peptidase activity	*YBR139W*, *HSP31*, *PRB1*, *LAP4*, *YPS1*, *YPS3*, *HSP32*
GO:0016853	Iisomerase activity	*PDI1*, *GPM2*, *EUG1*, *CPR6*, *PGM2*
GO:0016829	Lyase activity	*APN2*, *ENO1*, *ARG4*, *ENO2*
GO:0005198	Structural molecule activity	*TIP1*, *SED1*, *HSP150*, *YPS3*
GO:0016874	Ligase activity	*UBC8*, *PYC1*, *FAA1*
GO:0003677	DNA binding	*RPN4*, *PHD1*, *HHT2*
GO:0016779	Nucleotidyltransferase activity	*PCT1*, *UGP1*
GO:0030528	Transcription regulator activity	*RPN4*, *PHD1*
GO:0004672	Protein kinase activity	*PKP2*
GO:0004871	Signal transducer activity	*COS7*
GO:0004386	Helicase activity	*YHL050C*
GO:0003674	Molecular function unknown	*ATG8*, *YRO2*, *RTC2*, *TOS1*, *SDS24*, *YBR287W*, *YCL042W*, *YCR013C*, *HSP30*, *GPM2*, *STF1*, *YDR133C*, *EMI2*, *RGI1*, *SPI1*, *HSP12*, *YFL066C*, *YGL117W*, *STF2*, *YGR146C*, *RTS3*, *COS6*, *AIM17*, *PAU13*, *COS8*, *YJL016W*, *MPM1*, *YKL044W*, *YKL151C*, *YMR018W*, *ICY1*, *HOR7*, *YGP1*, *DDR2*, *YOL157C*, *SIA1*, *SRL1*, *RDL1*
Other	Other	*CYC7*, *FLO5*

[a] Based on Ogawa et al. (2000); Alexandre et al. (2001); Chandler et al. (2004); Marks et al. (2008); Dinh et al. (2009); and Ma and Liu (submitted)

stearic acid, respectively (Stukey et al. 1989, 1990). The important role of UFAs in ethanol tolerance was confirmed by synthetic mono-unsaturated fatty acids and expression of insect desaturase TniNPVE in yeast (You et al. 2003). On the other hand, no significant difference of mRNA abundance was observed for *OLE1* under ethanol challenges (Chandler et al. 2004; Ma and Liu (submitted)). It is possible that UFAs biosynthesis, in addition to transcription levels, is regulated by the levels of mRNA stability and translation as well as enzyme reactions with carbon and oxygen sources (Martin et al. 2007).

Ergosterol is another major component of cellular membranes associated with plasma membrane fluidity. Higher ergosterol content in yeast was found to be associated with higher ethanol tolerance (del Castillo Agudo 1992). Deletion mutants of *ERG2*, *ERG3*, *ERG5*, *ERG6*, *ERG24*, and *ERG28* involved in ergosterol biosynthesis showed defective growth under ethanol stress (Kubota et al. 2004; Fujita et al. 2006; Van Voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009). However, all these genes involved in ergosterol biosynthesis showed down-regulation under ethanol challenges (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)). These structural genes appeared to be important for cell tolerance functions but may not be regulated at transcriptional abundance levels.

Amino acids

In yeast, proline exhibits multiple functions during the fermentation process, including protection of cells from

damage by freezing, desiccation, or oxidative stress (Takagi 2008). It enhances stability of proteins and membranes, and inhibits protein aggregation during protein refolding (Rudolph and Crowe 1985; Samuel et al. 2000). When the wild-type *PRO1* gene was replaced by *pro1*^{D154N} allele, the yeast increased biosynthesis of proline and was more tolerant to ethanol stress (Takagi et al. 2005). The function of proline in ethanol tolerance is also supported by a *PRO1*-deletion strain which is more sensitive to ethanol stress (Kubota et al. 2004; Yoshikawa et al. 2009). Under ethanol stress, expressions of *PRO1*, *PRO2*, and *PRO3* involving de novo biosynthesis of proline were not significantly induced (Kaino and Takagi 2008; Ma and Liu (submitted)). A delayed accumulation of proline in yeast cells was observed and consistent with the up-regulated expression of *PUT4* encoding a high-affinity proline transporter (Kaino and Takagi 2008). However, over accumulation of intracellular proline is associated with reduced growth rate in *S. cerevisiae* and delayed yeast cell growth in the presence of ethanol (Maggio et al. 2002; Takagi et al. 2007). It appeared that the amount of intracellular proline needs to be at well-controlled levels to properly cope with ethanol stress and avoid side effects such as delayed growth.

Tryptophan is another amino acid that is believed to contribute to ethanol tolerance. Deletion of genes *TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5* in any step of tryptophan biosynthesis resulted in sensitive responses to ethanol stress (Kubota et al. 2004; Fujita et al. 2006; Hirasawa et al. 2007; Yoshikawa et al. 2009). A brewing yeast in general shows higher expression levels of tryptophan biosynthesis genes compared with a laboratory strain (Hirasawa et al. 2007). Higher levels of overexpression of either tryptophan biosynthesis genes (*TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5*) or tryptophan permease gene (*TAT2*), especially *TRP2* and *TRP5*, are closely related to the increased ethanol tolerance. Supplementation of tryptophan to culture medium also appeared to enhance yeast tolerant levels to ethanol.

V-ATPase and peroxisomal functions

It is known that ethanol increases membrane permeability to protons causing increased proton influx and intracellular acidification (Cartwright et al. 1987; Rosa and Sá-Correia 1996). The transport of intracellular H^+ into vacuoles by H^+ V-ATPase is important for yeast to maintain intracellular pH homeostasis and therefore counteracting ethanol stress. V-ATPase aids translocation of protons across the vacuolar membrane through hydrolysis of ATP (Forgac 1998; Inoue et al. 2005). The H^+ translocation is considered as a tolerance response to ethanol and supported by gene deletion assays. Deletion mutants of 14 genes encoding structural components of V-ATPase showed sensitive response to ethanol challenges, including *VMA1*, *VMA2*, *VMA4*, *VMA5*, *VMA6*, *VMA7*, *VMA8*, *VMA9*, *VMA10*, *VMA11*, *VMA13*, *VMA16*, *VPH1*, and *CUP5* (Kubota et al. 2004; Fujita et al. 2006; Van Voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009). Five other genes, *VMA12*, *VMA21*, *VMA22*, *RAV1*, and *RAV2*, involved in the assembly of the V-ATPase also appeared to be necessary for ethanol tolerance. Theoretically, transportation of intracellular H^+ into the vacuole by H^+ V-ATPase would cause vacuolar acidification. In fact, vacuolar acidification by ethanol was recently demonstrated to be dose-dependent (Teixeira et al. 2009). Although expression of H^+ V-ATPase genes were not induced by ethanol, a set of genes involved in ATPase regulation were up-regulated, such as *STF2* encoding an ATPase stabilizing factor (Alexandre et al. 2001). Many other genes related to vacuolar protein sorting machinery, vacuolar membrane structure and function were found to be essential for ethanol stress tolerance, including at least the following 44 genes: *FAB1*, *MEH1*, *VAC14*, *VAM1*, *VAM3*, *VPS2*, *VPS4*, *VPS5*, *VPS6*, *VPS9*, *VPS11*, *VPS15*, *VPS16*, *VPS18*, *VPS20*, *VPS22*, *VPS23*, *VPS24*, *VPS25*, *VPS27*, *VPS28*, *VPS29*, *VPS30*, *VPS31*, *VPS32*, *VPS33*, *VPS34*, *VPS35*, *VPS36*, *VPS37*, *VPS38*, *VPS39*, *VPS41*, *VPS43*, *VPS46*, *VPS52*, *VPS54*, *VPS60*, *VPS65*, *VPS66*, *VPS68*, *VPS71*, *VPS72*, and *VPS74* (Kubota et al. 2004; Fujita et al. 2006; Van Voorst et al. 2006; Auesukaree et al. 2009; Teixeira et al. 2009; Yoshikawa et al. 2009).

Peroxisomal function also appeared to be associated with ethanol tolerance in yeast. Strains with gene deletions of *PEX1*, *PEX2*, *PEX3*, *PEX4*, *PEX5*, *PEX8*, *PEX10*, *PEX12*, *PEX14*, *PEX15*, *PEX17*, *PEX19*, and *PEX22*, encoding proteins of peroxisome transport machinery and peroxisomal membrane protein import machinery required for peroxisome organization and biogenesis, were found to be sensitive to ethanol challenge (Teixeira et al. 2009; Yoshikawa et al. 2009). Functions of peroxisomes involved in metabolisms of peroxides and other reactive oxygen species (ROS) have been recently recognized (Schrader and Fahimi 2004; Du and Takagi 2007). Peroxisomal function is important for synthesis or degradation of membrane phospholipids in cell membrane remodeling (Lockshon et al. 2007) and cells deficient in peroxisomal functions are unable to effectively control fatty acid composition of membrane phospholipids.

Heat shock proteins

Genes encoding HSPs that induced under ethanol stress exhibited a broad overlapping with genes associated with heat stress response (Piper et al. 1994). These induced

expressions are reflected by typical up-regulated transcription response. Commonly identified significant up-regulated transcripts include at least 10 HSP genes *HSP12*, *HSP26*, *HSP30*, *HSP31*, *HSP32*, *HSP42*, *HSP78*, *HSP82*, *HSP104*, and *HSP150* (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Marks et al. 2008; Ma and Liu (submitted)). The ethanol-induced expression can be concentration-dependent or strain-dependent based on varied definitions of the tolerance. For example, the induced expression of *HSP26* was undetectable with the addition of 2% ethanol, barely detectable at 4% ethanol, and more abundant with further increased ethanol concentrations (Piper et al. 1994). Most HSP genes, for example, *HSP30*, *HSP42*, and *HSP104*, can be detected at earlier time points for yeast strains (Piper et al. 1994; Alexandre et al. 2001; Chandler et al. 2004; Marks et al. 2008; Ma and Liu (submitted)). However, the lack of continued function of these genes and interaction with other relevant gene expressions led to no further metabolic functions for a wild-type strain. Only those HSP genes continuously expressed under ethanol stress over time, for example, *HSP12*, *HSP32*, *HSP42*, *HSP78*, *HSP82* and *HSP150*, as observed for tolerant strain Y-500316, allowed yeast to establish a viable culture in the presence of 8% ethanol, displaying a meaningful tolerance (Ma and Liu (submitted)). This indicated that tolerance response of yeast is a series of dynamic metabolic events rather than a transient response by ethanol challenge (Fig. 1). Also, interactions of many genes at multiple loci over time are important for cell functions under the stress. Isolated induced gene expressions not leading to successfully reprogrammed metabolism for cell survival and ethanol production may not be accounted as a tolerance response. It is in fact merely a stress response. In addition, numerous genes of the tolerant strain demonstrated significantly enriched transcriptional abundance, including HSP genes such as *HSP31* and *HSP82*, without ethanol challenges (Ma and Liu (submitted)). These genes are regulons of commonly recognized transcription factors Msn4p, Msn2p, Yap1p, and Hsf1p.

HSPs, mainly acting as chaperones, can insure proper folding or refolding of nascent or denatured proteins and enzymes in order to maintain a functional conformation (Table 2) (Parsell et al. 1994; Ruepp et al. 2004; Young et al. 2004; McClellan et al. 2007; Gong et al. 2009). Besides HSPs, other genes encoding chaperones, such as *SSA1*, *SSA2*, *SSA3*, *SSA4*, *SSE1*, *SSE2*, *APJ1*, and *LHS1*, involved in protein folding and refolding, are also up-regulated under ethanol stress conditions (Alexandre et al. 2001; Chandler et al. 2004; Marks et al. 2008). Interactions between chaperones exist widely and correct folding or stability of certain proteins may require more than two chaperones in participation for efficient function (Gong et al. 2009). Therefore, induction of multiple chaperones may be necessary to counteract ethanol stress. At the same time, certain duel function chaperones are required for more difficult-to-fold proteins from nascent polypeptides into biologically active structures as well as for the refolding of denatured proteins back into native conformations (Fig. 1). For example, *HSP82* was up-regulated by ethanol in ethanol-tolerant strain Y-500316 (Ma and Liu (submitted)). Its protein Hsp82p has been reported to activate many other key proteins having cellular regulatory and signaling functions, including transcription factors and regulatory kinases (Picard 2002; Prodromou and Pearl 2003; Young et al. 2004; McClellan et al. 2007). Since ethanol perturbs protein conformation and causes accumulation of denatured proteins, protein repair functions mediated by multiple chaperones appear to be critical for yeast tolerance to ethanol.

In addition to functioning as chaperones, HSPs have numerous other functions contributing to ethanol tolerance. For example, Hsp30p, a hydrophobic plasma membrane protein, was reported as a regulator of H^+-ATPase Pma1p (Piper et al. 1997). Hsp31p and Hsp32p function as hydrolase and peptidase (Wilson et al. 2004). Hsp150 has been identified as a necessary protein for cell wall stability and remodeling (Moukadiri and Zueco 2001). Recently, *HSP31*, *HSP32*, and *HSP150* were newly identified as tolerance candidate genes (Ma and Liu (submitted)). Significantly enhanced expressions of these genes for tolerant yeast over time support their multi-functional roles under ethanol stress (Fig. 1). Many HSP genes induced by ethanol stress are present in the cytoplasm as well as in the nucleus and mitochondrion (Table 1). Up-regulated HSP genes influence cell functions at multiple locations including facilitating the functions of transcription factors in nucleus, improving ATP energy generation in metabolic processes, maintaining enzyme functions involving biosynthesis, catabolism, and ethanol production in cytoplasm. The subset of genes responsible for ethanol tolerance is likely to vary with strains and conditions studied. Investigations on comprehensive interactions at the genome level are needed to provide more detailed networks involving ethanol tolerance in yeast. However, a core set of genes for HSP-mediated functions along with the reprogrammed glucose metabolic pathways are likely a center piece interacting with many other genes supporting the ethanol tolerance in yeast.

Trehalose and glycogen metabolisms

Storage carbohydrates such as trehalose are compatible solutes that can prevent influx of excess salts into irreversible dehydration of yeast cells. Yeasts are able to accumulate trehalose up to 15% of cell dry mass when

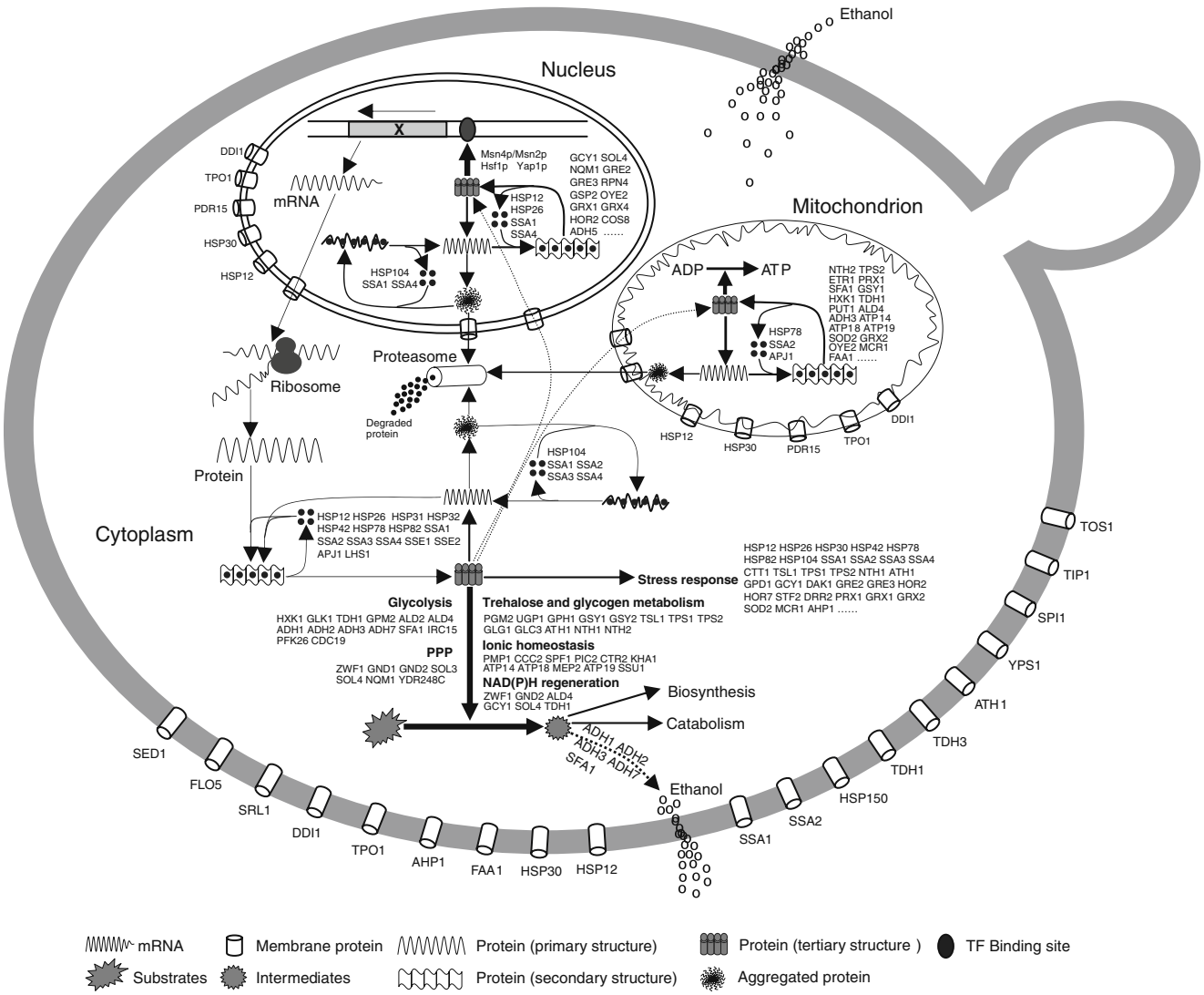

Fig. 1 A schematic diagram showing a prototype of ethanol tolerance mechanisms in *S. cerevisiae*. Proteins encoded by significantly up-regulated genes are located in cell wall, membrane, nucleus, mitochondrion, and cytoplasm. Heat shock proteins are mainly detailed as chaperones protecting and maintaining proteins functions at multiple loci. Function of gene products are classified based on gene ontology. A list of legends is provided under the illustration. This figure is based on Ma and Liu (submitted) with modifications using additional data from Ogawa et al. (2000), Alexandre et al. (2001), Chandler et al. (2004), Marks et al. (2008), and Dinh et al. (2009)

growing under a stress environment (Francois and Parrou 2001). Trehalose accumulation was observed under ethanol stress, and cells unable to accumulate trehalose displayed retarded growth under ethanol challenges (Mansure et al. 1994; Ogawa et al. 2000; Kaino and Takagi 2008). Accumulation of trehalose is reflected by up-regulated genes involved in trehalose synthesis, including *TPS1*, *TPS2*, *TSL1*, *PGM2*, and *UGP1*, under ethanol stress (Ogawa et al. 2000; Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)). Trehalose has been reported to function by reducing membrane permeability as well as ensuring proper folding of proteins (Mansure et al. 1994; Singer and Lindquist 1998). The co-induction of trehalose genes and HSP genes were commonly observed in yeast response to ethanol challenges (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)). In this close interplay, trehalose prevents protein denaturation as a first step, and subsequently HSPs stop protein aggregation and promote refolding into functional conformations (Singer and Lindquist 1998). Trehalose accumulation by enhanced gene expression responsible for trehalose synthesis may create an optimal intracellular environment under ethanol stress conditions.

The intermediate trehalose-6-phosphate is a regulator of yeast glycolysis that inhibits hexokinase (Blazquez et al. 1993). Such an inhibition can avoid depletion of intracel-

Table 2 Functional categories and terms of proteins for induced genes by ethanol challenge from *Saccharomyces cerevisiae* based on gene products classified according to functional catalogue described in Munich Information Center for Protein Sequences (MIPS) database

MIPS ID	Functionary category	*p* value	Protein[a]
01.05.02.04	Sugar, glucoside, polyol and carboxylate anabolism	2.70E−07	NTH2, TPS1, NTH1, NQM1, PFK26, UGP1, TSL1, PGM2, ATH1
01.05.02.07	Sugar, glucoside, polyol and carboxylate catabolism	2.26E−11	CDC19, NTH2, TPS1, PGK1, NTH1, SDH4, NQM1, TDH3, ENO1, GRE3, ENO2, PFK26, TDH1, UGP1, PGM2, CIT1, ATH1
01.05.03.01	Glycogen metabolism	1.94E−04	GSY1, GLG1, GSY2
2.01	Glycolysis and gluconeogenesis	1.96E−12	CDC19, GLK1, PGK1, ADH7, UBC8, HXK1, PYC1, TDH3, PDX1, ENO1, ENO2, PFK26, TDH1, PGM2, YNL134c, GRE2
2.07	Pentose phosphate pathway	3.77E−03	NQM1, SOL4, GND2, PGM2
2.11	Electron transport and membrane-associated energy conservation	2.30E−04	STF1, SDH4, CYC7, STF2, MCR1, ATP14, ATP18, ATP19
2.13	Respiration	3.69E−04	ETR1, STF1, SDH4, CYC7, YEL047c, COX15, STF2, MCR1, ATP14, ATP18, ATP19, ALD4
2.16	Fermentation	2.56E−04	ADH5, ADH7, ADH3, ALD2, ADH2, ADH1, ALD4
2.19	Metabolism of energy reserves (e.g. glycogen, trehalose)	4.79E−14	NTH2, TPS1, NTH1, TPS2, GLC3, GIP2, GSY1, UGP1, GLG1, GSY2, TSL1, PGM2, YOL157c, HSP82, ATH1, GPH1, GDB1
2.45	Energy conversion and regeneration	2.06E−05	STF1, STF2, OYE2, ATP14, ATP18, ALD2, ATP19, OYE3
14.01	Protein folding and stabilization	3.51E−10	SSA1, SSA3, HSP26, SSE2, PDI1, HSP42, HSP78, EUG1, SSA4, LHS1, SSA2, HSP104, CRP6, ERO1, APJ1, SSE1, HSP82
16.21	Complex cofactor/ cosubstrate/ vitamine binding	1.61E−04	GPD1, YDL124w, SDH4, SER3, GND2, OYE2, MCR1, OYE3
20.01.0×1	Ion transport	2.47E−03	PMP1, CCC2, SPF1, PIC2, CTR2, KHA1, ATP14, ATP18, MEP2, ATP19, SSU1
20.01.15	Electron transport	5.21E−10	GRX1, STF1, SDH4, GRX2, CYC7, YEL047c, GRX4, STF2, OYE2, MCR1, ATP14, ATP18, ERO1, ATP19, SIA1, OYE3
32.01	Stress response	1.48E−19	SSA1, PRX1, SSA3, NTH2, YRO2, TIP1, HSP26, TPS1, SSE2, GRX1, HSP30, GPD1, NTH1, TPS2, SED1, HSP42, HSP78, GRX2, HSP31, CYC7, PRB1, HOR2, SSA4, DDI1, GRX4, HSP12, STF2, NQM1, PAU13, COS8, SOD2, GRE3, HSP150, LHS1, MCR1, HSP104, UBI4, AHP1, CRP6, DAK1, TSL1, HOR7, APJ1, YGP1, DDR2, GRE2, GCY1, SSE1, HSP82, HSP32, ATH1
32.01.01	Oxidative stress response	1.84E−06	PRX1, GRX1, GRX2, GRX4, HSP12, NQM1, SOD2, MCR1, AHP1, GRE2
32.01.07	Unfolded protein response	2.75E−07	SSA1, HSP26, HSP42, HSP78, HSP31, SSA4, COS8, LHS1, CRP6, APJ1, SSE1, HSP32
32.07	Detoxification	1.61E−05	PRX1, ADH5, GRX1, SFA1, GRX2, GRX4, NQM1, SOD2, TPO1, AHP1, GRE2, SRL1, SSU1

[a] Based on data from Ogawa et al. (2000); Alexandre et al. (2001); Chandler et al. (2004); Marks et al. (2008); Dinh et al. (2009); and Ma and Liu (submitted). Proteins underlined indicate more than one function have been described

lular Pi and ATP by over-phosphorylation of glucose and hereby maintains ATP consuming-regeneration balance in glycolysis (Francois and Parrou 2001). Interestingly, genes involved in trehalose degradation, including *NTH1*, *NTH2*, and *ATH1*, were also induced by ethanol challenge (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)). The persistence of high levels of trehalose after recovery from stress may lead to the inactivation of important yeast enzymes, such as glutathione reductase, cytosolic pyrophosphatase and glucose 6-phosphate dehydrogenase (Sebollela et al. 2004). Thus, co-induction of trehalose degradation genes may mediate optimal concentrations of trehalose for its proper functions. Similarly, simultaneously induced expression of other genes involved in glycogen biosynthesis such as *GSY1* and *GSY2* and glycogen degradation such as *GPH1* were also observed under ethanol stress (Ma and Liu (submitted)). Whether variations of intracellular glycogen concentrations are

related to increased ethanol tolerance remains to be confirmed. However, these coincident observations may suggest a common adaptation response in yeast. With increased trehalose levels in cells, it is possible that yeast cells have to adapt the newly evolved intracellular environment by actively balancing trehalose or glycogen concentrations to backing proper cellular functions and enhanced glucose metabolism (Fig. 2). Since glycogen metabolism is very close to trehalose pathway, trehalose biosynthesis could be subtly affected by glycogen biosynthesis and catabolism.

NAD(P)H regeneration and redox balance

Cofactors NADH and NADPH play important roles in yeast metabolism and biosynthesis of amino acids, lipids, and nucleotides (Bruinenberg et al. 1983; Hou et al. 2009). Under ethanol stress, most genes involved in NADH/NADPH regeneration reaction steps were up-regulated, including *GND2*, *ZWF1*, *ALD4*, *GCY1*, *TDH1*, and *TDH3* (Fig. 2) (Alexandre et al. 2001; Chandler et al. 2004; Ma and Liu (submitted)).

Comparative analyses of expression dynamics and metabolic profiling between a tolerant strain and its parental wild-type strain uncovered pathway relationships under stressed conditions (Liu et al. 2009; Ma and Liu (submitted)). Highly integrated expression of genes involved in glycolysis and pentose phosphate pathways, especially after the exponential phase for the ethanol-tolerant Y-50316, appeared to promote accelerated glucose-to-ethanol conversion (Fig. 2). Strain Y-50316 maintained its tolerance to numerous fermentation inhibitors and displayed expression patterns very similar to its inhibitor-tolerance parental strain

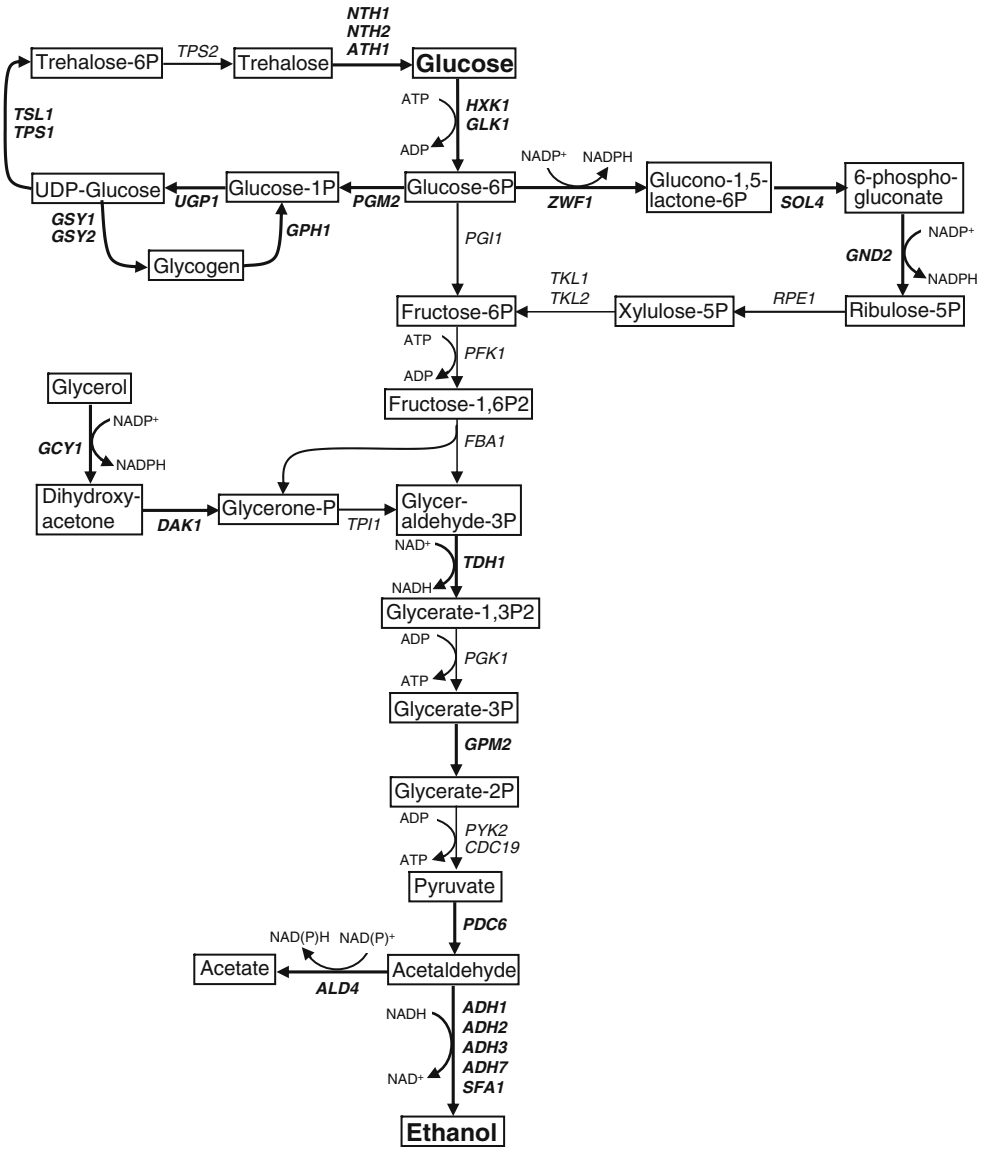

Fig. 2 Illustrative expression pathways of ethanol- and inhibitor-tolerant mutant *S. cerevisiae* NRRL Y-50316 involved in glycolysis, pentose phosphate pathway, glycerol metabolism, trehalose metabolism, and glycogen metabolism in response to ethanol challenges inferred by dynamic quantitative mRNA expression analysis and metabolic profiling analysis compared with its parental strain NRRL Y-50049. *Bold lines and letters* indicate the levels of expression are statistically significant at $P \leq 0.05$

(Liu et al. 2009; Ma and Liu (submitted)). Under ethanol stress condition, up-regulated *GND2* and *ZWF1* in oxidative phase of pentose phosphate pathway, *ALD4* in acetic acid production, and *GCY1* in glycerol metabolism were distinct characteristics of the tolerant yeast. Products of enhanced expression of *ZWF1*, *SOL4*, and *YDR248C* would also provide sufficient substrate and accelerate downstream decarboxylation reactions to regenerate more NADPH by 6-phosphogluconate dehydrogenase encoded by *GND2*. Similarly, highly induced *TDH1* is anticipated to support another necessary NADH regenerating step in the pathway. The enhanced expression of alcohol dehydrogenase genes *ADH1*, *ADH2*, *ADH3*, *ADH7*, and *SFA1*, together with other normal or near normally expressed genes in the intermediate steps of glycolysis are necessary to complete ethanol fermentation.

For the wild-type control, most of these genes were initially induced but failed to be expressed continuously after 6 h under the ethanol challenges (Ma and Liu (submitted)). As a result, the wild type strain was unable to establish a viable culture. Therefore, sufficient NAD(P)H supply and a well maintained redox balance appeared to be essential for active cell metabolism and inhibitor- and ethanol-tolerance functions in yeast (Liu et al. 2009; Ma and Liu (submitted)). It is important to distinguish a tolerance response from a stress response of yeast to the ethanol challenge. The commonly observed stress response is not always a tolerance characteristic and does not necessarily lead to a tolerance withstanding the ethanol stress. Only the tolerance response and active functional characteristics that are able to result in successful ethanol fermentation are accountable for a strain performance against ethanol stress.

Signal transduction pathways

Understanding signal transduction and transcriptional response is necessary in order to dissect the mechanisms of ethanol tolerance and aid tolerant strain development. The first signal transduction pathway implicated in Msn2p/Msn4p localization is cAMP-protein kinase pathway (PKA). The cAMP-PKA pathway has been studied extensively (Colombo et al. 1998; Estruch 2000; Costa and Moradas-Ferreira 2001; Hohmann 2002; Müller et al. 2003; Nikolaou et al. 2009) and a summary update is presented (Fig. 3). Adenylate cyclase encoded by *CYR1* can be activated by the G protein-coupled receptor system Gpr1p-Gpa2p or Ras1p/2p. Under normal physiological conditions, glucose triggers G protein-coupled receptor system to activate adenylate cyclase for higher levels of cAMP generation, which activates PKA and inhibits general stress response regulated by Msn2p/Msn4p, and specific stress responses regulated by Yap1p and Skn7p (reviewed in Estruch 2000; Costa and Moradas-Ferreira 2001). However, under stress conditions, cAMP levels are down-regulated through the Ras-cAMP pathways. Members of the HSP70 protein family, such as Ssa1p, can interact with Cdc25p to activate Ras1p/2p and cAMP-PKA pathway (Geymonat et al. 1998). These HSPs are recruited to participate in refolding proteins that were denatured by ethanol and to maintain the protein native conformation for normal functions (Young et al. 2004; McClellan et al. 2007; Gong et al. 2009). This process reduces interactions of HSPs with Cdc25p and thereafter slows down activities involved in the cAMP-PKA pathway (Thevelein and de Winde 1999). Down-regulation of the cAMP-PKA pathway releases inhibition to stress responses and causes transfer of Msn2p/Msn4p, as well as Yap1p, from the cytosol to the nucleus to trigger the stress responses.

Intracellular acidification is also a possible signal to trigger signal transduction pathways for ethanol tolerance response. Intracellular acidification and its interactions with Ira1/2p can also negatively affect the function of Ras proteins to trigger stress responses (Thevelein 1991). High culture ethanol concentrations cause osmotic stress to yeast cells. The HOG-MAPK signal transduction pathway may also be involved in ethanol stress response (Alexandre et al. 2001). It is simply because Msn2p/Msn4p can be activated by the HOG-MAPK pathway (Fig. 3). In addition, up-regulated expressions of *GPD1*, *HOR2*, *HOR7*, *DAK1*, and *GRE3* by ethanol were all dependent on the HOG-MAPK signal transduction pathways (Rep et al. 2000). Ethanol has also been shown to induce ROS, thus oxidative stress is also possibly imposed on cells indirectly (Du and Takagi 2007). The subsequently generated ROS may damage almost all cellular components, including DNA, protein, lipid, and membranes (Costa and Moradas-Ferreira 2001). Consequently, many genes associated with oxidative stress are up-regulated under the Yap1p transcription factor control under ethanol stress conditions.

An alcohol-sensitive ring/PHD finger 1 protein (Asr1p) of *S. cerevisiae* was thought as a signal transduction signal element but was documented later to have no significant affect on ethanol tolerance (Betz et al. 2004; Izawa et al. 2006). Recently, Asr1p has been demonstrated to be a RING finger ubiquitin-ligase that binds directly to RNA polymerase II via the carboxyl-terminal domain of the largest subunit and is associated with inactivation of polymerase function (Daulny et al. 2008).

Transcription factors and stress response element

Many genes up-regulated by ethanol challenges were found sharing transcription factor binding motifs of Msn2p/

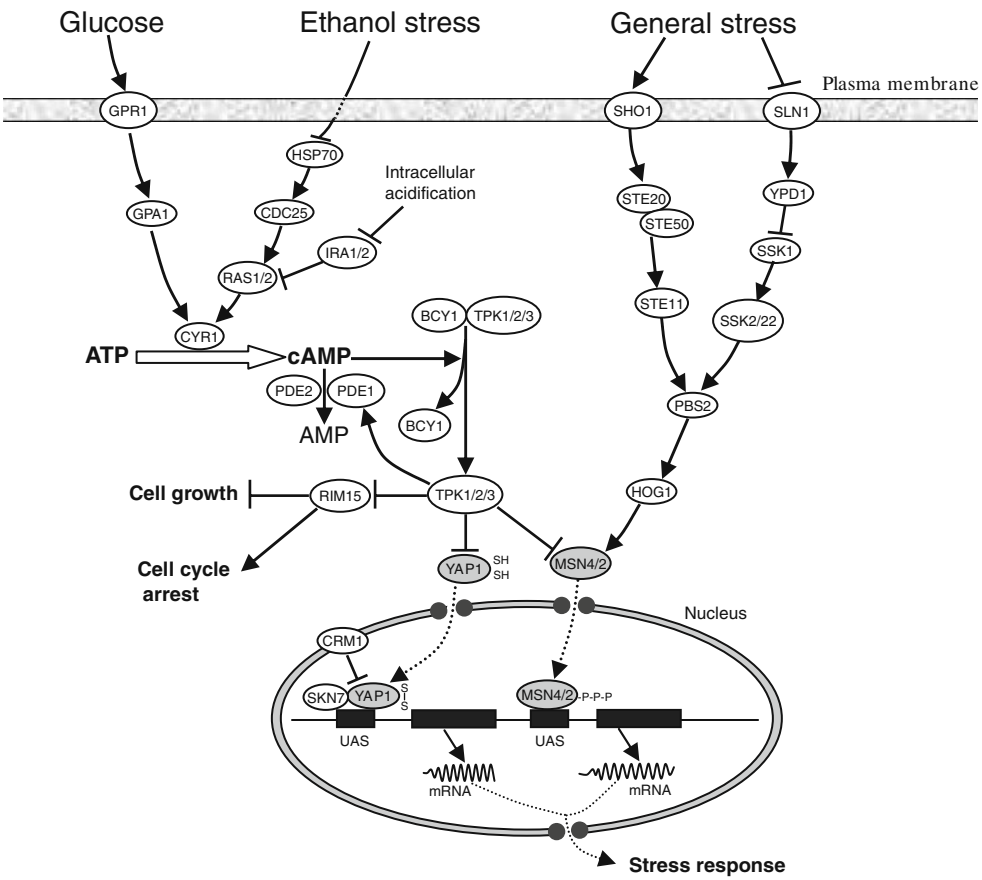

Fig. 3 A schematic diagram showing signal transduction pathways involved in ethanol-tolerant stress response in *S. cerevisiae* with transcription factors shaded. *A line ended with an arrow* indicates a positive interaction, and *with a bar*, as a negative interaction. This figure is adapted based on Colombo et al. (1998), Estruch (2000), Costa and Moradas-Ferreira (2001), Hohmann (2002), Müller et al. (2003), and Nikolaou et al. (2009)

Msn4p, Yap1p, and Hsf1p in their upstream sequence (Teixeira et al. 2006; Ma and Liu (submitted)). For nearly 200 up-regulated genes reported under the ethanol stress, a total of 58 genes were co-regulated by these three transcription factors (Table 1, Fig. 4). A recent study on quantitative transcription dynamics of an ethanol-tolerant yeast revealed Msn4p as a key regulator for ethanol tolerance and production under stress (Ma and Liu (submitted)). Transcription factors Msn2p, Msn4p, Yap1p, and Hsf1p are likely key regulators worthy of further investigation for ethanol tolerance in yeast.

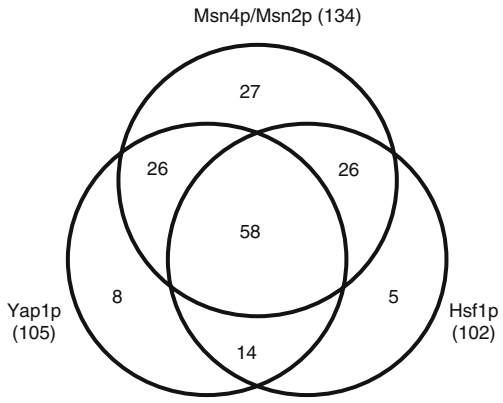

Fig. 4 A Venn diagram showing potential regulatory relationships for genes up-regulated under ethanol stress sharing common protein binding sites of transcription factors Msn4p, Msn2p, Yap1p, and Hsf1p based on data from Ogawa et al (2000), Alexandre et al (2001), Chandler et al (2004), Marks et al (2008), Dinh et al (2009), and Ma and Liu (submitted)

Yeasts are capable of adapting and developing general responses to adverse environmental conditions including ethanol, heat shock, chemical toxic, osmotic stress, oxidative, and numerous other factors. Under ethanol stress, activation of transcription factors Msn2p and Msn4p by signal transduction pathways can trigger a so-called environmental-stress response (Fig. 3). It has been demonstrated that Msn2p and Msn4p induces gene expression via a stress response element (STRE) and triggers transcriptional response of downstream genes (Marchler et al. 1993; Schuller et al. 1994). STRE has a core pentameric *cis*-acting sequence CCCCT and is located in promoter regions of the induced genes (Marchler et al. 1993; Kobayashi and McEntee 1993). STRE functions in both orientations of CCCCT or AGGGG and was found in most ethanol-induced genes. A recent comparative study showed that *MSN2* and *MSN4* actually displayed different expression responses over time for the tolerant yeast under ethanol stress (Ma and Liu (submitted)). This raises a concern that mechanism in tolerance to ethanol and regulatory functions

between the two genes may not be identical. Based on the unique transcription dynamics of *MSN4* in tolerant yeast, Msn4p is likely a key regulator accountable for the ethanol tolerance beyond an ethanol stress response. Further studies on functional mechanisms of Msn4p in regulatory interactions and networks are needed.

Although a single copy of STRE is sufficient to activate expression of a reporter gene by a stress factor, two or more copies of this sequence induced a greater expression of stress response genes (Kobayashi and McEntee 1993). However, more copy numbers of STRE do not necessarily lead to greater expression than low copy numbers do. For example, *HSP26* was highly induced by an ethanol challenge (Ma and Liu (submitted)) but has only four STRE elements in its upstream sequence, far less than the seven STRE elements found for *HSP12*, a less expressed HSP gene. Furthermore, the presence of a STRE-like element in a promoter region does not imply the functionality of this sequence either. A comprehensive evaluation should be undertaken to relate a STRE-containing gene in the context of the STRE position, its copy numbers and other associated transcription factors.

A double gene deletion *msn2msn4*-mutant showed hypersensitivity to environmental stress conditions including higher ethanol concentrations (Moskvina et al. 1998). Genetic disruption of *MSN2* decreases expression of the reporter gene *lacZ* under the control of STRE from the promoter region of *HSP12* implicating Msn2p as a necessary regulator for the STRE-controlled gene expression (Watanabe et al. 2007). Overexpression of *MSN2* under the control of constitutive promoters such as *TDH3* has been shown to increase tolerance to ethanol (Cardona et al. 2007; Watanabe et al. 2009). However, cell growth is usually affected by either activation of Msn2p or its overexpression under the control of a constitutive promoter (Martínez-Pastor et al. 1996; Durchschlag et al. 2004). Ethanol-tolerant strain K11 and Y-50316 in fact also showed slower growth than their parental strains (Hara et al. 1976b; Ma and Liu (submitted)). The difference between the tolerant strains and the wild type is their capability to grow continuously under higher ethanol concentrations (Dinh et al. 2008; Ma and Liu (submitted)). Cell growth inhibited by ethanol is partly mediated via the cAMP-PKA signal transduction pathway to negatively regulate *RIM15* gene expression and influenced by Yak1p kinase through transcription factor Msn2p (Hartley et al. 1994; Reinders et al. 1998; Herman 2002). Recent discovery of induced expression of Msn2p under *SPI1* gene promoter control appears interesting (Cardona et al. 2007). *SPI1* is a regulon of Msn2p having three STRE sequences in its promoter region (Puig and Pérez-Ortín 2000). When a promoter of *MSN2* was replaced by that of *SPI1* allowing self-induction of *MSN2* expression, the harboring strain showed improved resistance to multiple stress conditions and nearly normal growth.

As discussed above, oxidative stress is indirectly imposed on cells under ethanol stress (Costa et al. 1997; Du and Takagi 2007). Yap1p is a basic leucine zipper transcription factor required for oxidative stress tolerance that binds to several motifs including TTASTMA and TTAGTMAGC (Fernandes et al. 1997; Harbison et al. 2004; Nguyên et al. 2001). These transcription factor binding motifs exist in the upstream sequence of many ethanol-induced genes. Functions of Yap1p in ethanol tolerance are not well documented to date. Another transcription factor Hsf1p could be a potential candidate in regulation of ethanol tolerance since it is also associated with many ethanol-induced genes overlapping with Msn2p and Msn4p. It has been reported to regulate transcription of hundreds of targets including genes involved in protein folding, detoxification, energy generation, carbohydrate metabolism, and cell wall organization (Hahn et al. 2004; Eastmond and Nelson 2006). Hsf1p recognizes and binds to conserved heat shock elements consisting of inverted NGAAN repeats in promoter regions of its target genes (Bonner et al. 1994; Harbison et al. 2004). In unstressed cells, Hsf1p is constitutively phosphorylated, but under certain stresses, it becomes hyperphosphorylated and adopts to activate transcription of target genes (Hashikawa et al. 2006; Lee et al. 2000). An *HSF1*-deletion mutant showed repressed expression for its target genes usually induced by ethanol (Takemori et al. 2006).

Evolutionary engineering

Since yeast tolerance, including tolerance to ethanol, is associated with several hundreds of genes involving multiple quantitative trait loci, a well programmed integration upon the stress at genome level is required (Liu 2006; Hu et al. 2007; Liu et al. 2008; 2009; Ma and Liu (submitted)). Evolutionary engineering is a common approach used to improve microbial performance using methods of various types of mutagenesis and recombination or gene shuffling and selection of desirable phenotypes (Sauer 2001; Nevoigt 2008). For improved ethanol tolerance, several methods of evolutionary engineering have been studied. Stepwise adaptation was reported to select ethanol-tolerant mutants producing a higher concentration of ethanol (Hara et al. 1976a; Cakar et al. 2005; Dinh et al. 2008). Since ethanol tolerance is also associated with general stress tolerance, freeze–thaw treatment appeared to enhance tolerant levels to ethanol and multiple stress conditions (Cakar et al. 2005; Wei et al. 2007). Using combined stepwise adaptation and the freeze–thaw method, an ethanol-tolerant yeast Y-50316 was obtained that

maintained fermentation inhibitor-tolerance as well (Ma and Liu (submitted)). As mentioned earlier, a slower growth rate that commonly accompanies ethanol tolerance needs to be addressed. A recently developed global transcription machinery engineering (gTME) method showed interesting results. Components of general factor RNA Pol II transcription factor D including TATA-binding protein (SPT15) and 14 other associated factors (TAFs) are thought to be the main DNA binding proteins regulating promoter specificity in yeast (reviewed by Hahn 2004). Using the gTME method, mutation libraries were generated from either *SPT15* encoding TATA-binding protein or one of the TATA-binding protein-associated factors *TAF25* by random mutagenesis and expressed in the standard laboratory strain BY4741 (Alper et al. 2006). Mutant spt15-300 with three amino acid substitutions of Phe177 to Ser, Tyr 195 to His, and Lys218 to Arg exhibited a phenotype of improved ethanol tolerance. However, when the expression of the *SPT15-300* allele was examined for industrial significance, it did not show improved ethanol tolerance for several yeasts including *S. cerevisiae* (Baerends et al. 2009).

Conclusions and future prospects

It is clear that ethanol tolerance in yeast is not an isolated event but involves interplay of many genes in complex networks at the genome level. Many genes induced by ethanol are associated with and overlapping with genes involving other environmental factors such as osmotic, heat shocking, chemical toxic, and oxidative stress. As presented in our prototype model (Fig. 1), heat shock protein genes and transcription factor Msn4p and Msn2p appeared to be important for yeast to cope with the ethanol stress. Multiple functions of a tolerance candidate gene at multiple loci cannot be overlooked in dissection of ethanol tolerance. Mechanisms of ethanol tolerance can only be better understood when a comprehensive view of pathways and network events are considered as functional dynamics. A transient gene expression response to ethanol challenge does not necessarily imply a functional characteristic of ethanol tolerance in yeast. Our improved understanding on the mechanisms of ethanol tolerance in yeast uncovered new aspects in dynamic studies of tolerance over time. For example, snap shots of expression responses by tolerant and susceptible yeasts to ethanol may be similar, particularly at the earlier stage. However, the tolerant yeast demonstrated distinct dynamics of gene expression that lead to establishment of viable cultures reflecting a tolerance phenotype to ethanol. On the other hand, wild-type strains are unable to survive regardless of how many genes were induced in the earlier responses. Yeast tolerance is a processing procedure of numerous interacting living events over time. It becomes apparent that comparative studies on time-course based studies reveal more relevant information to dissect mechanisms of ethanol tolerance. With many useful tools in genomics, molecular biology and computational biology, it will be interesting to see more systematic approaches and models, addressing more detailed dynamics of pathway interplay and network interactions. Every valid piece of knowledge will be required to resolve the puzzle of ethanol tolerance.

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