รายงานวิจัยฉบับสมบูรณ์

โครงการ: Genome-wide analysis of alcohol resistance mechanism in *Saccharomyces cerevisiae*

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ชื่อโครงการ : การศึกษากลไกการต้านอัลกอฮอล์ในระดับ genome-wide ในยีสต์ *Saccharomyces cerevisiae*

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ในระหว่างกระบวนการหมักเซลล์ยีสต์จะพบความเครียดชนิดต่างๆ เช่น ความเครียดจากความเข้มข้นของอัลกอฮอล์ที่สูงขึ้น แรงดันออสโมติกที่สูงขึ้น และการเปลี่ยนแปลงของอุณหภูมิ เป็นต้น ซึ่งกลไกในการตอบสนองต่อความเครียดต่างๆ เหล่านี้คาดว่าอาจมีบางส่วนที่ใช้กลไกเดียวกัน ในการศึกษานี้ได้นำชุดยีสต์ *S. cerevisiae* สายพันธุ์กลายซึ่งขาดยีน nonessential แต่ละยีนมาคัดเลือกสายพันธุ์กลายที่มีความไวต่ออัลกอฮอล์ (เอทานอล เมทานอล และ 1-โพรพานอล) ความร้อน แรงดันออสโมติก และความเครียดชนิดออกซิเดทีฟเพื่อศึกษายีนที่จำเป็นในการทนทานต่อความเครียดแต่ละชนิด พบว่าสายพันธุ์กลายจำนวน 95, 54, 125, 178, 42 และ 30 สายพันธุ์มีความความไวต่อเอทานอล เมทานอล 1-โพรพานอล ความร้อน แรงดันออสโมติก และความเครียดชนิดออกซิเดทีฟตามลำดับ เมื่อนำยีนที่ขาดหายไปในมาจัดกลุ่มตามหน้าที่การทำงานและวิเคราะห์การทับซ้อนของยีนที่จำเป็นต่อความเครียดแต่ละชนิด พบว่ายีนจำนวนมากที่เกี่ยวข้องกับการทำงานของ H^+-ATPase ที่แวคิลโอล (V-ATPase) การสร้างโครงสร้างของเซลล์และความแข็งแรงของผนังเซลล์จำเป็นในการทนทานต่ออัลกอฮอล์ทุกชนิดที่ศึกษา จึงคาดว่ากลไกเหล่านี้เป็นกลไกสำคัญในการปกป้องเซลล์จากอัลกอฮอล์ แม้ว่าการทำงานของไมโทคอนเดรียจะพบว่าจำเป็นในการทนทานต่อความเครียดเกือบทั้งหมดที่ศึกษา แต่กลับพบว่าไม่เกี่ยวข้องกับการทนทานต่อความร้อน ซึ่งยีนที่คัดเลือกได้จากการศึกษานี้จะเป็นประโยชน์อย่างมากในการศึกษากลไกการตอบสนองต่อความเครียดต่างๆ ของเซลล์ในระดับโมเลกุลต่อไป

คำหลัก : genome-wide อัลกอฮอล์ ความร้อน *Saccharomyces cerevisiae*

Abstract

Project Code : MRG4980127

Project Title : Genome-wide analysis of alcohol resistance mechanism in *Saccharomyces cerevisiae*

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During fermentation, yeast cells are exposed to a number of stresses, such as high alcohol, high osmotic pressure and temperature fluctuation, and overlap of some mechanisms involved in the response to these stresses has been suggested. To identify the genes required for tolerance to alcohol (ethanol, methanol, and 1-propanol), heat, osmotic stress, and oxidative stress, genome-wide screening was performed using a set of yeast deletion mutants. Our screens identified 95, 54, 125, 178, 42, and 30 deletion mutants sensitive to ethanol, methanol, 1-propanol, heat, NaCl, and H_2O_2, respectively. These deleted genes were then classified based on their cellular functions, and cross-sensitivities between each stress were determined. A large number of genes involved in vacuolar H^+-ATPase (V-ATPase) function, cytoskeleton biogenesis, and cell wall integrity were required for tolerance to all kinds of alcohol, suggesting their protective role against alcohol stress. Although mitochondrial function is likely involved in tolerance to several stresses, it was found to be less important for thermotolerance. The genes identified in this study should be helpful for future research into the molecular mechanisms of stress response.

Keywords : genome-wide, alcohol, heat stress, *Saccharomyces cerevisiae*

EXECUTIVE SUMMARY

The yeast *Saccharomyces cerevisiae* has been widely used in several industries such as in alcoholic fermentation. During fermentation, yeast cells are exposed to a number of stresses including high alcohol, high osmotic pressure, and temperature fluctuation. All these stresses reduce growth rates and affect cellular viability; it is therefore essential that yeast cells possess appropriate mechanisms to respond and adapt rapidly to each stress condition for survival.

It has been suggested that some mechanisms required for ethanol tolerance may also be responsible for thermotolerance. In addition to the overlap between ethanol and heat stress responses, the ethanol stress response may also overlap with the high osmotic stress response because high ethanol concentration may cause high osmotic stress to yeast cells. Moreover, ethanol stress has been found to induce antioxidant defences and ethanol toxicity is correlated with the production of ROS in the mitochondria. However, the mechanism of tolerance to each stress and the overlap of genes required for each stress have not been studied. We therefore analyzed the Research Genetics collection of yeast deletion mutants to identify genes required for tolerance to alcohol (ethanol, methanol, and 1-propanol), heat, osmotic stress, and oxidative stress. Genes required for each stress tolerance were classified by the function of gene products based on the database of the Munich Information Center for Protein Sequences (MIPS) and the *Saccharomyces* Genome Database (SGD).

The appropriate conditions for evaluation of sensitivity to ethanol, methanol, 1-propanol, NaCl, H_2O_2 and heat which caused approximately equal levels of stress to yeast cells were determined by the serial-dilution spot test. We found that the minimal inhibitory concentrations and temperature were 10% ethanol, 16% methanol, 7% 1-propanol, 1 M NaCl, 5 mM H_2O_2, and 37 ^{o}C. Because the log P_{ow} values of methanol, ethanol, and 1-propanol are −0.74, −0.30, and 0.25, respectively, it is clear that alcohols

with high log P_{ow} values are more toxic than those with low log P_{ow} values. Our results are consistent with previous observations showing that the toxicity of alcohols is correlated with their lipophilicity

Our screens identified 95, 54, 125 deletion mutants sensitive to ethanol, methanol, 1-propanol, respectively, suggesting a correlation between the number of genes required for tolerance to alcohols and the lipophilicity, namely the toxicity, of alcohols. The functional classes that contained a large number of genes required for tolerance to all three kinds of alcohol were the vacuolar function class, especially V-ATPase function, and cytoskeleton biogenesis and organization class. These results suggest that V-ATPase, and the cytoskeleton are important for tolerance to alcohol stress. On the other hand, the functional class containing the large number of genes specifically required for ethanol tolerance was mitochondrial function, while those for 1-propanol were vacuolar function, transcription, and protein fate. Furthermore, a number of genes involved in ubiquitin-dependent proteolysis were found to be required for 1-propanol tolerance.

178 deletion mutants were found to be sensitive to heat stress. The classification results showed that these genes were frequently classified into transcription and biogenesis of cellular components classes. These results suggest that a defect in general transcription activity and in the cytoskeleton will lead to reduced tolerance to stress, such as heat stress. For high osmotic stress, 42 mutants exhibiting apparently reduced growth were identified. In addition to genes that were reported to be sensitive to high osmolarity, a large number of genes involved in vacuolar protein sorting and ATP production, suggesting that these functions are required for tolerance to high osmotic stress. For oxidative stress, 30 mutants were sensitive to H_2O_2. In addition to genes involved in oxidative stress response, the functional class that contained the highest number of genes was the mitochondrial function class, suggesting that functional mitochondria are important for tolerance to oxidative stress. This result is consistent

with previous data suggesting that a defect in mitochondria leads to more ROS production and oxidative damage.

Our screens found that *SOD1* gene encoding superoxide dismutase and *TPS2* gene encoding trehalose-6-phosphate phosphatase was required for tolerance to almost all stresses. These findings suggest that superoxide dismutase and trehalose, which are known to be involved in protecting cells from several stresses, are commonly essential for stress tolerance. Furthermore, our results revealed that approximately 40% of genes required for tolerance to alcohols were also required for thermotolerance. These findings emphasize the idea that the defect in fundamental structure, such as in the cell wall and actin cytoskeleton, may result in increased sensitivity to stress. However, only few genes essential for tolerance to high osmolarity and oxidative stress were found to be required for tolerance to other stresses. Although mitochondrial function is likely involved in tolerance to several stresses, it was found to be less important for thermotolerance. In contrast, the functional classes containing a large number of heat-specific genes were the transcription, and cell cycle and DNA processing classes, suggesting that the proper regulation of general transcription activity and the cell cycle are more important for thermotolerance. The genes identified in this study should be helpful for future research into the molecular mechanisms of stress response.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been widely used in several industries such as in alcoholic fermentation. During fermentation, yeast cells are exposed to a number of stresses including high alcohol, high osmotic pressure, and temperature fluctuation. All these stresses reduce growth rates and affect cellular viability; it is therefore essential that yeast cells possess appropriate mechanisms to respond and adapt rapidly to each stress condition for survival. These mechanisms involve the rapid activation and synthesis of protective molecules, and the activation of signal transduction pathways (Estruch 2000).

Ethanol is a major stress factor for yeast during fermentation processes. This compound is known to inhibit yeast growth and viability, and to affect several transport systems such as glucose and amino acid transports (Arneborg et al. 1995, Ibeas and Jimenez 1997, Ingram and Buttke 1984, Leao and van Uden 1982, Salmon et al. 1993). Ethanol also changes plasma membrane fluidity (Alexandre et al. 1994a, Jones and Greenfield 1987, Lloyd et al. 1993) and activates plasma membrane H^+-ATPase (Alexandre et al. 1994b, Monteiro and Sa-Correia 1998, Rosa and Sa-Correia 1991), leading to the idea that the main target of ethanol stress is the plasma membrane. In addition, it has been shown that other straight-chain alcohols, such as 1-propanol and 1-butanol, affect cell growth and cell morphology in a similar manner to ethanol, and the toxicity of these alcohols is strongly correlated with their lipophilicity (Fujita et al. 2004). Heat stress is known to result in accumulation of misfolding and aggregation of proteins. When cells are exposed to high temperature, the expression of a set of heat shock proteins (HSPs) is induced to protect cells from damage by either refolding or degrading misfolded proteins (Morano et al. 1998). Osmotic stress may occur at the beginning of the alcoholic fermentation process due to a high concentration of sugars. This stress causes shrinkage of cells because of loss of intracellular water, which leads to loss of

turgor (Hohmann 1997). The response of yeast to osmotic stress is regulated by the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway (Chen and Thorner 2007, Gustin et al. 1998, Hohmann 2002). Oxidative stress is caused by reactive oxygen species (ROS) generated during respiration and oxidation of nutrients. High aeration and aerobic metabolism during the fermentation process are supposed to lead to oxidative stress (Costa and Moradas-Ferreira 2001, Ikner and Shiozaki 2005). The ROS damage almost all cell components including DNA, protein, and lipid membrane (Costa and Moradas-Ferreira 2001). In response to oxidative stress, yeast cells sense and induce expression of various anti-oxidant genes through the specific signal transduction pathway (Costa and Moradas-Ferreira 2001, Ikner and Shiozaki 2005).

It has been suggested that heat stress and ethanol stress cause similar effects to plasma membrane and induce some identical stress response (Piper 1995). Global gene expression also indicates that most of the genes coding for HSPs and genes involved in trehalose synthesis were up-regulated during ethanol stress, similar to that described for heat treatment (Alexandre 2001, Singer and Lindquist 1998). The increase of ethanol concentration during fermentation may cause osmotic stress to yeast cells; it is therefore possible that the ethanol stress response overlaps with the osmotic stress response in some mechanisms. However, it has been shown that ethanol does not stimulate the HOG pathway responsible for signaling in response to osmotic stress (Tamas et al. 2000). It has been reported that ethanol stress induces antioxidant defences, such as mitochondrial superoxide dismutase (MnSOD), and that ethanol toxicity is correlated with the production of ROS in the mitochondria (Costa et al. 1993, Costa et al. 1997).

In an effort to explore the mechanisms of stress tolerance, we analyzed the Research Genetics collection of yeast deletion mutants to identify genes required for tolerance to alcohol (ethanol, methanol, and 1-propanol), heat, osmotic stress, and

oxidative stress. Our results suggest that V-ATPase and the cytoskeleton are essential for tolerance to all kinds of alcohol, and that the RNA polymerase II mediator complex is specifically important for thermotolerance. However, no significant overlap of genes required for osmotic stress and oxidative stress with those required for other stresses were observed.

MATERIALS AND METHODS

Strains and media

The 4,828 nonessential haploid *S. cerevisiae* deletion strains generated by the Saccharomyces Genome Deletion Project (Winzeler et al. 1999) were obtained from Research Genetics/Invitrogen. These strains are on a BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) background (Brachmann et al. 1998). YPD medium (1% yeast extract, 2% peptone, 2% glucose), with the optional addition of 200 mg/l geneticin (Sigma-Aldrich), was used as a rich medium for yeast growth.

Screening for stress-sensitive deletion mutants

To determine appropriate concentrations of ethanol, methanol, 1-propanol, sodium chloride (NaCl), and hydrogen peroxide (H_2O_2) for screening, which cause approximately equal levels of stress to yeast cells, the parent-type BY4742 strain grown in YPD medium to an OD_{600} of 1.0 were diluted in 10^{-1} steps. Aliquots (3 μl) of 10-fold serial dilution were spotted onto YPD plates containing ethanol, methanol, 1-propanol, NaCl and H_2O_2 of various concentrations as described below, and incubated at 30 °C for 3 days. For determination of the appropriate high temperature, 10-fold serial dilutions were spotted onto YPD plates and incubated at different temperatures up to 39 °C. High temperature and concentrations of ethanol, methanol, 1-propanol, NaCl and H_2O_2 that did not inhibit growth and caused approximately equal levels of stress were used to screen for stress-sensitive deletion mutants.

The deletion mutants were transferred from thawed 96-well microtiter plate stocks to YPD plates supplemented with 200 mg/liter of geneticin using a 48-pin replicator. After incubation at 30 °C for 2 days, strains were stamped onto YPD plates and YPD plates containing 10% ethanol, 16% methanol, 7% propanol, 1M NaCl and 5mM H_2O_2 and grown at 30 °C for a further 3 days. The screening for heat-sensitive deletion mutants was performed at 37 °C. All experiments for the identification of stress-sensitive mutants were performed in three replicate plates.

Classification of genes by gene function

Genes required for each stress tolerance were classified by the function of gene products based on the database of the Munich Information Center for Protein Sequences (MIPS) and the *Saccharomyces* Genome Database (SGD).

RESULTS

Determination of appropriate concentrations of ethanol, methanol, 1-propanol, NaCl, and H_2O_2, and appropriate high temperature for screening

The appropriate conditions for evaluation of sensitivity to ethanol, methanol, 1-propanol, NaCl, H_2O_2 and heat which caused approximately equal levels of stress to yeast cells were determined by the serial-dilution spot test. 10-fold serial dilutions of the wild-type strain BY4742 were spotted onto YPD plates containing 8%, 10%, or 12% ethanol; 10%, 12%, 14%, 16%, or 18% methanol; 6%, 8%, 10%, or 12% 1-propanol; 0.8 M, 0.9 M, 1 M, or 1.2 M NaCl; and 4 mM, 4.5 mM, or 5 mM H_2O_2. For determination of the appropriate high temperature for analysis, the serial-dilution spot test was performed at 35, 37 or 39 °C. We found that the minimal inhibitory concentrations and temperature were 10% ethanol, 16% methanol, 7% 1-propanol, 1 M NaCl, 5 mM H_2O_2, and 37 °C (Fig. 1). Thus, we used YPD plates containing 10% ethanol, 16% methanol, 7% 1-propanol, 1 M NaCl, 5 mM H_2O_2, and an incubation temperature of 37 °C to screen

for stress-sensitive deletion mutants.

Identification of deletion mutants sensitive to ethanol, methanol, and 1-propanol

Ethanol is one of the main stress factors during fermentation. To identify genes required for growth under ethanol stress, we systematically screened a collection of 4,828 haploid yeast deletion mutants of nonessential genes grown onto YPD plates containing 10% ethanol for ethanol-sensitive deletion mutants. We identified 95 mutants showing significant sensitivity to ethanol (Fig. 2). The genes deleted in these mutants were defined as essential genes required for ethanol tolerance, and were classified into functional categories using the MIPS and the SGD database (Table 1). Our results revealed that the functional class that contained the highest number of genes was the vacuolar function class. This class contained several genes involved in vacuolar H^+-ATPase (V-ATPase) function, viz. *VMA2*, *VMA5*, *VMA6*, *VMA7, VMA8, VMA10*, *VMA11*, and *VMA21*, suggesting the protective role of V-ATPase against ethanol stress.

It has been shown that alcohols with high log P_{ow} values, the logarithm of the octanol and water partition coefficient of a solvent, are more toxic to yeast cells than those with low log P_{ow} values, supporting the idea that the toxicity of lipophilic alcohols is correlated with their lipophilicity (Fujita et al. 2004, Weber and de Bont 1996). To assess the cross-sensitivities of ethanol (log P_{ow}, −0.30) to other straight-chain alcohols, we examined methanol (log P_{ow}, −0.74) and 1-propanol (log P_{ow}, 0.25), which have sizes of molecules similar to ethanol but have lower and higher log P_{ow} values than ethanol, respectively (Fujita et al. 2004). We screened the same set of haploid deletion mutants in 16% methanol and 7% 1-propanol to identify the genes required for tolerance to these alcohols. Our screens identified 54 and 125 mutants exhibiting clear sensitivity to methanol and 1-propanol, respectively (Fig. 2). Based on the classification, the functional class that contained a large number of genes required for methanol and 1-propanol tolerance was the vacuolar function class (Tables 1, 2 and 3). Interestingly,

most of the identified genes in this functional class are involved in V-ATPase function as was observed in the screening for ethanol tolerance (Tables 1, 2 and 3), suggesting the important role of V-ATPase in protecting yeast cells from alcohol stress. Of the mutants identified, only 21 mutants were sensitive to all alcohols, whereas 45, 16, and 68 mutants exhibited specific sensitivity to ethanol, methanol, and 1-propanol, respectively (Fig. 2). The genes required for tolerance to all three kinds of alcohol included *VMA6* and *VMA8* genes encoding the structural components of V-ATPase, *GIM5*, *SLA2* and *SPC72* genes involved in cytoskeleton biogenesis and organization, and *OCH1* and *MNN11* genes encoding mannosyltransferases involved in maintaining cell wall integrity (Tables 1, 2 and 3). These results suggest that V-ATPase, the cytoskeleton, and cell wall integrity are important for tolerance to alcohol stress. On the other hand, the functional class containing the large number of genes specifically required for ethanol tolerance was mitochondrial function, such as *MDM12*, *MDM30*, *MMM1*, and *TOM5* genes involved in mitochondrial organization and protein import into mitochondria (Table 1). In our screens, the metabolism, vacuolar function, transcription, and protein fate classes were found to contain a high number of genes specifically essential for 1-propanol tolerance (Table 3). These findings raise the possibility that, in addition to the common mechanism essential for tolerance to all alcohols such as V-ATPase and the cytoskeleton, yeast cells may also possess other specific mechanisms for tolerance to ethanol and 1-propanol. However, only few genes were found to be specifically necessary for methanol tolerance and no predominant functional class was observed (Table 2). Interestingly, we observed that a number of genes involved in ubiquitin-dependent proteolysis were required for 1-propanol tolerance, such as *UBI4* encoding ubiquitin, *UBP5*, *DOA1*, *DOA4*, and *TOM1* encoding ubiquitin-specific enzymes, *STP22* and *VPS25* involved in sorting ubiquitinated proteins. These results suggest that ubiquitin-dependent proteolysis is important for 1-propanol tolerance.

Identification of deletion mutants sensitive to heat stress

To identify genes required for growth under heat stress, we screened a set of deletion mutants grown at 37 °C, and identified 178 mutants showing a clear reduced growth (Table 4). The classification results showed that these genes were frequently classified into transcription and biogenesis of cellular components classes (Table 4). The transcription class contained several genes involved in general transcription activities, such as *CSE2*, *GAL11*, *MED1*, *MED2*, *PAF1*, *PGD1*, and *SRB2*, coding for subunits of the RNA polymerase II mediator complex, and genes involved in RNA processing, such as *CCR4*, *ISY1*, *LEA1*, *LSM6*, and *REF2* (Table 4). The biogenesis of cellular components class contained several genes involved in the actin cytoskeleton, such as *BNI1*, *CAP2*, *END3*, *SAC6*, *SLA1*, and *SLA2*, and genes involved in cell wall integrity, such as *GAS1*, *GET2*, *LDB7*, and *SMI1* (Table 4). According to our results, it is conceivable that a defect in general transcription activity and in the cytoskeleton will lead to reduced tolerance to stress, such as heat stress.

Identification of deletion mutants sensitive to high osmolarity

To identify mutants sensitive to high osmolarity, a set of deletion mutants was grown onto YPD plates containing 1 M NaCl, and 42 mutants exhibiting apparently reduced growth were identified (Table 5). As expected, genes that were reported to be sensitive to high osmolarity, such as *HOG1* and *PBS2* (Hohmann 2002), were identified in this screen. Our classification results revealed that the functional classes containing a large number of genes required for growth under high osmotic stress were the vacuolar function and mitochondrial function classes (Table 5). The vacuolar function class included *VPS1*, *VPS3*, *VPS16*, and *VPS45* involved in vacuolar protein sorting, while the mitochondrial function contained *OXA1*, *PET100*, and *SCO1* involved in oxidative phosphorylation responsible for ATP production (Table 5). These results suggested that vacuolar protein transport and energy metabolism are required for tolerance to high

osmotic stress.

Identification of deletion mutants sensitive to oxidative stress

To identify mutants sensitive to oxidative stress, we screened a set of deletion mutants in YPD plates supplemented with 5 mM H_2O_2, and found 30 mutants showing clear sensitivity (Table 6). As expected, the mutants lacking genes involved in oxidative stress response, such as *YAP1*, *SOD1*, *HYR1*, and *SKN7*, were sensitive to H_2O_2. Based on the classification, the functional class that contained the highest number of genes required for tolerance to oxidative stress was the mitochondrial function class (Table 6). This class contained *MRPL7*, *RSM19*, *MST1*, and *NAM2* involved in mitochondrial protein synthesis, and *OXA1* and *PET100* involved in oxidative phosphorylation machinery, suggesting that functional mitochondria are important for tolerance to oxidative stress (Table 6). This result is consistent with previous data suggesting that a defect in mitochondria leads to more ROS production and oxidative damage (Bandy and Davison 1990, Bonawitz et al. 2006).

The overlaps of genes required for each stress

Both heat and ethanol stresses have been shown to cause similar changes to plasma membrane lipid compositions and decrease plasma membrane H^+-ATPase activity (Alexandre et al. 1994b, Piper 1995), and most genes coding for HSPs were up-regulated by ethanol stress (Alexandre et al. 2001). These data suggest that some mechanisms required for ethanol tolerance may also be responsible for thermotolerance. In addition to the overlap between ethanol and heat stress responses, the ethanol stress response may also overlap with the high osmotic stress response because high ethanol concentration may cause high osmotic stress to yeast cells. Moreover, ethanol stress has been found to induce antioxidant defences and ethanol toxicity is correlated with the production of ROS in the mitochondria (Costa et al. 1993, Costa et al. 1997). To assess the overlaps of response to alcohol stress (i.e. ethanol, methanol and 1-propanol)

with heat, osmotic and oxidative stresses, we compared the genes required for tolerance to alcohol stresses with those necessary for growth during heat, osmotic or oxidative stress. We found that *SOD1* gene encoding superoxide dismutase was required for tolerance to all stresses examined, and *TPS2* gene encoding trehalose-6-phosphate phosphatase was required for tolerance to several stresses, except for methanol (Tables 1, 2, 3 and 4). These findings suggest that superoxide dismutase and trehalose, which are known to be involved in protecting cells from several stresses (Elbein et al. 2003, Liochev and Fridovich 2005, Singer and Lindquist 1998), are commonly essential for stress tolerance, at least for all stresses examined in this study. Furthermore, our results revealed that 35 of 95 (37%) (Fig. 3a), 23 of 54 (43%), and 58 of 125 (44%) (Fig. 3b) genes required for tolerance to ethanol, methanol, and 1-propanol, respectively, were also required for thermotolerance. Of 19 genes required for growth during methanol, 1-propanol, and heat stresses, 12 genes were also important for tolerance to ethanol (Fig. 3). Among these, *MNN11* and *OCH1* genes encoding mannosyltransferase involved in cell wall integrity, and several genes involved in the actin cytoskeleton, such as *BEM4*, *SAC6*, *SHE4*, *SLA2*, *SPC72*, and *YKE2*, were found to be required for both alcohol tolerance and thermotolerance (Tables 1, 2, 3 and 4). These findings emphasize the idea that the defect in fundamental structure, such as in the cell wall and actin cytoskeleton, may result in increased sensitivity to stress. Of the 95 genes essential for ethanol tolerance, only 12 and 7 genes were also indispensable for growth during high osmolarity and oxidative stress, respectively (Fig. 4). Moreover, only few genes essential for tolerance to high osmolarity and oxidative stress were found to be required for tolerance to other stresses (Tables 2, 3, 4, 5, and 6). In addition to *SOD1* and *TPS2*, *AFT1* encoding transcription factor involved in iron homeostasis, *SIT4* encoding protein phosphatase involved in mitotic cell cycle regulation, and *SRV2* encoding adenylate cyclase-associated protein were required for cotolerance to ethanol, high osmolarity, and

oxidative stress (Tables 1, 5 and 6).

The number of genes specifically required for tolerance to ethanol, methanol, 1-propanol, heat, osmotic stress, and oxidative stress was 31 (33%), 13 (24%), 37 (30%), 102 (57%), 17 (40%), and 14 (48%), respectively (Tables 1, 2, 3, 4, 5 and 6). The specific genes for ethanol and oxidative stresses were frequently classified into the mitochondrial function class. It has been suggested that mitochondria are a target for ethanol damage (Aguilera and Benitez 1985), and defects in mitochondria may result in faulty respiration and lead to more ROS production (Bandy and Davison 1990). These data thus emphasize the important role of mitochondria against ethanol and oxidative stresses. Interestingly, although a large number of genes in the mitochondrial function class were essential for tolerance not only to ethanol and oxidative stresses but also methanol, 1-propanol, and osmotic stresses, only 3 genes were found to be required for thermotolerance (Table 4). These results suggest that mitochondrial function is important for tolerance to several stresses, except for heat stress. In contrast, the functional classes containing a large number of heat-specific genes were the transcription, and cell cycle and DNA processing classes. Several heat-specific genes in the transcription class encode subunits of the RNA polymerase II mediator complex required for regulation of general transcription, viz. *CSE2*, *GAL11*, *MED1*, *MED2*, *PAF1*, *PGD1*, and *SRB2*, while most of those in the cell cycle and DNA processing class are involved in control of the cell cycle, viz. *CDC26*, *CTF18*, *KAR3*, *SIC1*, *SPO7*, and *UME1*. These data suggest that the proper regulation of general transcription activity and the cell cycle are more important for thermotolerance. However, no functional class was found to contain a significant number of specific genes for methanol, 1-propanol, osmotic stress, and oxidative stress. Interestingly, our results revealed that *HOG1* encoding MAP kinase (MAPK) and *PBS2* encoding MAP kinase kinase (MAPKK) involved in the HOG pathway were required for growth during osmotic stress, but not the other stresses used

in this study, suggesting that this pathway is mainly essential for signaling in response to osmotic stress. Moreover, we found that *YAP1* and *SKN7* encoding transcription factors involved in the oxidative stress response were specifically essential for oxidative stress tolerance, suggesting that these two transcription factors are mainly required to regulate the expression of oxidative stress-responsive genes.

DISCUSSION

During fermentation, yeast cells are exposed to several stresses, such as high alcohol, high osmotic pressure and temperature fluctuation, and the overlap of some mechanisms involved in the response to these stresses has been suggested. In this study, we systematically screened a set of yeast deletion mutants for identification of genes required for tolerance to alcohol, heat, osmotic stress, and oxidative stress. We observed that the minimal inhibitory concentrations which cause approximately equal levels of stress to yeast cells were 16% methanol (log P_{ow}, −0.74), 10% ethanol (log P_{ow}, −0.30), and 7% 1-propanol (log P_{ow}, 0.25) (Fig. 1), indicating that alcohols with high log P_{ow} values are more toxic than those with low log P_{ow} values. Our results are consistent with previous observations showing that the toxicity of alcohols is correlated with their lipophilicity (Fujita et al. 2004, Fujita et al. 2006). Our screens revealed that 54, 95, and 125 mutants were sensitive to methanol, ethanol, and 1-propanol, respectively (Fig. 2), suggesting a correlation between the number of genes required for tolerance to alcohols and the lipophilicity, namely the toxicity, of alcohols. Recently, a set of homozygous diploid yeast deletion mutants has been screened for the genes required for growth on 12.5% ethanol (Fujita et al. 2006). However, only 31 of 95 (33%) genes important for tolerance to ethanol in our screen were identified in the screen of Fujita et al. This discrepancy may be due to differences in the ploidy level of the strains used or the concentration dependence of mechanisms of ethanol sensitivity.

Although the vacuolar function class contained a high number of genes required for tolerance to all stresses examined, genes involved in V-ATPase function are mainly found to be essential for alcohol tolerance (Tables 1, 2 and 3). V-ATPase functions in translocating protons across the vacuolar membrane through the hydrolysis of ATP, thereby playing an important role in the maintenance of intracellular pH homeostasis through vacuolar acidification (Forgac 1998, Inoue et al. 2005), whereas, ethanol is known to increase membrane permeability to protons, which then cause increased proton influx and intracellular acidification (Cartwright et al. 1987, Rosa and Sa-Correia 1996). Our results therefore suggest that V-ATPase is required for recovery from intracellular acidification caused by alcohol. In contrast, our screens revealed that the genes involved in vacuolar protein sorting, such as *VPS1*, *VPS4*, *VPS16*, and *VPS45*, are not only required for tolerance to alcohol but also essential for tolerance to heat, osmotic stress, and oxidative stress. Many *vps* mutants have been shown to exhibit sensitivity to calcofluor white, which is used to examine impairment in the cell wall (Koning et al. 2002), suggesting that the maintenance of cell wall integrity is important for tolerance to several stresses. Taken together, our results strongly suggest that the biogenesis and functions of vacuoles are important for stress tolerance.

According to our data, it is likely that, in addition to the common mechanism essential for tolerance to several stresses, yeast cells may also possess specific mechanisms required for tolerance to only one type of stress. For instance, all genes encoding components of the Mdm10p-Mdm12p-Mmm1p complex localized to the mitochondrial outer membrane were required for tolerance to ethanol stress, especially *MDM12* and *MMM1* that are ethanol-specific genes. This complex is required to link mitochondrial membranes and DNA to the cytoskeleton-based segregation machinery, and for the maintenance of mitochondrial DNA and morphology (Boldogh et al. 2003), suggesting that defects in mitochondrial morphology and inheritance result in increased

sensitivity to ethanol stress. In addition to this complex, 3 of 4 genes encoding components of the THO complex, viz. *HPR1*, *RLR1*, and *THP2*, were required for tolerance to only ethanol stress. The THO complex is required for efficient transcription elongation and mRNA export (Jimeno et al. 2002, Rondon et al. 2003). Therefore, it is possible that the Mdm10p-Mdm12p-Mmm1p complex and the THO complex are the targets for ethanol stress. Moreover, we identified *YAP1*, *SKN7*, and *HYR1*, as genes required only for tolerance to oxidative stress. *YAP1* and *SKN7* encode transcription factors involved in regulating the transcription of anti-oxidant genes in response to oxidative stress (Kuge et al. 1997, Raitt et al. 2000), while *HYR1* encodes thiol peroxidase that functions in sensing intracellular hydroperoxide levels and transducing a redox signal to Yap1p (Delaunay et al. 2002). Our data further confirm that Yap1p and Hyr1p play a cooperative role in signal transduction in response to oxidative stress. Since the regulation of Skn7p activity has not been clearly determined, further studies, such as identification of the regulator of Skn7p, are necessary.

HSPs are known to play crucial roles in protein folding and preventing protein aggregation and their expressions are up-regulated under several stress conditions, especially heat stress (Bukau et al. 2006, Morano et al. 1998). None of the mutants of HSPs, however, were sensitive to heat stress and other stresses in our screens, possibly due to redundant functions of HSPs. Moreover, the transcription factors Msn2p and Msn4p are involved in regulating the expression of genes in response to stresses, including heat stress, osmotic stress, and oxidative stress (Causton et al. 2001, Gasch et al. 2000, Martinez-Pastor et al. 1996). Neither the *msn2* nor the *msn4* mutants exhibited sensitivity to all stresses examined. These results were consistent with the previous observation showing that the *msn2msn4* double mutants, but not the single mutants of *msn2* and *msn4*, were sensitive to several stresses, such as heat shock, osmotic stress, and oxidative stress (Martinez-Pastor et al. 1996), and further support the

idea that Msn2p and Msn4p may play a cooperative role in regulating the expression of stress-responsive genes.

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Table 1 Classification of genes whose deletions result in ethanol sensitivity

Vacuolar function (16)	*PEP3*, *PEP5*, *STP22*, *VAM3*, *VMA2*, *VMA5*, *VMA6*, *VMA7*, *VMA8*, *VMA10*, *VMA11*, *VMA21*, *VPS4*, *VPS20*, *VPS24*, *VPS34*
Transcription (13)	*AFT1*, *BDF1*, *DEP1*, *HPR1*, *NOT5*, *RLR1*, *RPA12*, *RPB4*, *SIN3*, *SPT10*, *SPT20*, *TAF14*, *THP2*
Mitochondrial function (12)	*ATP11*, *HMI1*, *MDJ1*, *MDM10*, *MDM12*, *MDM30*, *MMM1*, *MSD1*, *SCO1*, *TIM13*, *TOM5*, *YDJ1*
Biogenesis of cellular components (9)	*BEM4*, *BUD16*, *BUD27*, *NUP120*, *SAC6*, *SHE4*, *SLA2*, *SPC72*, *TPD3*
Metabolism (9)	*ARD1*, *ERG24*, *ERG28*, *LIP5*, *PFK2*, *PHO80*, *SUR4*, *TCO89*, *TYR1*
Protein fate (8)	*DOA4*, *GIM4*, *GIM5*, *MNN11*, *OCH1*, *PAC10*, *PPM1*, *YKE2*
Cellular transport (7)	*DRS2*, *FPS1*, *SEC28*, *SEC66*, *SNF7*, *SPF1*, *TRS33*
Signal transduction (6)	*AKR1*, *SIT4*, *SLG1*, *SNF1*, *SRV2*, *STE3*
Cell cycle and DNA processing (4)	*BUB1*, *MFT1*, *RSC2*, *UME6*
Cell rescue, defense and virulence (4)	*RAD17*, *RAD27*, *SOD1*, *TPS2*
Protein synthesis (1)	*RPS26B*
Unknown function (6)	*YER038w-A*, *YER077c*, *YKL037w*, *YLR144c*, *YLR204w*, *YLR322w*

Table 2 Classification of genes whose deletions result in methanol sensitivity

Vacuolar function (11)	*CHC1*, *FEN1*, *VAM3*, *VMA6*, *VMA8*, *VMA21*, *VPH2*, *VPS1*, *VPS4*, *VPS16*, *VPS25*
Transcription (7)	*AFT1*, *MOT2*, *RPA12*, *RPB4*, *RPB9*, *SNF6*, *SPT7*
Mitochondrial function (4)	*FMP13*, *MRPL19*, *YDJ1*, *YME1*
Biogenesis of cellular components (5)	*SLA1*, *SLA2*, *SPC72*, *SVL3*, *TPD3*
Metabolism (2)	*ERG24*, *PFK2*
Protein fate (5)	*ALG3*, *ANP1*, *GIM5*, *MNN11*, *OCH1*
Cellular transport (2)	*CCS1*, *SEC66*
Signal transduction (2)	*SRV2*, *STE3*
Cell cycle and DNA processing (4)	*BUB1*, *CIK1*, *RSC2*, *SWM1*
Cell rescue, defense and virulence (5)	*AFT2*, *FLC1*, *FYV6*, *RAD27*, *SOD1*
Protein synthesis (1)	*RPL23A*
Unknown function (7)	*YJL131c*, *YLR144c*, *YLR315w*, *YLR322w*, *YNL254c*, *YOR331c*, *YOR364w*

Table 3 Classification of genes whose deletions result in 1-propanol sensitivity

Vacuolar function (16)	*ARP5*, *BRO1*, *FAB1*, *PEP12*, *PIB2*, *PPA1*, *STP22*, *TFP1*, *VMA2*, *VMA6*, *VMA7*, *VMA8*, *VMA22*, *VPS16*, *VPS25*, *VPS45*
Transcription (15)	*AFT1*, *CTK3*, *ELP2*, *ELP3*, *KEM1*, *NSR1*, *REF2*, *RPA12*, *RPA49*, *RPB4*, *RPB9*, *RPD3*, *SIN3*, *SNF6*, *STO1*
Mitochondrial function (9)	*CAF17*, *FMP13*, *MDM10*, *MRPL11*, *PET494*, *SHY1*, *TIM13*, *YDJ1*, *YME1*
Biogenesis of cellular components (18)	*ALF1*, *BEM4*, *BUD27*, *CDC10*, *ECM33*, *EMI1*, *END3*, *GAS1*, *LDB7*, *NUM1*, *NUP120*, *PEX6*, *RVS161*, *SHE4*, *SLA1*, *SLA2*, *SPC72*, *TPM1*
Metabolism (12)	*ALD6*, *ARO1*, *ARO2*, *CHO2*, *DDI3*, *ERG2*, *ERG24*, *IRC15*, *LIP5*, *PFK2*, *TYR1*, *YGR012w*
Protein fate (12)	*ANP1*, *DOA1*, *DOA4*, *GIM5*, *MNN11*, *NBP2*, *OCH1*, *PRE9*, *TOM1*, *UBI4*, *UBP5*, *YKE2*
Cellular transport (6)	*CCS1*, *FPS1*, *HXT4*, *SEC28*, *SEC66*, *SNF7*
Signal transduction (11)	*AKR1*, *GIS3*, *GPB2*, *KCS1*, *MCK1*, *RAM1*, *SIT4*, *SLT2*, *SNF1*, *SRV2*, *STE3*
Cell cycle and DNA processing (5)	*BUB1*, *CIK1*, *MFT1*, *RSC2*, *SCP160*
Cell rescue, defense and virulence (7)	*GLR1*, *KTI12*, *PIM1*, *PTC1*, *SOD1*, *SSD1*, *TPS2*
Protein synthesis (2)	*RPL23A*, *RPS26B*
Unknown function (12)	*YBL031w*, *YDR149c*, *YGR026w*, *YHR029c*, *YKL037w*, *YKR035c*, *YLR021w*, *YLR144c*, *YLR322w*, *YOR331c*, *YOR364w*, *YPL073c*

Table 4 Classification of genes whose deletions result in heat sensitivity

Vacuolar function (16)	*BRO1*, *CUP5*, *DID4*, *PEP7*, *PEP12*, *PPA1*, *VID21*, *VMA7*, *VPS4*, *VPS16*, *VPS25*, *VPS30*, *VPS45*, *VPS66*, *VPS69*, *VPS74*
Transcription (30)	*BDF1*, *CAF130*, *CCR4*, *CSE2*, *DEP1*, *ELP4*, *GAL11*, *ISY1*, *LEA1*, *LEO1*, *LSM6*, *MED1*, *MED2*, *MSS11*, *NGL1*, *PAF1*, *PGD1*, *RAI1*, *REF2*, *RNY1*, *RPA12*, *RPB9*, *RRN10*, *SRB2*, *STO1*, *SWI3*, *SWI6*, *TRM1*, *TRM8*, *YGR272c*
Mitochondrial function (3)	*MDM10*, *YDJ1*, *YME1*
Biogenesis of cellular components (24)	*BEM4*, *BNI1*, *BUD16*, *BUD31*, *CAP2*, *CDC10*, *CIN8*, *ELM1*, *END3*, *GAS1*, *GET2*, *LDB7*, *NUP120*, *PEX6*, *PRM8*, *RVS161*, *SAC6*, *SHE4*, *SHS1*, *SLA1*, *SLA2*, *SLM1*, *SMI1*, *SPC72*
Metabolism (16)	*ADH1*, *ADK1*, *ARD1*, *ARG82*, *ARO1*, *CYS4*, *DDI3*, *GCR2*, *OPI3*, *PDC1*, *PFK2*, *PRS3*, *RTS1*, *SUR4*, *TRP3*, *YEL047c*
Protein fate (11)	*BUL1*, *DOA1*, *LAS21*, *MNN11*, *NAT3*, *NBP2*, *OCH1*, *OST4*, *PPM1*, *PRE9*, *YKE2*
Cellular transport (9)	*ARN1*, *COG1*, *FPS1*, *GTR1*, *NUP84*, *NUP133*, *SEC66*, *TRS33*, *YPT6*
Signal transduction (10)	*AKR1*, *BCK1*, *GIS3*, *GPB2*, *IRA2*, *KCS1*, *MCK1*, *RAM1*, *SIT4*, *SLG1*
Cell cycle and DNA processing (14)	*BUB1*, *CDC26*, *CIK1*, *CTF18*, *HOF1*, *IWR1*, *KAR3*, *MFT1*, *PAT1*, *SIC1*, *SPO7*, *SWM1*, *UME1*, *YKU80*
Cell rescue, defense and virulence (14)	*ASF1*, *CPR6*, *FYV6*, *KTI12*, *LSP1*, *PIM1*, *PTC1*, *RAD27*, *RAD52*, *SOD1*, *SSD1*, *RVS167*, *TPS2*, *YKU70*
Protein synthesis (5)	*ASC1*, *FMT1*, *RPL34B*, *RPS28A*, *RSA1*
Unknown function (26)	*YBR063c*, *YDL032w*, *YDL151c*, *YDL204w*, *YEL044w*, *YEL045c*, *YEL059w*, *YER084w*, *YER087w*, *YGL072c*, *YHR100c*, *YJL077c*, *YJR129c*, *YKR035c*, *YLR021w*, *YLR114c*, *YLR125w*, *YLR204w*, *YLR261c*, *YLR322w*, *YNL215w*, *YNR068c*, *YOL019w*, *YOR141c*, *YOR364w*, *YPL144w*

Table 5 Classification of genes whose deletions result in NaCl sensitivity

Vacuolar function (7)	*TFP1*, *VMA8*, *VPS1*, *VPS3*, *VPS16*, *VPS45*, *YCK3*
Transcription (3)	*AFT1*, *CTK1*, *CTK3*
Mitochondrial function (7)	*ATP2*, *MRPL51*, *OXA1*, *PET100*, *PET494*, *SCO1*, *TOM5*
Biogenesis of cellular components (4)	*RVS161*, *SLA1*, *TPD3*, *VRP1*
Metabolism (3)	*AYR1*, *DCS1*, *THR4*
Protein fate (1)	*DOA4*
Cellular transport (2)	*SWF1*, *TRK1*
Signal transduction (5)	*MCK1*, *PBS2*, *SIT4*, *SNF1*, *SRV2*
Cell rescue, defense and virulence (6)	*HOG1*, *RVS167*, *SOD1*, *SSQ1*, *TPS2*, *ZUO1*
Unknown function (4)	*YDR506c*, *YER077c*, *YIL039w*, *YKR041w*

Table 6 Classification of genes whose deletions result in H_2O_2 sensitivity

Vacuolar function (4)	*CUP5*, *PEP*, *VPS1*, *VPS27*
Transcription (2)	*AFT1*, *RPB4*
Mitochondrial function (8)	*FMC1*, *MRP7*, *MST1*, *NAM2*, *OXA1*, *PET100*, *RSM19*, *YDJ1*
Biogenesis of cellular components (1)	*VRP1*
Protein fate (1)	*UBP6*
Signal transduction (4)	*MCK1*, *SIT4*, *SKN7*, *SRV2*
Cell rescue, defense and virulence (4)	*HYR1*, *SOD1*, *TPS2*, *YAP1*
Protein synthesis (1)	*PMT5*
Unknown function (5)	*YAR029w*, *YGL168w*, *YLR346c*, *YNR042w*, *YOR331c*

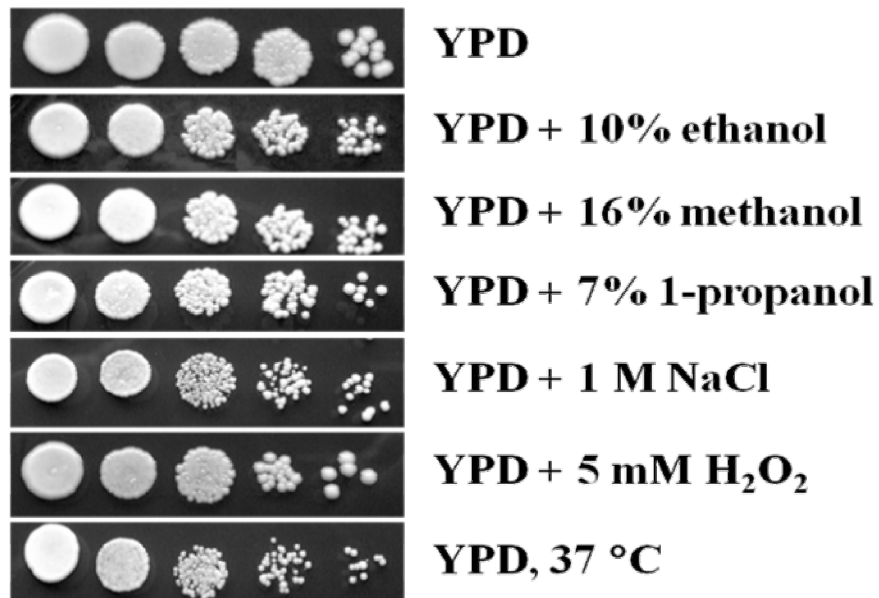

Fig. 1 Growth of wild-type strain (BY4742) during stress conditions. Aliquots (3 μl) of 10-fold serial dilutions of the BY4742 strain were spotted onto YPD plates or YPD plates supplemented with 10% ethanol, 16% methanol, 7% 1-propanol, 1 M NaCl, or 5 mM H_2O_2, and grown at 30 °C for 3 days. For heat stress, the serial dilutions were spotted onto YPD plates and incubated at 37 °C for 3 days.

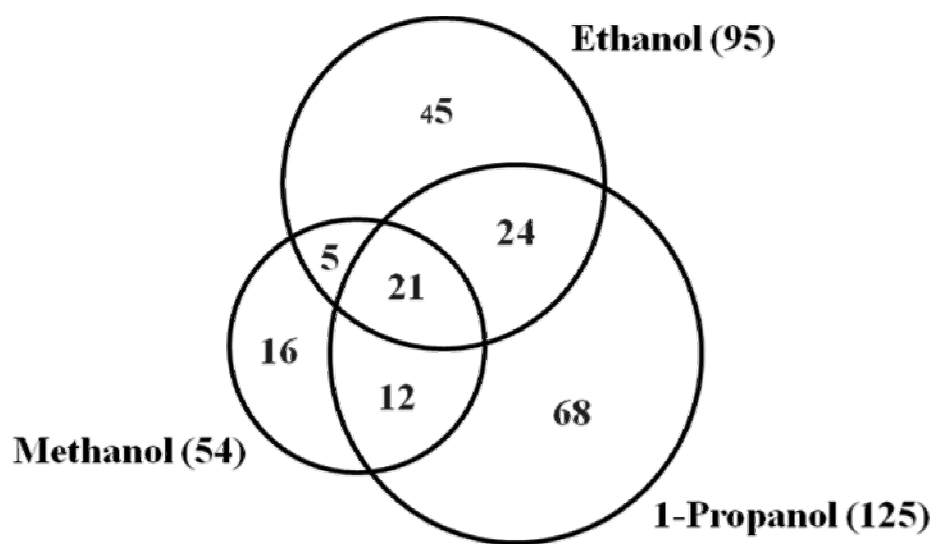

Fig. 2 Diagrammatic representation of the distribution of deletion mutants sensitive to methanol, ethanol, and 1-propanol.

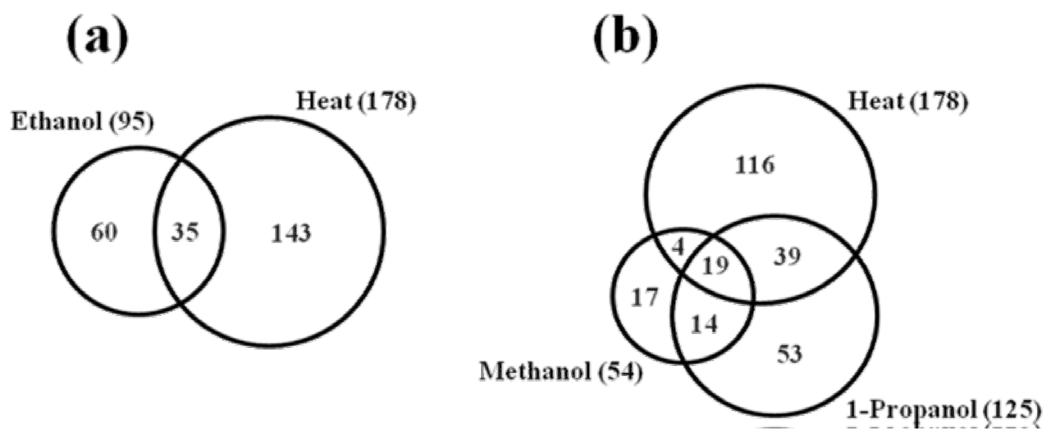

Fig. 3 Diagrammatic representations of (**a**) the distribution of deletion mutants sensitive to ethanol and heat, and (**b**) that of deletion mutants sensitive to methanol, 1-propanol, and heat.

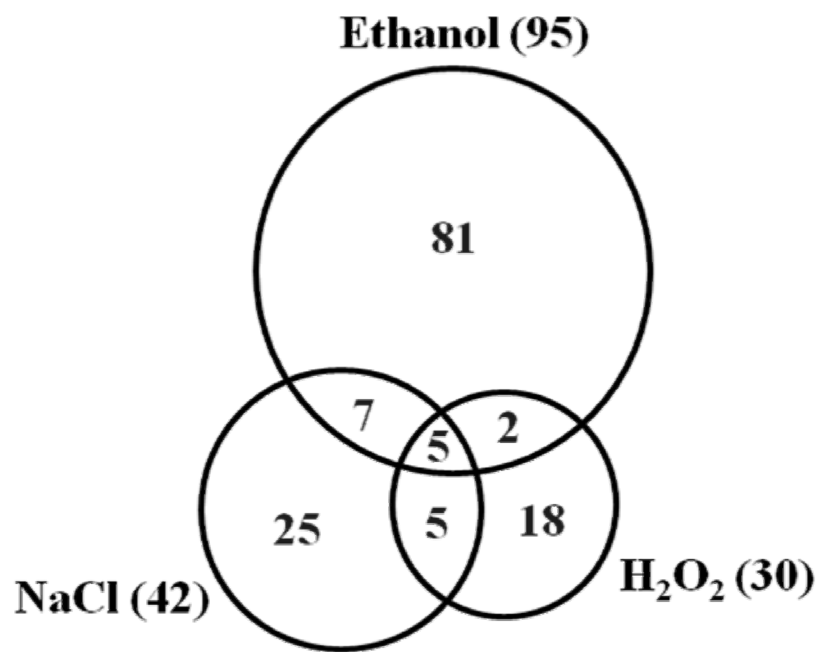

Fig. 4 Diagrammatic representation of the distribution of deletion mutants sensitive to ethanol, NaCl, and H_2O_2.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   1.1 **Choowong Auesukaree**, Alisa Damnernsawad, Maleeya Kruatrachue, Prayad Pokethitiyook, Chuenchit Boonchird, Yoshinobu Kaneko, and Satoshi Harashima. Genome-wide identification of genes involved in tolerance to different environmental stresses in *Saccharomyces cerevisiae*. (under submission, 2008)

2. การนำเสนอผลงานในที่ประชุมวิชาการ

   2.1 **Choowong Auesukaree**, Alisa Damnernsawad, Maleeya Kruatrachue, Prayad Pokethitiyook, Chuenchit Boonchird, Yoshinobu Kaneko, and Satoshi Harashima. Genome-wide Identification of Genes Required for Ethanol Tolerance in *Saccharomyces cerevisiae*. การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. Pattaya, Thailand. October 11-13, 2007.