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ORIGINAL PAPER

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Genome-wide expression analysis of yeast response during exposure to 4°C

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Abstract Adaptation to temperature fluctuation is essential for the survival of all living organisms. Although extensive research has been done on heat and cold shock responses, there have been no reports on global responses to cold shock below 10°C or near-freezing. We examined the genome-wide expression in *Saccharomyces cerevisiae*, following exposure to 4°C. Hierarchical cluster analysis showed that the gene expression profile following 4°C exposure from 6 to 48 h was different from that at continuous 4°C culture. Under 4°C exposure, the genes involved in trehalose and glycogen synthesis were induced, suggesting that biosynthesis and accumulation of those reserve carbohydrates might be necessary for cold tolerance and energy preservation. The observed increased expression of phospholipids, mannoproteins, and cold shock proteins (e.g., *TIP1*) is consistent with membrane maintenance and increased permeability of the cell wall at 4°C. The induction of heat shock proteins and glutathione at 4°C may be required for revitalization of enzyme activity, and for detoxification of active oxygen species, respectively. The genes with these functions may provide the ability of cold tolerance and adaptation to yeast cells.

Keywords Yeast · DNA microarray · Hierarchical cluster · Gene expression profiling · Cold shock protein · Trehalose · Glycogen · Membrane maintenance

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Introduction

Ambient temperature is an important environmental factor for all living organisms and various adaptive biochemical and physiological properties have evolved in response to temperature variations. For instance, while increased expression of heat shock proteins (HSPs) is the hallmark of high temperature stress, under low temperature conditions, permeability of the cellular membrane decreases, protein synthesis is diminished, protein folding slows, and enzyme activities decrease. Regulation of gene expression and responses in low temperature have been studied extensively in some organisms (Guy 1999; Thieringer et al. 1998).

When an exponentially growing culture of *Escherichia coli* is shifted from 37°C to low temperature, such

as 15°C, a number of cold shock proteins are greatly induced. The CspA, cold shock protein A, is a major cold shock protein in the Csp family of *E. coli*, and some Csps bind single-stranded nucleic acids and function as RNA chaperones favoring efficient translation of mRNA at low temperature (Fang et al. 1998; Phadtare et al. 2004). In *Bacillus* and *Cyanobacteria*, enhanced synthesis of desaturase and catalyzed unsaturation of membrane fatty acids has been reported for cells exposed to low temperature conditions (Aguilar et al. 1999; Inaba et al. 2003; Suzuki et al. 2001). However, in eukaryotes, no proteins homologous to bacterial Csp have been isolated, but genes encoding cold shock-inducible proteins such as *TIP1* (temperature inducible protein), *TIR1*, and *TIR2* are induced in budding yeast, *Saccharomyces cerevisiae* (Kondo and Inouye 1991). The predicted protein products of these genes have sequences that are consistent with extensive *O*-mannosylation as well as glycosyl phosphatidyl inositol (GPI) membrane anchoring (Kowalski et al. 1995). *NSR1*, a cold-induced gene, is involved in ribosomal RNA processing and ribosomal synthesis at low temperature (Kondo and Inouye 1992). *OLE1* is the *Δ*9-fatty acid desaturase gene, and its expression is affected by the presence of unsaturated fatty acids (UFAs) in the medium (Nakagawa et al. 2001). The genetic responses in *S. cerevisiae* during transfer from 30°C to 10°C have recently been explored by genome-wide expression analysis using a yeast cDNA microarray (Sahara et al. 2002).

However, there have been no studies on cold responses in yeast at temperatures below 10°C. Many organisms may be exposed to near-freezing or freezing temperatures in natural environments, such as day–night change or over-wintering. Microorganisms used in research, such as yeast and *E. coli*, may be exposed to temperatures in the range of 0–4°C during experimental preparation. There have been some investigations of cellular responses to 4°C or freezing in a few organisms (Kandror and Goldberg 1997; Odani et al. 2003; Zarka et al. 2003). In studies of the adaptation of *E. coli* or *S. cerevisiae* to near-freezing temperatures, it is surprising to note that the production of trehalose is actually more critical for survival than major cold shock proteins (Kandror et al. 2002). Because we are part of a culture collection consortium involved in the preservation of many types of organisms, we are interested in low temperature adaptation, especially at temperatures below 10°C, typically used for preservation. In this paper, we report global yeast gene-expression profiles during 4°C exposure using cDNA microarray. We show by hierarchical cluster analysis that gene expression at 6–48 h after 4°C exposure is different from those of 35°C or 4°C maintained cultures. Induction of genes involved in energy preservation, phospholipid synthesis, and cell rescue, such as HSPs or detoxification enzymes was observed after 4°C exposure. Here, we reveal the cellular responses of yeast that are adapted to 4°C or able to maintain viability at 4°C.

Materials and methods

Strain and growth conditions

Saccharomyces cerevisiae S288c (*MATα SUC2 mal mel gal2 CUP1*) was grown in YPD medium (1% bacto yeast extract, 2% polypeptone, 2% glucose) at 25°C and shaken at 120–130 rpm. Yeast cultures were grown to a mid-log phase ($A_{660} = 0.5$), and then transferred to a water bath kept at 0°C and cooled to 4°C. The cold cells were aerobically cultured at 4°C at 120–130 rpm. Cells were harvested at 6, 12, 24, and 48 h after the cold shock by centrifugation at 2,300 *g* for 3 min at 4°C. After harvesting, the cells were flash-frozen and stored at −80°C until RNA preparation. Yeast cultures grown to late log phase ($A_{660} = 1.0$) at 25°C were used as control cells.

RNA extraction and mRNA purification

Total RNA was extracted from cells by the hot-phenol method (Murata et al. 2002). The poly A mRNA was purified from total RNA with an Oligotex-dT30 mRNA purification kit (Takara, Shiga, Japan).

Probe preparation and labeling

Probe preparation was performed using the CyScribe first strand cDNA labeling kit (Amersham Biosciences, Buckinghamshire, UK). Briefly, 1–2 μg of mRNA were mixed with oligo(dT) primer, random primer, and dNTP nucleotide mix, and the reverse transcription was performed in the presence of 100 U CyScribe reverse transcriptase (Amersham Biosciences), 50 μM Cy3 or Cy5. In all experiments, Cy3-UTP (Amersham Biosciences) and Cy5-UTP (Amersham Biosciences) were used to label the control ($A_{660} = 1.0$) and experimental samples (cold-shocked samples), respectively. Each probe was purified using AutoSeq G-50 column (Amersham Biosciences).

Microarray hybridization and scanning

We used *S. cerevisiae* cDNA microarrays (DNA Chip Research, Kanagawa, Japan) containing targets for about 5,952 genes. We mixed 20 μl of the labeled cDNA, 40 μl of DEPC-treated water, and 60 μl 2× hybridization solution (CyScribe first strand cDNA labeling kit). After 3 min at 90°C, the mixture was placed onto the array and hybridized overnight at 65°C. After hybridization, the microarray was washed and dried. Microarrays were scanned with a Scanarray 4000 scanner, and, the GenePix 4000 software was used to locate spots on the chip. To correct for the variations (poor quality of some spots, background subtraction, and the different labeling efficiency between Cy3 and Cy5 on the same DNA microarray), cut off was done at an average background

value of +2SD. A Lowess curve was fit to the log-intensity vs log-ratio plot. A total of 20% of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than ten, then ten was used instead. These transcriptional data can be seen at the web site (http://kasumi.nibh.jp/~iwahashi/). DNA microarray experiments were performed using at least five independent culture experiments. The genes with hybridization ratios greater than 2.0 and less than 0.5 in at least three of five experiments were considered to be, respectively, up-regulated and down-regulated during 4°C exposure. The ratio values used in tables are the average expression profiles from the five independent experiments.

Hierarchical cluster analysis

In this study, we used the GeneSpring (version 6.1) hierarchical cluster algorithm (GeneSpring, Slicon Genetics, USA). The similarity between transcriptional data after exposure to 4°C was measured by standard correlation with adequate parameters (separation ratio: 1.0; minimum distance: 0.001). We referred to the Munich Information Center for Protein Sequences (MIPS) functional categories and other databases to determine functional relationships among genes in each cluster using the GeneSpring analysis software.

Results

Growth at 4°C

Saccharomyces cerevisiae, S288c was grown in YPD at 25°C until exponential phase, and was cultured for 48 h after 4°C exposure. Figure 1 shows a growth curve for S288c after 4°C exposure. When yeast cells are cultured continuously at 4°C for several months, the doubling time is about 50 h (Homma et al. 2003). Yeast cells in our experiments grew slowly with a doubling time of 50 h or more after transfer to 4°C (Fig. 1). Thus, yeast cells grew slowly or extremely slowly at 4°C, yet retained the ability to form colonies (data not shown). Because exposure to 4°C is growth limiting for yeast cells, we examined the influences of low temperature at 4°C on global gene expression. Yeast cells were harvested by centrifugation at 6, 12, 24, and 48 h after transfer from 25°C to 4°C, and total RNAs were prepared from harvested cells as described in section "Material and methods".

Global gene expression analysis of response at 4°C by cDNA microarray

The mRNA purified from control cultures at 25°C was labeled with the fluorescence dye, Cy3-UDP. The mRNA purified from yeast cells collected at each time point after 4°C exposure were labeled with the fluorescence dye, Cy5-UDP. The resulting cDNA probes were mixed and hybridized competitively onto a yeast cDNA microarray.

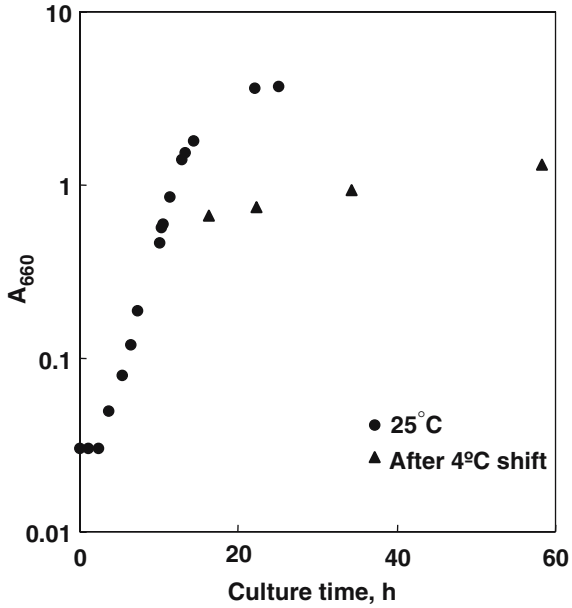

Fig. 1 The growth of yeast cells at 25°C (*closed circles*) and after 4°C exposure (*closed triangles*). Control yeast cells were kept in culture at 25°C until they reached stationary phase (15 h) (*closed circles*). When the density of yeast cells was 0.5 at A_{660}, the culture was transferred to a water bath kept at 0°C, and was cooled down from 25 to 4°C (*closed triangles*). The cold cells were kept in culture at 4°C up to 48 h

We first examined the variation of the procedure and the preparation among samples. The mRNA purified from total RNA from the same source was divided in two, and labeled with Cy3 or Cy5, respectively, and used in competitive hybridization on a cDNA microarray. As because the ratio values (Cy5/Cy3) of signal intensities for ≥ 95% of all genes on cDNA microarray were within twofold, we defined significant gene expression change as greater than or equal to twofold ratio (data not shown). Moreover, as two or more independent microarray experiments are required for the acquisition of reliable data from DNA microarray experiment and for the selection on induced genes (Mizukami et al. 2004), we carried out five independent experiments. Genes with more than twofold increase in at least three of the five experiments were considered to be up-regulated, and genes with less than 0.5-fold in at least three of the five experiments were considered to be down-regulated genes. Up- and down-regulated genes are shown in Table 1a and b. After 4°C exposure, the number of up-regulated genes was 398 at 6 h, 370 at 12 h, 394 at 24 h, and 403 at 48 h (Table 1a). The number of down-regulated genes was 441 at 6 h, 370 at 12 h, 362 at 24 h, and 365 at 48 h (Table 1b). The up- and down-regulated genes after 4°C exposure were classified into functional categories according to MIPS.

Table 1 Expression changes of yeast genes at each time point after 4°C exposure

	Total ORFs	6 h	(%)	12 h	(%)	24 h	(%)	48 h	(%)
a. Greater than twofold up-regulated genes		**398**		**370**		**394**		**403**	
Metabolism	984	**77**	7.8	**77**	7.8	**82**	8.3	**78**	7.9
Energy	260	**28**	10.8	**29**	11.2	**32**	12.3	**32**	12.3
Cell cycle and DNA processing	688	**25**	3.6	**23**	3.3	**22**	3.2	**20**	2.9
Transcription	836	**27**	3.2	**19**	2.3	**23**	2.8	**19**	2.3
Protein synthesis	380	**5**	1.3	**4**	1.1	**5**	1.3	**10**	2.6
Protein fate (folding, modification, and destination)	630	**36**	5.7	**26**	4.1	**30**	4.8	**39**	6.2
Protein with binding function or co-factor requirement (structural or catalytic)	39	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Protein activity regulation	27	**2**	7.4	**0**	0.0	**2**	7.4	**1**	3.7
Cellular transport, transport facilitation, and transport routes	718	**39**	5.4	**37**	5.2	**35**	4.9	**27**	3.8
Cellular communication, signal transduction mechanism	93	**4**	4.3	**6**	6.5	**6**	6.5	**7**	7.5
Cell rescue, defense, and virulence	294	**49**	16.7	**48**	16.3	**47**	16.0	**40**	13.6
Interaction with the cellular environment	331	**17**	5.1	**20**	6.0	**18**	5.4	**13**	3.9
Interaction with the environment (systemic)	2	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Transposable elements, viral, and plasmid proteins	118	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Development	1	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Biogenesis of cellular components	447	**15**	3.4	**15**	3.4	**11**	2.5	**14**	3.1
Cell type differentiation	339	**13**	3.8	**13**	3.8	**8**	2.4	**17**	5.0
Subcellular localization	11	**1**	9.1	**3**	27.3	**2**	18.2	**1**	9.1
Classification not yet clear-cut	114	**9**	7.9	**9**	7.9	**11**	9.6	**9**	7.9
Unclassified proteins	2490	**151**	6.1	**143**	5.7	**161**	6.5	**169**	6.8
b. Less than 0.5-fold down-regulated genes		**441**		**370**		**362**		**365**	
Metabolism	984	**89**	9.0	**73**	7.4	**82**	8.3	**113**	11.5
Energy	260	**11**	4.2	**13**	5.0	**8**	3.1	**15**	5.8
Cell cycle and DNA processing	688	**45**	6.5	**33**	4.8	**27**	3.9	**39**	5.7
Transcription	836	**58**	6.9	**28**	3.3	**45**	5.4	**33**	3.9
Protein synthesis	380	**90**	23.7	**118**	31.1	**93**	24.5	**71**	18.7
Protein fate (folding, modification, and destination)	630	**56**	8.9	**28**	4.4	**25**	4.0	**36**	5.7
Protein with binding function or co-factor requirement (structural or catalytic)	39	**8**	20.5	**5**	12.8	**3**	7.7	**4**	10.3
Protein activity regulation	27	**3**	11.1	**3**	11.1	**1**	3.7	**1**	3.7
Cellular transport, transport facilitation, and transport routes	718	**41**	5.7	**25**	3.5	**28**	3.9	**35**	4.9
Cellular communication and signal transduction mechanism	93	**4**	4.3	**4**	4.3	**2**	2.2	**5**	5.4
Cell rescue, defense and virulence	294	**20**	6.8	**17**	5.8	**13**	4.4	**26**	8.8
Interaction with the cellular environment	331	**24**	7.3	**20**	6.0	**20**	6.0	**18**	5.4
Interaction with the environment (systemic)	2	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Transposable elements, viral, and plasmid proteins	118	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Development	1	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Biogenesis of cellular components	447	**35**	7.8	**21**	4.7	**20**	4.5	**24**	5.4
Cell type differentiation	339	**20**	5.9	**11**	3.2	**11**	3.2	**18**	5.3
Subcellular localization	11	**0**	0.0	**0**	0.0	**0**	0.0	**0**	0.0
Classification not yet clear-cut	114	**5**	4.4	**1**	0.9	**2**	1.8	**6**	5.3
Unclassified proteins	2490	**76**	3.1	**55**	2.2	**61**	2.4	**40**	1.6

Total ORFs represent the total number of classified genes in each category according to the MIPS functional database (http://mips.gsf.de/genre/proj/yeast/index.jsp)
The number of parentheses indicate the percentage of up- or down-regulated genes in each functional category
Bold numbers represent up- and down-regulated genes in each condition

Many genes involved in cell rescue, defense and virulence, energy and metabolism are up-regulated (Table 1a), while many genes were involved in protein synthesis, proteins with binding functions or cofactor requirements, protein activity regulation, and protein fate are down-regulated (Table 1b). This suggests a global response to 4°C exposure, as is seen in response to other stresses such as chemicals (e.g., straight-chain alcohols, herbicides, etc.) (http://kasumi.nibh.jp/~iwahashi/), heat shock, osmotic stresses, and low temperature (Causton et al. 2001; Fujita et al. 2004; Gasch et al. 2000; Kitagawa et al. 2002; Sahara et al. 2002). We applied hierarchical cluster analysis to an aggregation of data at 6–48 h of 4°C exposure (Fig. 2). As shown in Fig. 2, the gene expression up to 48 h after 4°C exposure showed cellular responses for low temperature adaptation, and the difference of gene expression profiling patterns at each time point revealed the variation of responses to 4°C exposure. We also tried to compare the aggregation data up to 48 h after 4°C exposure with gene expression profiles at the continuous growth at 4°C and at 35°C using the hierarchical cluster analysis. As shown in Fig. 2, the transcriptional profiling data in continuous growth at 35°C and 4°C were different from those data after 4°C exposure. It is believed that the differences of gene expression profiling patterns indicate the variation of the cellular responses between 4°C exposure and continuous growth at 4°C. We further analyzed those genes that showed induction or repression between 6 and 48 h post-exposure in order

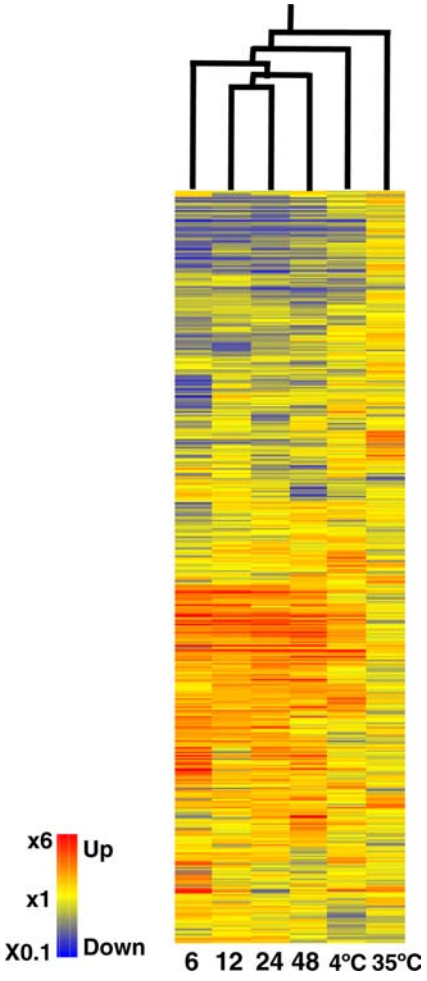

Fig. 2 Hierarchical cluster analysis at 6–48 h after 4°C exposure, culture at 4°C, and culture at 35°C. The hierarchical clustering was performed using GeneSpring. The gene expression profiles at 6–48 h after 4°C exposure were compared with cultures grown at 4°C or at 35°C. These data were cited at http://kasumi.nibh.jp/~iwahashi/. The variations in abundance of transcripts for each gene are depicted by means of a color scale, red, blue, and yellow. The gene's relative intensity is represented as logarithmic scale ($\log_2 X$) of the fold increase or decrease as shown in the scale bar. *Red* indicates expression levels more than two fold greater in 4°C exposure compared with that of the control ($A_{660} = 1.0$ at 25°C) and *blue* indicates expression levels less than 0.5-fold after 4°C exposure compared with that of the control. *Yellow* denotes no significant difference at 4°C exposure compared with control

to elucidate the physiological responses to 4°C conditions.

Characterization of transcriptional profiles 6–48 h after 4°C exposure

When the induced genes at 4°C exposure were classified into functional categories according to the MIPS database, the most prevalent categories were energy and metabolism, and cell rescue, defense and virulence. The repressed genes at 4°C exposure more commonly fell into categories involved in protein synthesis, binding, activity regulation, and protein fate.

Energy

Genes included in the energy category have functions involved in glycolysis/gluconeogenesis (*TPI1*, *TDH1*, *TDH2*, *TDH3*, *GPM1*, *GPM2*, *ENO1*, *ENO2*, *PYC1*, *PDC5*, and *PDC6*), trehalose biosynthesis/degradation (*TPS1*, *TPS2*, *ATH1*, *HXK1*, and *GLK1*), glycogen biosynthesis/degradation (*GAC1*, *GPH1*, *GDB1*, and *GLC3*), and arylalchol dehydrogenase (*AAD*) (Table 2a–c). Trehalose and glycogen are the two major forms of carbohydrate energy reserves in *S. cerevisiae*, and they are known to accumulate under various stress conditions (Francois and Parrou 2001). The induction of trehalose or glycogen synthesis genes is observed under heat shock, osmotic shock, and oxidative stress conditions (Iwahashi et al. 1998). Because trehalose or glycogen biosynthesis genes were induced by 4°C exposure, accumulation of trehalose and glycogen may also protect cells exposed to extreme low temperature. The up-regulated genes involved in glycolysis/glyconeogenesis (*TPI1*, *TDH1*, *TDH2*, *TDH3*, *GPM1*, *GPM2*, *ENO1*, *ENO2*, *PYC1*, *PDC5*, and *PDC6*) may then be needed for utilization of trehalose and glycogen (Table 2b).

Surprisingly, the genes involved in glycogen degradation (*GDB1* and *GPH1*), together with glycogen synthesis genes (*GAC1* and *GLC3*) were simultaneously induced by 4°C exposure (Table 2a). This has also been observed in other conditions, such as heat shock, and low temperature (10–15°C) exposure (Parrou et al. 1997; Sahara et al. 2002). The intracellular trehalose is mostly degraded by *NTH1*, however, the fold of *NTH1* after 4°C exposure was 0.98 at 6 h, 1.05 at 12 h, 1.59 at 24 h, and 1.29 at 48 h, respectively. Because significant induction of *NTH1* was not observed after 4°C exposure, trehalose but not glycogen degradation may not occur by 4°C cold stress.

The genes encoding arylalcohol dehydrogenase (AAD) (*AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD14*, and *AAD15*) were also induced after 4°C exposure (Table 2c). It has been reported that AAD genes are induced under oxidative stress (Delneri et al. 1999), but the function of AAD in yeast is unclear.

Metabolism

Genes involved in phospholipid synthesis (*INO1*, *PSD1*, *CDS1*, *CHO1*, *CHO2*, and *OPI3*) and methionine biosynthesis (*MET3*, *MET6*, *MET13*, *MET16*, *MET17*, and *SAM2*) were induced (Table 3). These included key genes involved in phospholipid synthesis, such as *INO1* and *OPI3*, (Table 3a), suggesting that phospholipid synthesis was stimulated and probably, de novo

Table 2 Genes involved in energy

ORFs	Name	Ratio of Cy5/Cy3				Description
		6 h	12 h	24 h	48 h	
a.The genes for trehalose or glycogen synthesis and degradation						
YBR126C	TPS1	2.0	1.8	2.6	1.2	Trehalose-phosphate synthase
YDR074W	TPS2	2.4	2.6	2.5	1.6	Trehalose-6-phosphate phosphatase
YHR093W	AHT1		1.8	1.9	2.5	Vacuolar acid trehalose
YEL011W	GLC3	5.3	4.9	7.1	4.5	Glycogen branching enzyme
YPR160W	GPH1	3.4	3.1	5.8	4.8	Releases glucose-1-phosphate from glycogen
YOR178C	GAC1	4.9	4.9	6.6	7.4	Regulatory subunit for Glc7p (protein phosphatase I) for glycogen synthesis
YPR184W	GDB1	3.4	1.7	2.6	2.2	Glycogen debranching enzyme
b. Glycolysis and gluconeogenesis						
YDR050C	TPI1	2.7	3.8	2.1	2.6	Induced under stress conditions
YJL052W	TDH1	3.1	3.1	2.7	2.0	Glyceraldehyde-3-phosphate dehydrogenase 1
YJR009C	TDH2	2.7	2.0	1.7	1.4	Glyceraldehyde-3-phosphate dehydrogenase 2
YGR192C	TDH3	3.0	2.5	1.9	1.9	Glyceraldehyde-3-phosphate dehydrogenase 3
YKL152C	GPM1	2.8	2.5	1.9	1.2	Phosphoglycerate mutase 2
YDL021W	GPM2	1.7	2.3	2.6	4.0	Phosphoglycerate mutase 1
YGR254W	ENO1	2.2	2.3	1.8	1.7	Enolase 1
YHR174W	ENO2	2.3	2.8	1.9	1.8	Enolase 2
YGL062W	PYC1	2.5	2.3	1.7	1.7	Converts pyruvate to oxaloacetate
YLR134W	PDC5	1.6	1.7	1.5	2.0	Pyruvate decarboxylase
YGR087C	PDC6	1.6	1.5	2.3	1.6	Minor isozyme of pyruvate decarboxylase
YKR097W	PCK1			2.1	2.4	Phosphoenolpyruvate carboxylkinase
c. Aryl-alcohol dehydrogenase (AAD)						
YCR107W	AAD3	1.5	2.7	1.9	2.1	High degree of similarity with the AAD of *P. chrysosporium*
YDL243C	AAD4	2.3	2.4	2.1	2.3	High degree of similarity with the AAD of *P. chrysosporium*
YFL056C	AAD6	2.0	2.4	1.4	2.7	High degree of similarity with the AAD of *P. chrysosporium*
YJR155W	AAD10	2.3	3.0	3.0	3.8	High degree of similarity with the AAD of *P. chrysosporium*
YNL331C	AAD14	2.1	2.2	2.1	2.2	AAD located on chromosome 14
YOL165C	AAD15	1.9	2.0	1.8	1.7	High degree of similarity with the AAD of *P. chrysosporium*

Table 3 Genes involved in metabolism

ORFs	Name	Ratio of Cy5/Cy3				Description
		6 h	12 h	24 h	48 h	
a. The genes involved in phospholipid synthesis pathway						
YJL153C	INO1	6.2	6.0	4.4	6.0	Involved in the rate-limiting step of inositol biosynthesis
YJR073C	OPI3	13.1	14.7	13.0	8.1	Second and third steps of methylation pathway for phosphatidylcholine biosynthesis
YOL108C	INO4	2.3	2.1	1.9	3.0	Transcription factor required for derepression of inositol-choline-regulated genes involved in phospholipid synthesis
YBR029C	CDS1	1.7	1.7	1.4	1.2	CDP-diacylglycerol synthase
YER026C	CHO1	5.4	4.9	3.0	2.4	phosphatidylserine synthase
YGR157W	CHO2	1.6	1.4	1.8	2.3	First step in the methylation pathway for phosphatidylcholine biosynthesis
YNL169C	PSD1	6.7	2.4	3.2	3.1	Converts phosphatidylserine to phosphatidylethanolamine.
b. The genes for methionine biosynthesis pathway						
YDR502C	SAM2	2.5	2.6	1.0	0.7	Methionine biosynthesis regulation
YER091C	MET6	3.2	2.9	2.0	1.5	Cobalamine-independent methionine synthase
YGL125W	MET13	6.8	4.8	4.5	5.3	Putative methylenetetrahydrofolate reductase (mthfr)
YJR010W	MET3	3.0	2.3	1.3	1.2	One of three genes essential for the assimilatory reduction of sulfate to sulfide (with sulfite as an intermediate product)
YLR303W	MET17	2.7	1.9	1.0	0.6	*O*-acetylhomoserine-*O*-acetylserine sulfhydralase
YPR167C	MET16	3.1	3.2	2.5	1.7	3′-Phosphoadenylylsulfate reductase

membrane synthesis was stimulated. The induction of methionine biosynthesis genes (*MET6*, *MET17*, and *SAM2*, etc.; Table 3b) may indicate a need for *S*-adenosylmethionine (AdoMet) as a methyl donor in the cold acclimation process, because methionine gives rise to AdoMet, which is used for numerous transmethylation reactions, such as nucleic acid and lipid synthesis (Mountain et al. 1991; Thomas et al. 2000).

Cell rescue, defense, and virulence

The genes included in cell rescue, defense, and virulence in MIPS functional categories were cold shock-inducible genes (*TIP1*, *TIR1, TIR2*, *TIR3*, and *TIR4*), *PAU* genes (*PAU1*, *PAU2*, *PAU4*, *PAU5*, *PAU6*, and *PAU7*), HSPs (*HSP12*, *HSP104*, *HSP30*, *SSE2*, and *SSA4*), and detoxification genes (*TSA2*, *TTR1*, *AHP1*, *TRX3*, *GTT1*, *GTT2*, *GPX1*, *MXR1*, and *CTT1*) (Table 4).

The cold-inducible proteins (Tip1, Tir1, Tir2, Tir3, and Tir4) are serine and threonine-rich mannoproteins localized in the cell wall (Table 4a). However, their functions and mode of regulation are largely unknown (Kondo and Inouye 1991; Kowalski et al. 1995). The *PAU* genes encode seripauperin (Srp) proteins, and are low in serine content compared with related proteins, such as Tip (Table 4b). Seripauperins are generally considered to be stress proteins, and share homology with the N-terminal region of TIP proteins (Rachidi et al. 2000). It is thought that the genes encoding seripauperin proteins are induced, as well as related TIP or SRP family genes, under various stress conditions, i.e., heat shock and cold shock.

Genes encoding HSPs (*HSP12*, *HSP104*, *HSP30*, *SSE2*, and *SSA4*) were induced by 4°C exposure (Table 4c). The induction of these HSPs, except for *SSE2* and *HSP30*, has been previously observed at 0°C as part of the near-freezing response (Kandror et al. 2004). In general, HSPs are induced under various stress conditions, such as oxidative stress, high pressure, and heat shock, and they play an important role in the process of protein folding when proteins are denatured under stress conditions (Godon et al. 1998; Iwahashi et al. 1998). Interestingly, most of the major HSPs (*CIS3*, *HMS2*, *HSC82*, *HSP30*, *HSP60*, *HSP78*, *HSP82*, *HSP150*, *SSA1*, *SSA2*, *STI1*, and *YDJ1*) are strongly repressed after transferring cells to 10–18°C (Lashkari et al. 1997; Sahara et al. 2002). The induction of several genes encoding HSPs after 4°C exposure as shown in Table 4c, suggests that the cellular response at 4°C is distinct from that seen at 10°C.

Many genes involved in detoxification (*TSA2*, *AHP2*, *TTR1*, *TRX3*, *GTT1*, *GTT2*, *GPX1*, *MXR1*, and *CTT1*) were induced after 4°C exposure (Table 4d). *GTT1* and *GTT2* encode functional glutathione *S*-transferase (GST) enzymes in yeast, and play an important role in drug resistance (Choi et al. 1998). *TSA2* and *AHP2* encode thioredoxin peroxidases and *GPX1* encodes a glutathione peroxidase (Avery and Avery 2001). These peroxidases together with the *CTT1* gene product (catalase) play an important role in removing hydrogen peroxide and alkylhydroperoxide. *TTR1* encodes glutaredoxin, which functions as a glutathione *S*-transferase (Collinson and Grant 2003). The *TRX3* gene encodes a

Table 4 Genes involved in cell rescue, defense, and virulence after 4°C exposure

ORFs	Gene	Ratio of Cy5/Cy3				Description
		6 h	12 h	24 h	48 h	
a. Cold shock-inducible protein						
YBR067C	TIP1	11.6	7.5	3.2	2.7	Cold and heat shock-induced protein of the Srp1p/Tip1p family
YER011W	TIR1	9.0	7.9	7.3	3.2	Cold shock-induced protein of the Tip1p family
YOR010C	TIR2	6.2	4.6	6.0	1.9	Cold shock-induced protein of the Srp1p/Tip1p family
YIL011W	TIR3	3.3	2.0	1.8	0.5	TIP1-related protein
YOR009W	TIR4	3.8	3.4	2.8	1.2	Tir4p
b. Seripauperin protein						
YJL223C	PAU1	5.4	2.9	3.4	3.3	Member of the seripauperin protein/gene family
YEL049W	PAU2	2.7	2.5	2.8	2.4	Member of the seripauperin protein/gene family
YLR461W	PAU4	3.3	2.8	2.7	2.0	Member of the seripauperin protein/gene family
YFL020C	PAU5	2.5	2.2	2.0	1.7	Member of the seripauperin protein/gene family
YNR076W	PAU6	3.1	2.7	2.0	2.0	Member of the seripauperin protein/gene family
YAR020C	PAU7	3.5	2.8	2.6	2.2	Member of the seripauperin protein/gene family
c. Heat shock proteins (HSPs)						
YCR021C	HSP30	3.5	2.5	4.1	2.9	Heat shock protein
YER103W	SSA4	2.1	2.4	2.5	2.0	Member of 70 kDa heat shock protein family
YFL014W	HSP12	9.1	29.3	43.5	54.8	Heat shock protein
YLL026W	HSP104	2.9	2.5	2.9	2.1	Heat shock protein
YBR169C	SSE2	1.9	1.8	3.2	2.4	Member of 70 kDa heat shock protein family
d. Detoxification of active oxygen species						
YDR453C	TSA2	1.7	2.5	2.6	2.4	Thioredoxin peroxidase
YDR513W	TTR1	1.8	2.0	1.6	2.0	Glutaredoxin (glutathione reductase)
YCR083W	TRX3	2.2	2.2	3.0	4.9	Mitochondrial thioredoxin
YLR109W	AHP1	3.6	3.2	3.2	1.3	Thioredoxin peroxidase
YIR038C	GTT1	2.0	2.4	2.5	2.5	Glutathione transferase
YKL026C	GPX1	5.9	4.5	8.3	13.6	Glutathione peroxidase paralogue
YLL060C	GTT2	2.0	2.8	3.4	3.0	Glutathione transferase
YER042W	MXR1	4.4	5.0	3.2	3.3	Peptide methionine sulfoxide reductase 1
YGR088W	CTT1	3.1	3.3	4.6	3.7	Cytoplasmic catalase T

14 kDa protein, which is located in mitochondria and contains a characteristic thioredoxin active site (Pedrajas et al. 1999). *MXR1* encodes methionine sulfoxide reductase, which is an antioxidant under methionine oxidation conditions (Moskovitz et al. 1997). Together, these genes are active as a defense system against reactive oxygen species (Causton et al. 2001; Gasch et al. 2000). Several genes involved in detoxification of reactive oxygen are also induced after 10°C exposure (Sahara et al. 2002), suggesting that both low (10°C) and extreme low (4°C) temperature exposures result in oxidative stress.

Repressed genes

Many of the genes that are repressed at 4°C have functions related to protein synthesis (148 of 782 down-regulated genes) (Table 5). These comprise ribosomal protein large subunit (RPL) genes (58 genes), ribosomal protein small subunit (RPS) genes (42 genes), and others genes (48 genes). Both RPL genes and RPS genes were repressed during 4°C exposure (Table 5a, b). The latter category includes genes (*RPP0*, *RPP1A*, *RPP1B*, *RPP2A* and *RPP2B*) encoding subunits of nuclear RNase P (Table 5c). RNase P is a ubiquitous endoribo-

Table 5 Repressed genes in protein synthesis, 148 ORFs

ORFs	Name	Ratio of Cy5/Cy3				Description
		6 h	12 h	24 h	48 h	
a. Ribosomal protein large subunit (RPL) 15/58 ORFs						
YDL136W	RPL35B	0.4	0.4	0.6	0.6	Homology to rat L35
YDR012W	RPL4B	0.5	0.4	0.4	0.2	Highly similar to ribosomal protein L4A
YDR471W	RPL27B	0.3	0.3	0.4	0.6	Homology to mammalian L27
YGL030W	RPL30	0.4	0.4	0.4	0.5	Homology to rat and mouse L30
YGL076C	RPL7A	0.3	0.3	0.3	0.4	SNR39
YGL147C	RPL9A	0.4	0.3	0.3	0.4	Homology to rat L9
YHL033C	RPL8A	0.4	0.3	0.4	0.4	Homology to human L7a, mouse L7a, and rat L7a
YLR029C	RPL15A	0.3	0.3	0.6	0.5	Homology to rat L15
YMR121C	RPL15B	0.3	0.3	0.5	0.5	Homology to rat L15
YNL067W	RPL9B	0.5	0.3	0.4	0.5	Homology to rat L9
YNL301C	RPL18B	0.5	0.3	0.4	0.4	Homology to rat ribosomal protein L18
YOL120C	RPL18A	0.4	0.4	0.4	0.4	Homology to rat ribosomal protein L18
YOR063W	RPL3	0.3	0.2	0.4	0.3	Homology to rat L3
YPL131W	RPL5	0.5	0.2	0.4	0.4	Homology to rat ribosomal protein L5
YPL198W	RPL7B	0.3	0.3	0.3	0.4	SNR59
b. Ribosomal protein small subunit (RPS) 14/42 ORFs						
YBR048W	RPS11B	0.4	0.3	0.4	0.4	Homology to rat S11, human S11, and *E. coli* S17
YBR181C	RPS6B	0.5	0.3	0.4	0.5	Homology to rat, human, and mouse S6
YBR189W	RPS9B	0.5	0.3	0.4	0.4	Homology to rat S9 and *E. coli* S4
YDR447C	RPS17B	0.5	0.4	0.5	0.6	Homology to rat ribosomal protein S17
YGL123W	RPS2	0.5	0.3	0.5	0.4	Homology to rat S2 and E. coli S5
YHL015W	RPS20	0.3	0.2	0.4	0.4	Homology to rat S20, human S20, *Xenopus* S22, and *E. coli* S10
YHR203C	RPS4B	0.3	0.4	0.3	0.3	Homology to rat S4 and human S4
YJL136C	RPS21B	0.4	0.4	0.4	0.5	Homology to rat S21
YLR167W	RPS31	0.5	0.3	0.6	0.7	Homology to rat S27a
YNL096C	RPS7B	0.3	0.4	0.4	0.3	Homology to human S7 and *Xenopus* S8
YNL178W	RPS3	0.3	0.3	0.4	0.6	Homology to mammalian S3
YOR293W	RPS10A	0.5	0.4	0.5	0.5	Homology to rat S10
YPL081W	RPS9A	0.3	0.3	0.3	0.4	Homology to rat S9 and *E. coli* S4
YPL090C	RPS6A	0.3	0.3	0.3	0.4	Homology to rat, mouse, and human S6
c. Others 17/48 ORFs						
YLR340W	RPP0	0.4	0.2	0.5	0.4	Homology to rat P0, human P0, and *E. coli* L10e
YDL081C	RPP1A	0.5	0.3			Homology to rat P1, human P1, and *E. coli* L12eIIA
YOL039W	RPP2A		0.4	0.6	0.5	Homology to rat P2, human P2, and *E. coli* L12eIB
YDL130W	RPP1B	0.5	0.4		0.3	Homology to rat P1, human P1, and *E. coli* L12eIIB
YDR382W	RPP2B		0.6	0.6		Homology to rat P2, human P2, and *E. coli* L12eIA
YBR121C	GRS1	0.4	0.4	0.3	0.4	Glycyl-tRNA synthase
YDR023W	SES1	0.4	0.4	0.3	0.4	Seryl-tRNA synthetase
YGR264C	MES1	0.4	0.5	0.4	0.5	Methionyl tRNA synthetase
YLR060W	FRS1	0.5	0.5	0.3	0.3	Phenylalanyl-tRNA synthetase
YPL160W	CDC60	0.2	0.3	0.3	0.3	Cytosolic leucyl tRNA synthetase
YHR066W	SSF1	0.4	0.5	0.4		Putative involvement in mating
YIL078W	THS1	0.4	0.4	0.3		Threonyl-tRNA synthetase, cytoplasmic
YIL093C	RSM25	0.4	0.5		0.5	Mitochondrial ribosome small subunit component
YBL076C	ILS1		0.4	0.5	0.5	Cytoplasmic isoleucyl-tRNA synthetase
YDR037W	KRS1		0.4	0.5	0.3	Lysyl-tRNA synthetase
YDR385W	EFT2		0.4	0.3	0.2	Translation elongation factor 2 (EF-2)
YHR019C	DED81		0.5	0.4	0.2	Asparaginyl-tRNA synthetase

nuclease that cleaves 5′ terminal leader sequences of precursor tRNAs to generate 5′ mature termini, and is an essential enzyme for biosynthesis of tRNA (Stolc et al. 1998). It has been shown that transcription is reduced at low temperature (15°C)(Jones and Inouye 1996). Repression of key genes involved in protein synthesis in the present study suggests that protein synthesis in yeast cells is down-regulated after 6–48 h at 4°C. The repression of these genes at 4°C exposure is reflected in the delayed growth of yeast cells (Fig. 1), because growth delays typically precede adaptation to stress conditions and proper fitness under a new environment occurs during delayed growth.

Discussion

When yeast cells were cultured at 25°C then transferred to 4°C, the cell growth estimated by A_{660} was very slow (Fig. 1). Colony forming ability could be maintained for more than 1 year, when yeast cells grown to stationary phase were stored in 4°C (data not shown). Thus, it was thought that low temperature is suitable to preservation of microorganisms. As because we are responsible in maintenance of viability as the culture collection consortium, we are interested in low temperature nearing 4°C as the condition for the strain preservation.

We have previously performed an analysis of gene expression for continuous growth at 4°C (Homma et al. 2003). In the present study, we have examined gene expression following a 25°C to 4°C shift. As because it was found that the transcriptional patterns after 4°C shift were different from that of continuous growth at 4°C depending on the hierarchical cluster analysis (Fig. 2), it was considered that the cellular responses were the difference between 4°C shift and the continuous growth at 4°C. We regarded these genes as the transcriptional response necessary for adaptation or maintenance of viability to 4°C.

Genes involved in phospholipid synthesis were induced in our experiments (Table 3a), suggesting that membrane biogenesis was stimulated. In general, because the fluidity and permeability of the cellular membrane decreases at low temperature, fatty acid desaturase is induced to maintain membrane fluidity and permeability (Aguilar et al. 1999; Inaba et al. 2003; Nakagawa et al. 2002). In *S. cerevisiae*, *OLE1* encodes *Δ*9-fatty acid desaturase, and this enzyme catalyzes the introduction of the initial double bond between ninth and tenth carbons of palmitoyl CoA and stearoyl CoA (Nakagawa et al. 2001). We did not observe a large induction of *OLE1* (the hybridization ratio was 1.19 at 6 h, 0.8 at 12 h, 0.16 at 24 h, and 1.0 at 48 h). However, *OLE1* transcript level confirmed by northern blot is transiently activated and afterward repressed by low temperature (10°C) (Nakagawa et al. 2002), therefore, it is speculated that transcription of *OLE1* may have been induced at an early stage (< 6 h after 4°C transfer), and subsequently repressed.

We also observed induction of methionine synthesis genes (Table 3b). Methionine can give rise to AdoMet, the methyl donor in hundreds of transmethylation reactions of nucleic acid, proteins, and lipids (Mountain et al. 1991; Thomas et al. 2000). In the phospholipid synthesis pathway, AdoMet is needed for methylation of PE to PC (Carman and Zeimetz 1996). It has been reported that phopholipid synthesis is integrated with methionine biosynthesis (Murata et al. 2003); consequently, the observed induction of methionine synthesis may also be related to membrane biogenesis.

Trehalose synthesis in yeast is catalyzed by two enzymes, trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase (*TPS2*), which are part of the trehalose synthase complex (Bell et al. 1998). The induction of trehalose synthesis genes, *TPS1* or *TPS2*, was observed at various times after 4°C exposure (Table 2a). Trehalose is accumulated at high levels in yeast in response to heat shock, osmotic shock, and high pressure (Iwahashi et al. 2000; Kandror et al. 2002), and it plays a major role in protection against environmental stresses, such as thermotolerance (Singer and Lindquist 1998). Trehalose synthesis has previously been shown to be important at very low temperatures; *ΔTPS1 ΔTPS2* mutants are less viable than wild-type cells at 0–4°C (Kandror et al. 2004).

In Kandror's microarray data, *TPS1* and *TPS2* were induced up to 5.6- and 5.9-fold after 24 h at 0°C incubation (Kandror et al. 2004). However, we observed only 2- to 2.6-fold induction through 6–48 h after 4°C exposure (Table 2a). This apparent discrepancy may be the result of the different exposure times (24 or 48 h) or different carbon sources. Kandror et al. (2004) used galactose as a carbon source (YPGal medium), while we used YPD medium containing glucose.

Genes involved in glycogen biosynthesis were also induced after 4°C exposure (Table 2a), including *GLC3*, the branching enzyme that introduces α(1,6) branches in mature glycogen, and *GAC1*, the regulatory subunit for phosphoprotein phosphatase type 1 (PP-1), which specifically activates glycogen synthase by dephosphorylation. Glycogen and trehalose serve as major reserve carbohydrates in yeast cells and the accumulation of these products is induced by stressful environmental conditions (Lillie and Pringle 1980). In our study, the expressed ratio of glycogen synthesis genes was higher than that of the genes for trehalose synthesis (the ratio is three- to five fold as shown in Table 2a). Both glycogen and trehalose may be required for growth and survival at 4°C.

As shown in Table 2a, both the glycogen phosphorylase gene (*GPH1*), which encodes the enzyme that breaks down glycogen into glucose-1-phosphate (*Glc1P*) and free glucose, was induced after 4°C exposure. The gene involved in glycogen degradation was up-regulated simultaneously with the genes for glycogen synthesis (Table 2a). The genes involved in the metabolism of this

reserve carbohydrate contain between one and several copies of the STRE (stress responsive element; core consensus CCCCT) in their promoters (Varela et al. 1995) and the induction of these genes is mediated by interaction of STRE and Msn2p/Msn4p transcriptional activators (Parrou et al. 1997). We also observed induction of Msn2p transcriptional activators (as shown in http://kasumi.nibh.jp/~iwahashi/), consistent with Msn2p activation of the genes for glycogen metabolism by this pathway after 4°C exposure.

NTH1 was not significantly induced after 4°C exposure (0.98 at 6 h, 1.05 at 12 h, 1.59 at 24 h, and 1.29 at 48 h, respectively). This gene was not induced in the case of continuous growth at 4°C (1.59). Thus, trehalose degradation may not occur at 4°C and this phenomenon is completely different from high temperature, where trehalose degradation and synthesis are shown to be activated.

Induction of the cold shock proteins *TIP1*, *TIR1*, *TIR2*, *TIR3*, and *TIR4* was observed after 4°C exposure (Table 4a). These cold shock proteins are serine/threonine-rich mannoproteins localized in cell wall (Abramova et al. 2001). *PAU* genes were also induced at 4°C (Table 4b). *PAU* genes encode Pau proteins (seripauperines), which have N-terminal sequences similar to cold shock proteins. The function of *PAU* genes remains unknown. However, *PAU* genes are found on all of the 16 yeast chromosomes and form the largest multigene family in *S. cerevisiae*; the substantial multiplicity of these genes suggests that they have an important role in yeast (Rachidi et al. 2000).

It is believed that mannoproteins may play a role in controlling cell wall porosity and increasing the permeability of the cell wall (Zlotnik et al. 1984). Therefore, the induction of mannoproteins, including cold shock proteins, may help to compensate for reduced membrane permeability at low temperatures.

The *DAN* and *TIR* genes encoding cell wall mannoproteins are expressed during anaerobiosis (Abramova et al. 2001). *PAU* genes are also induced under anaerobic condition (Rachidi et al. 2000). Why these genes are induced under both anaerobic and low-temperature conditions is not yet clear.

Several genes encoding HSPs (*HSP12*, *HSP104*, *HSP26*, *HSP30*, *SSE2*, and *SSA4*) were induced at 6–48 h after 4°C exposure (Table 4c). It has been recently

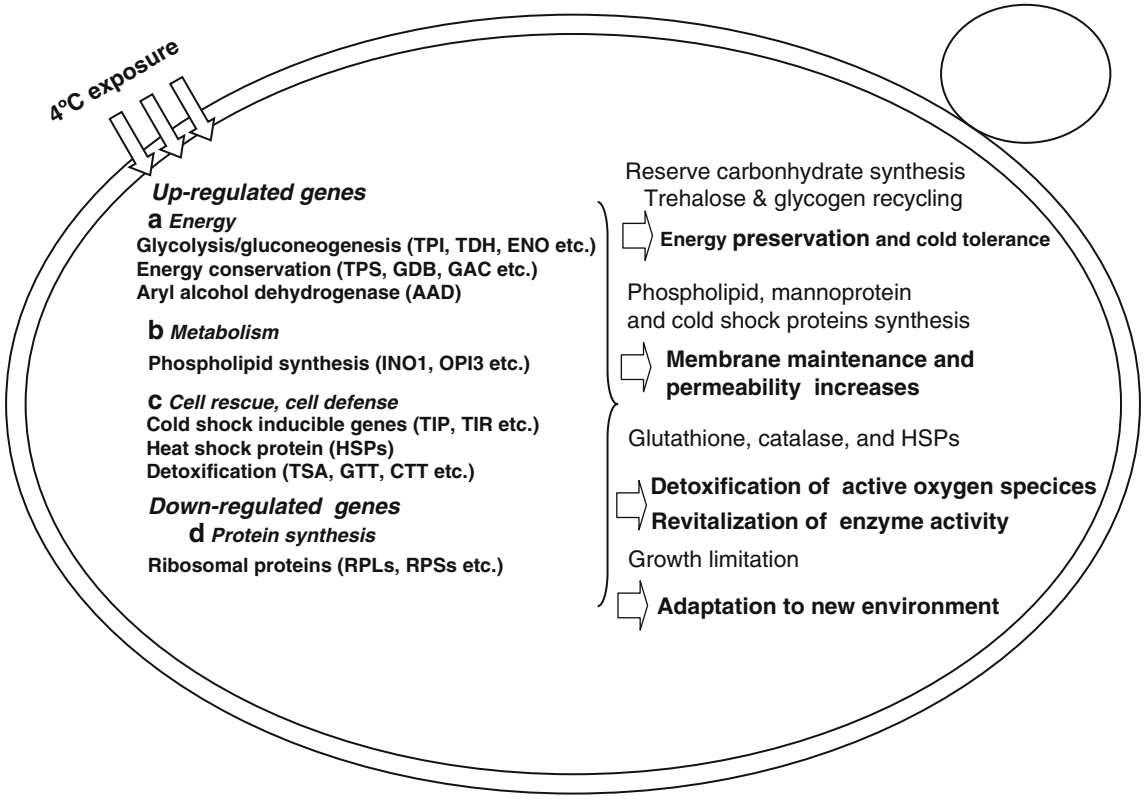

Fig. 3 The cellular responses within 6–48 h after 4°C exposure. In 4°C exposure, the induction of the genes (*TPS1*, *GDB1*, *GAC1*, etc.) involved in trehalose and glycogen synthesis may suggest that biosynthesis and accumulation of those reserve carbohydrates are necessary for cold tolerance and energy preservation. The observed increased expression of phospholipids (*INO1*, *OPI3*, etc.), seripauperin proteins (*PAU1*, *PAU2*, etc.), and cold shock proteins (*TIP1*, *TIR1*, etc.) is consistent with membrane maintenance and increased permeability of the cell wall at 4°C. The induction of HSPs (*HSP12*, *HSP104*, *SSA4* etc,) and glutathione (*TTR1*, *GTT1*, *GPX1*, etc.) at 4°C may be required for revitalization of enzyme activity, and for detoxification of active oxygen species, respectively. Down-regulation of protein synthesis (*RPL3*, *RPS3*, etc.) represent the drop of cell growth, and the delayed growth may reflect the preparation for adjustment to new environment

reported that these HSPs, except for *SSE2* and *HSP30*, are induced at 0°C (Kandror et al. 2004). However, only *HSP12* and *HSP26* are up-regulated at 4–8 h after down-shift from 30 to 10°C (Sahara et al. 2002). In general, HSPs are induced under various conditions, such as oxidative stress, high pressure, and heat shock, and play an important role in the process of protein folding when proteins are denatured under stress conditions (Godon et al. 1998; Iwahashi et al. 1998). A number of HSPs are also induced when yeast cells are cultured at 4°C for several months (Homma et al. 2003), suggesting that the induction of genes encoding various HSPs might be necessary for adjustment to 4°C or living at 4°C. Thus, 4°C may be more suitable for long-term storage than 10°C because enzyme activity would be maintained by the folding function of HSPs induced at 4°C.

We also observed induction of genes involved in detoxification of active oxygen (Table 4d). These gene products play an important role in detoxification of peroxidates or reactive oxygen species formed during metabolism. Glutathione is especially noteworthy in this group (Table 4d). The genes for methionine synthesis were induced by 4°C exposure (Table 4b). *MET3* encodes ATP sulfurylase, and *MET16* encodes 3′-phosphoadenylylsulfate reductase, and these genes are involved in sulfur assimilation (Thomas and Surdin-Kerjan 1997), which may be necessary for glutathione synthesis following 4°C exposure.

The cellular responses through 6–48 h after 4°C exposure are summarized in Fig. 3. It is thought that trehalose and glycogen were synthesized for cold tolerance and energy preservation, and the synthesis of phospholipid and mannoproteins was for maintenance of cell membranes and permeability of cell wall. It is also thought that the induction of the genes encoding glutathione, thioredoxin, and catalase was for detoxification of active oxygen species caused by 4°C, and the induction of HSPs was for vitalization of enzyme activity in 4°C. It is suggested that the delayed growth reflected the preparation for adjustment to new environment, and down-regulation of protein synthesis represented the drop of cell growth. Thus, yeast cells might acquire the ability to adapt and to grow in 4°C through these cellular responses.

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