Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Chemical Pollutants, Eds., Y. Murakami, K. Nakayama, S.-I. Kitamura, H. Iwata and S. Tanabe, pp. 123–132.

Yeast OMICS System for Environmental Toxicology

Yoshihide TANAKA, Tetsuji HIGASHI, Randeep RAKWAL, Junko SHIBATO, Shin-ichi WAKIDA and Hitoshi IWAHASHI

Health Engineering Research Center,
National Institute of Advanced Industrial Science and Technology (AIST),
Midorigaoka-1-8-31, Ikeda, Osaka 563-8577, Japan; and
Tsukuba west, Tsukuba, Ibaraki 305-8569, Japan

(Received 18 May 2008; accepted 29 July 2008)

Abstract—Bioassays are used for the assessment of environmental pollution and risk assessment of environmental stresses. In bioassay systems, harmless is estimated by monitoring biological responses to environmental stress. An extensive literature exists on bioassay systems that include tests by Ames, Microtox, Umu, and others. These traditional bioassays did contribute to prevent adverse effect of chemicals in environment. However, the information that can be estimated from traditional bioassay is limited to the degree of toxicity or mutagenicity. For advanced control of environmental toxicology, our challenging is now how extract the information concern to character of toxicity from bioassay.

Recent advance of biotechnology established OMICS technology. OMICS technology is made up of genomics, proteomics, and metabolomics. Genomics enables genome-wide analyses of cellular responses at the transcriptional levels, proteomics inform those of protein levels, and metabolomics of metabolite levels. These technologies may contribute a great opportunity for understanding mechanisms of action by chemicals and environmental stress. It was suggested that genomics and proteomics often result in conflicting features, however, genomics and metabolomics did not show conflicting but supplementing each other. Here, we would like to show that genomics and metabolomics suggest the different aspects and thus genomics and metabolomics are not parallel technology but should be integrated.

Keyword: OMICS, genomics, metabolomics, yeast, toxicity

INTRODUCTION

At present, more than 25 million materials are registered in the Chemical Abstract database (http://www.cas.org/), and it is estimated that more than 10,000 synthetic chemicals are accumulating in the environment every year. Despite the fact that these industrial chemicals have given us numerous benefits, there is no doubt that they have damaged the environment. The chemicals being dispersed on the earth should be carefully controlled to prevent their adverse effects. In fact, many

chemicals can be detected from environmental samples; however, only 10% of those chemicals can be identified by current technology (Suzuki and Utsumi, 1998). Ten percent is an inadequate number to protect the environment. Furthermore, not only chemical but also physical and biological stresses including radiation, temperature, and pathogens impact ecological systems. Thus, we have to develop the systems that can evaluate environmental toxicity not only by analytical methods but also by biological impact.

Bioassays are used for the assessment of environmental pollution and risk assessment of environmental stresses (Suzuki and Utsumi, 1998). In bioassay systems, safety is estimated by monitoring biological responses to environmental stress. One of these studies is the Multicenter Evaluation of *in vitro* Cytotoxicity program, organized by the Scandinavian Society for Cell Toxicology (Ekwall *et al*., 1998). The investigators compared LD50 data obtained *in vivo* (whole organism) and IC50 obtained *in vitro* (bioassay). They found a correlation between these parameters and defined the concept of "basal cytotoxicity" (Ekwall *et al*., 1998). "Basal cytotoxicity" can be understood as the generalized toxic effect to cellular components, functions and biosynthesis that are universal to all cell lines. On the other hand, the Ames test is well-known as one of the most powerful methods for monitoring the mutagenicity of environmental samples (Suzuki and Utsumi, 1998). In this system, mutants of *Salmonella typhimurium* are grown in a minimum medium and mutagenicity is estimated according to the frequency of back mutation. As the frequency of back mutation is dependent on DNA damage, we can estimate the mutagenicity of chemicals or environmental stress.

An extensive literature exists on bioassay systems that include tests by Ames, Microtox, Umu, and others (Suzuki and Utsumi, 1998). Each system can be used for estimating effects by environmental stress; however, the information that can be estimated is limited to the degree of toxicity or mutagenicity. Information concerning the nature of the environmental stress remains unavailable. In addition, bioassay systems sometimes mistakenly identify natural products as the toxic substance (data not shown). Although it is important to quantify the degree of effects in the environment, information concerning the nature of stress is essential for risk assessment and prevention. Bioassay systems are required that can be used for predicting the mechanism of environmental stress.

We proposed "multiple-end-point bioassays" several years ago (Iwahashi, 2000). The report introduces "multiple-end-point bioassay" systems that are based on stress sensitivities of microorganisms, responses by one kind of organism, and microarray technology. Microorganisms are screened to identify strains that are sensitive to specific stresses and the sensitivity of the isolated strain is then used for characterizing unknown chemicals or environmental samples. The "multiple-end-point bioassay" based on one kind of organisms are system using one organism and many kinds of endpoints such as growth inhibition, viability, induction of stress proteins, prion curing mutagenicity, cytoplasmic mutagenicity, and chromosomal mutagenicity. Using these endpoints we tried to

characterize chemicals and environmental stresses (Iwahashi, 2000).

In recent years, DNA microarray technology has developed rapidly and been widely adopted as a tool for understanding biological systems at the genomic level (Iwahashi, 2006). Furthermore, this technology can be combined with proteomics and metabolomics technology. Proteomics is essentially based on the analysis of proteins using 2-dimentional electrophoresis (Tanaka *et al*., 2008). This technology provides information on the expression levels of hundreds of proteins as well as protein modifications. However, it was suggested that genomics and proteomics often result in conflicting (Tanaka *et al*., 2008). This can be the reason that the rate of protein degradation is not so fast as those of mRNA (Tanaka *et al*., 2008).

Metabolomics is an emerging new omics science analogous to genomics, transcriptomics and proteomics, and can be regarded as the end point of the "omics" cascade (Tanaka *et al*., 2007, 2008). The advantage of metabolomic analysis is that the biochemical consequences of mutations and stress response mechanisms can be observed directly. As the metabolome represents a wide variety of chemical compounds, it is logical that numerous high-throughput analytical techniques are being used for metabolomics. Being non-destructive, nuclear magnetic resonance (NMR) spectroscopy is highly beneficial as a metabolomics technique (Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008), but it also possesses one major disadvantage, which is that it is relatively insensitive compared to mass spectrometry (MS) (Higashi *et al*., 2008). In the rapidly growing field of metabolomics, MS (Mass Spectrometry) coupled to a chromatographic separation technique is a useful method used to profile low molecular weight compounds (Soga *et al*., 2002, 2003; Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008). Capillary electrophoresis (CE)-mass spectrometry (CE/MS) has been considered a highly promising technique for comprehensive metabolomics analysis because most of the metabolites are polar and ionic compounds. It gives high-resolution separations of cationic metabolites, anionic metabolites and nucleotides/CoA in a reasonable time, and requires a minimum amount of samples (Soga *et al*., 2002, 2003; Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008).

It will be obtained new findings by combining genomic analysis with metabolomic analysis. Thus, we would like to discuss the development of a combined omics approach for environmental monitoring of chemicals using yeast system. As the model chemicals, we selected cadmium. Cadmium is one of the most widely characterized chemicals. In Japan, cadmium is well known as a chemical that may cause Itai-Itai (which means "ouch-ouch") disease, which leads to nephrotoxicity (Shibasaki *et al*., 1993), hepatotoxicity (Hussain *et al*., 1987), serious damage to the nervous system (Figueiredo-Pereira *et al*., 1998), and high frequency of chromatid aberrations in Japan (Shiraishi, 1975). In this report, we describe the development of a combined omics approach (genomics and metabolomics) for environmental toxicicology.

MATERIALS AND METHOD

Strains and growth conditions

Saccharomyces cerevisiae

S288C (*MATalfa*, *SUC2 mal mel gal2 CUP1*) was grown in YPD medium (1% Bacto Yeast Extract, 2% polypeptone, 2% glucose) at 25°C according as described previously (Momose and Iwahashi, 2001). Yeast cells were incubated with 0.3 mM cadmium chloride (Iwahashi *et al*., 2007) for 2 h and were supplied for DNA microarray and CE/MS analysis.

DNA microarray analysis

Each microarray, spotted on a glass slide for hybridization with labeled mRNA probes, represented almost all ORFs of yeast (5809~5819 genes; DNA Chip Research Inc. Yokohama, Japan). Extraction of total RNA, mRNA purification, labeling with Cy3 or Cy5, and hybridization were described previously (Momose and Iwahashi, 2001). A Scan Array 4000 laser scanner (GSI Lunomics, Billeria, MA, USA) was used to acquire hybridization signals. Array images were analyzed with Gene Pix 4000 (Inter Medical, Nagoya, Japan) (Iwahashi *et al*., 2007).

Capillary electrophoresis/mass spectrum (CE/MS) analysis

A Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Tokyo, Japan) was connected to an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Yokohama, Japan) through an electrospray ionization (ESI) source (Agilent Technologies Japan, Tokyo, Japan) (Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008). Yeast extracts were prepared after the stress conditions by filtration of yeast cells as described previously (Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008).

RESULTS AND DISCUSSION

Yeast genomics for the assessment of cadmium toxicology

Figure 1 shows cluster analysis of expression profiles obtained after the stress treatments (Tanaka *et al*., 2008). This calculation is based on the correlation factors between the treatments. We may select calculation methods and the calculations were mainly based on the Euclidean distance (distance between the treatments) or Pearson CC (direction or angle between the treatments). In any calculation what we can obtain is the similarities among the expression profiles of environmental toxicities. Expression profiles reflect the effect of toxicities on cells and the effect must be specific to the toxic treatments. We may speculate that clustering represents similar responses among stresses. Correlation factors for the expression profiles must be high among treatments that cause similar damages or responses. For example, zineb, maneb, and thiuram belong to dithiocarbamate fungicides and they have similar chemical structures (Kitagawa *et al*., 2003).

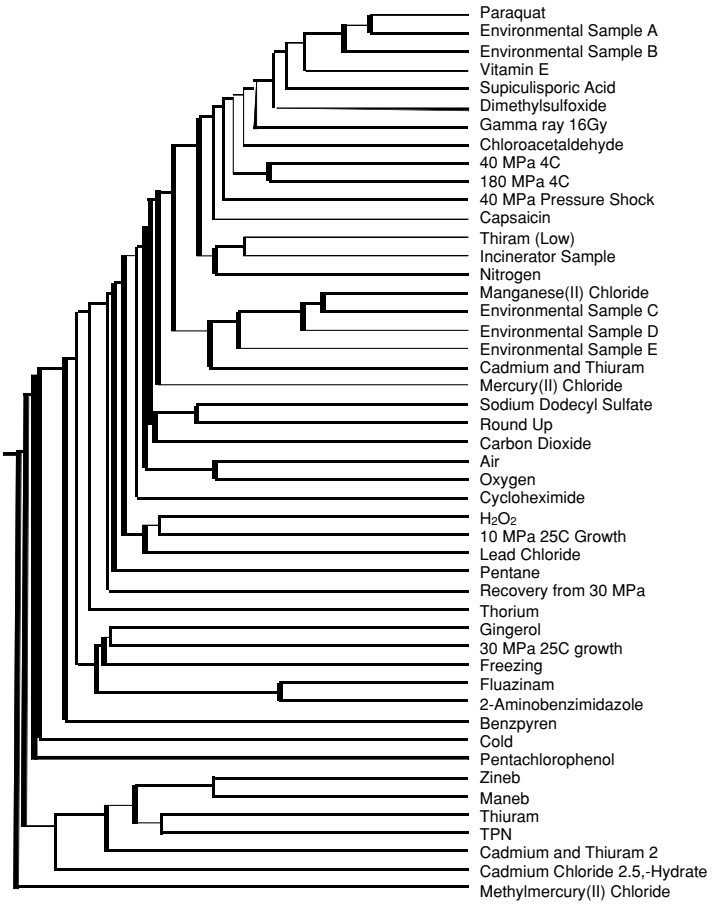

Fig. 1. Cluster analysis of the various environmental stress treatments to yeast cells. Each stress conditions was described previously (Tanaka *et al*., 2008).

Thus, these chemicals were expected to cause similar damages or similar cellular response (expression profile). As shown in Fig. 1, Zineb, Mneb, and Thiuram were clustered and we may conclude that these chemicals cause similar damages or responses. Thus, cluster analysis allows us to understand the effect of toxicities on yeast cells. The expression profile by the cadmium treatment (0.3 mM for 2 h at 30°C) had the cluster with those of dithiocarbamate fungicides and this suggests the similar toxicity of cadmium with those fungicides. This similarity may come from the similar oxidative stress to yeast cells as discuss later.

The list of induced genes by toxic treatments, help us to understand the toxic action to yeast cells. DNA microarray analyses of the global transcriptional response after exposure of cells to cadmium (0.3 mM for 2 h at 30°C) showed 310

Table 1. List of METgenes with the induction values after the cadmium treatment.

Systematic name	Fold induction	Gnene namae	Function
YKR069W	5.8	MET1	siroheme synthase
YNL277W	10.2	MET2	homoserine *O*-trans-acetylase
YJR010W	6.7	MET3	ATP sulfurylase
YNL103W	3.6	MET4	member of the leucine zipper family of transcriptiona
YER091C	3.8	MET6	isozyme of methionine synthase
YOR241W	0.6	MET7	folylpolyglutamate synthetase
YBR213W	1.5	MET8	effector of PAPS reductase and sulfite reductase
YFR030W	4.6	MET10	subunit of assimilatory sulfite reductase
YPL023C	1.1	MET12	putative methylenetetrahydrofolate reductase (mthfr)
YGL125W	3.1	MET13	putative methylenetetrahydrofolate reductase (mthfr)
YKL001C	16.3	MET14	adenylylsulfate kinase
YPR167C	4.8	MET16	3′phosphoadenylylsulfate reductase
YLR303W	16.3	MET17	*O*-Acetylhomoserine-*O*-Acetylserine Sulfhydralase
YIL128W	1.5	MET18	regulator of TFIIH
YOL064C	2.1	MET22	3′(2′)5′-bisphosphate nucleotidase
YIR017C	5.1	MET28	transcriptional activator in the Cbf1p-Met4p-Met28p
YIL046W	2.4	MET30	nteracts with and regulates Met4p
YPL038W	1.4	MET31	regulator of sulfur amino acid metabolism
YDR253C	7.3	MET32	regulator of sulfur amino acid metabolism

genes respond with increased mRNA levels (Momose and Iwahashi, 2001). These genes were annotated using functional categories assigned by the Munich Information Center for Protein Sequences (MIPS http://www.mips.biochem.mpg.de/). The highly up-regulated categories are cell rescue, defence, cell death and ageing (13%), transport facilitation (9%), energy (9%), metabolism (8%), and ionic homeostasis (7%). Among the subcategories, nitrogen and sulfur transport (50%), amino acid transporters (23%), nitrogen and sulfur utilization (22%), nitrogen and sulfur metabolism (23%), allatoin and allatonate transporters (22%), and other protein-destination activity (29%) are highly induced. These results strongly suggested that cadmium caused oxidative stress and induction of the pathway of sulphur amino acid (Momose and Iwahashi, 2001) and this aspect agree with the results by cluster analysis. In particular, almost all the genes involved in sulfur amino acid metabolism (*MET* genes) of which the final product is glutathione, were particularly induced (Table 1). These results indicate that glutathione synthesis is activated via whole sulfur amino acid synthesis. Recently it is also reported the strong induction of 9 enzymes of the sulfur amino acid biosynthetic pathway by cadmium (Dormer *et al.*, 2000). Thus, the addition of cadmium made yeast cells use glutathione. Consequently, cells needed to activate the sulfur salvage pathway via sulfur amino acid metabolism to have de novo synthesis of glutathione molecules for a defence system to oxidative stress.

Metabolomics technology for understanding flows of metabolites

Genomics is mainly evaluation systems for induced functions but not the products by induced functions. In contrast, metabolomics is a system for evaluating

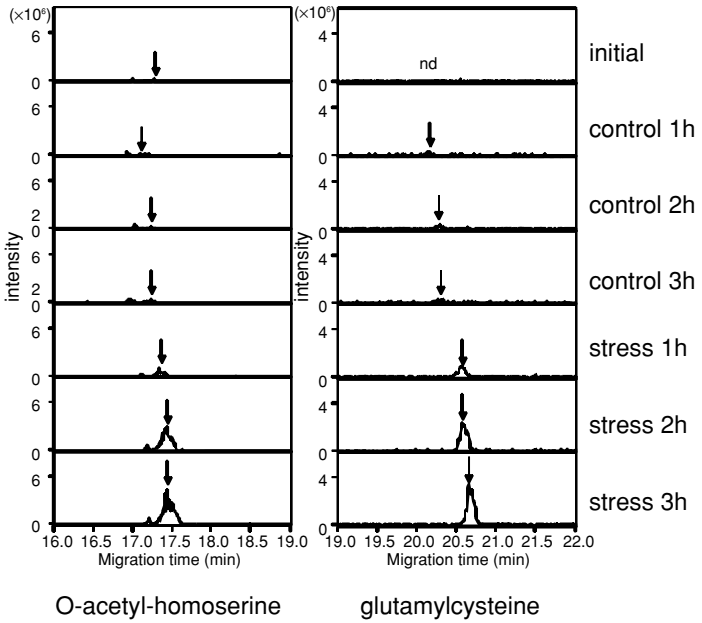

Fig. 2. Electropherograms of *O*-acetyl-L-homoserine and glutamylcysteine during the cadmium stress treatment obtained by CE/MS.

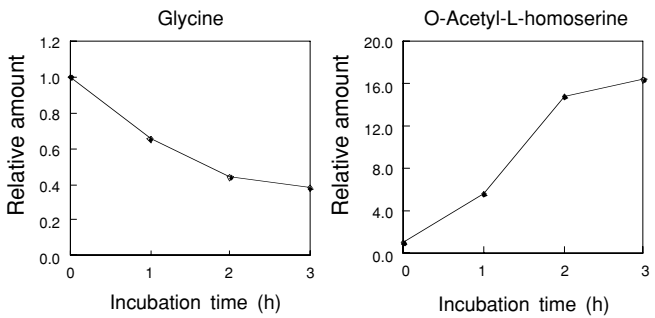

Fig. 3. Negative and positive accumulation of glycine and *O*-acetyl-L-homoserine during the cadmium stress conditions.

substances as the products of induced functions. Thus, metabolomics possibly yield direct evidence of cellular stress response. We are constructing a metabolomics system using CE/MS equipment (Tanaka *et al*., 2007, 2008; Higashi *et al*., 2008). CE/MS was selected because this system is suitable for analysis of low molecular weight and ionic substances. The majority of metabolites are considered as ionic and small substances.

Table 2. Relative amount of metabolites during cadmium treatment.

Metabolite	M.W.	Incubation time (h)			
		0	1	2	3
Glycine	75	1	0.65	0.44	0.38
Serine	105	1	0.28	0.24	0.53
Proline	115	1	0.98	1.56	1.78
Valine	117	1	1.04	1.38	1.53
Homoserine	119	1	0.71	0.92	1.02
Threonine	119	1	0.49	0.34	0.31
Iso-leucine	131	1	0.89	0.94	0.94
Leucine	131	1	0.79	0.71	0.73
Asparagine	132	1	1.65	1.86	1.79
Aspartic acid	133	1	1.54	1.22	1.21
Lysine	146	1	1.37	1.48	1.21
Glutamine	146	1	2.32	2.30	1.97
Glutamic acid	147	1	1.04	1.24	1.32
Methionine	149	1	0.27	0.33	0.39
Histidine	155	1	0.91	0.98	1.08
O-Acetyl-L-homoserine	161	1	5.56	14.78	16.29
Phenylalanine	165	1	0.70	0.63	0.63
Arginine	174	1	1.20	1.32	1.37
Tyrosine	181	1	0.78	0.83	0.85
Tryptophan	204	1	1.17	0.99	1.00
O-Succinyl-L-homoserine	219	1	1.33	1.67	1.33
Cystathionine	222	1	0.80	0.59	0.49
Glutamylcysteine	250	1	9.10		
Adenosine	267	1	0.96	0.76	0.66
Glutathione, reduced	307	1	2.09	2.66	2.58

The extracted metabolites, which were injected hydrodynamically into the capillary inlet, migrated toward the MS instruments separately according to their electrophoretic mobilities (Soga *et al*., 2002, 2003; Tanaka *et al*., 2007, 2008). Thus we can monitor metabolites according to their ionic character and molecular weight. We applied 37 kinds of metabolites as candidates for analysis. These materials were selected according to the results obtained by genomics analysis (Tanaka *et al*., 2007, 2008). For example, we selected sulfur-containing metabolites as the stress treatment frequently induced genes related to sulfur amino acid metabolism. From group of compounds we could detect 17 metabolites in a cationic mode using a low pH electrolyte buffer (Tanaka *et al*., 2007, 2008). As the example, we showed the MS electropherograms of *O*-acetyl-L-homoserine and glutamylcysteine in Fig. 2. Under the cadmium stress conditions, we could detect the accumulating feature of these metabolites. Table 2 showed the relative amount of 25 metabolites according to stress exposure time. The significantly accumulated metabolites were reduced glutathione, glutamate, *O*-acetyl-L-homoserine and glutamylcysteine. This suggests the direction of the metabolite

flow to glutathione and thus we can conclude that genomics and metabolomics agree very well.

Combination of genomics and metabolomics show new feature for stress response

Glutathione is produced from glutamate, cysteine, and glycine. The first step is biosynthesis of glutamylcysteine from glutamate and cysteine, and the second step is glutathione from glutamylcysteine and glycine. As shown in Table 2, we could detect the significant accumulation of glutamate, cysteine, glutamylcysteine, and glutathione. However, we could not detect the accumulation of glycine. Figure 3 summarized the time course of relative amount of glycine and *O*-acetyl-L-homoserine. Glycine was decreased and *O*-acetyl-L-homoserine was increased during the stress conditions. Thus, the metabolomics results suggest that glycine was not accumulated by the cadmium treatment. This could be explained because of consumption of glycine to glutathione synthesis. Thus, we focused on genes for glycine biosynthesis. The key enzyme of glycine biosynthesis is homoserine kinase that phosphorylates homoserine and is encoded by *THR1*. Phosphorylated homoserine of *O*-phospho-homoserine will be glycine through threonine. The expression level of this gene was 0.36 fold compared to that of control. The key enzyme was not induced and glycine was not biosynthesized actively by the cadmium treatment. While, the acetylation of homoserine to *O*-acetyl-L-homoserine was activated to 10 times higher than that of control (Table 1). This suggests that homoserine mainly goes to cysteine through *O*-acetyl-L-homoserine. Thus, positive biosynthesis of *O*-acetyl-L-homoserine and negative biosynthesis of glycine were shown by genomics and metabolomics. Glycine is essential metabolite for the production of glutathione, thus we cannot conclude that cadmium accumulates glutathione. So far, no one will agree this conclusion. This shows the possibility that combination of genomics and metabolomics may develop new feature of biology. The work we have to do is now to prove this conclusion.

REFERENCES

Dormer, U. H., J. Westwater, N. F. McLaren, N. A. Kent, J. Mellor and D. J. Jamieson (2000): Cadmium-inducible expression of the yeast *GSH1* gene requires a functional sulfur-amino acid regulatory network. *J. Biol. Chem.*, **275**, 32611–32616.

Ekwall, B., C. Clemedson, B. Crafoord, B. Ekwall, S. Hallander, E. Walum and I. Bondesson (1998): MEIC Evaluation of Acute Systemic Toxicity. *Alter. Lab. Anim.*, **26**, 571–658.

Figueiredo-Pereira, M. E., S. Yakushin and G. Cohen (1998): Disruption of the intracellular sulfhydryl homeostasis by cadmium-induced oxidative stress leads to protein thiolation and ubiquitination in neuronal cells. *J. Biol. Chem.*, **273**, 12703–12709.

Higashi, T., Y. Tanaka, R. Rakwal, J. Shibato, E. Kitagawa, S. Murata, S. Wakida and H. Iwahashi (2008): Environmental monitoring by use of genomics and metabolomics technologies. In *Atmospheric and Biological Environmental Monitoring*, ed. by Y. J. Kim *et al.*, Springer, Dordrecht (in press).

Hussain, T., G. S. Shukla and S. V. Chandra (1987): Effects of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats—*in vivo* and *in vitro* studies. *Pharmacol. Toxicol.*, **60**, 355–358.

Iwahashi, H. (2000): Multiple-end-point bioassay using microorganisms. *Biotech. Biop. Eng.*, **5**,

400–406.
Iwahashi, H. (2006): Yeast genes that star in the cross protection against environmental stress. *Cryobiol. Cryotech.*, **52**, 55–59.
Iwahashi, H., E. Ishidou, E. Kitagawa and Y. Momose (2007): Combined cadmium and thiuram show synergistic toxicity and induce mitochondrial petite mutants. *Environ. Sci. Technol.*, **41**, 7941–7946.
Kitagawa, E., Y. Momose and H. Iwahashi (2003): Correlation of the structures of agricultural fungicides to gene expression in *Saccharomyces cerevisiae* upon exposure to toxic doses. *Environ. Sci. Technol.*, **15**, 2788–2793.
Momose, Y. and H. Iwahashi (2001): Bioassay of cadmium using a DNA microarray: Genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. *Environ. Sci. Technol.*, **20**, 2353–2360.
Shibasaki, T., I. Ohno, F. Ishimoto and O. Sakai (1993): Characteristics of cadmium-induced nephrotoxicity in Syrian hamsters. *Nippon Jinzo Gakkai Shi*, **8**, 913–917.
Shiraishi, Y. (1975): Cytogenetic studies in 12 patients with itai-itai disease. *Human Genetics*, **27**, 31–44.
Soga, T., Y. Ueno, H. Naraoka, Y. Ohashi, M. Tomita and T. Nishioka (2002): Simultaneous determination of anionic intermediates for Bacillus subtilis metabolic pathways by capillary electrophoresis electrospray ionization mass spectrometry. *Anal. Chem.*, **74**, 2233–2239.
Soga, T., Y. Ohashi, Y. Ueno, H. Naraoka, M. Tomita and T. Nishioka (2003): Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *J. Proteome Res.*, **2**, 488–494.
Suzuki, M. and H. Utsumi (1998): Introduction. p. III–IV. In *Bioassay for the Control of Chemicals*, ed. by M. Suzuki and H. Utsumi, Koudannsya Press, Tokyo.
Tanaka, Y., T. Higashi, R. Rakwal, S. Wakida and H. Iwahashi (2007): Quantitative analysis of sulfur-related metabolites in yeast by capillary electrophoresis-mass spectrometry and its application of cadmium stress response. *Journal of Pharmaceutical and Biomedical Analyisis*, **44**, 608–613.
Tanaka, Y., T. Higashi, R. Rakwal, J. Shibato, E. Kitagawa, S. Murata, S. Wakida and H. Iwahashi (2008): *Saccharomyces cerevisiae* OMICS as the tools of environmental monitoring for chemicals, radiation, and physical stresses. p. 325–337. In *Advanced Environmental Monitoring*, ed by Y. J. Kim and U. Platt, Springer, Dordrecht.

Y. Tanaka, T. Higashi, R. Rakwal, J. Shibato, S. Wakida and H. Iwahashi (e-mail: hitoshi.iwahashi@aist.go.jp)