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Bioassay of Pesticide Lindane Using Yeast-DNA Microarray Technology

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Abstract

We analysed the gene expression pattern of *Saccharomyces cerevisiae* in response to lindane, a strong toxicant widely used as a pesticide in agriculture and by public health services. cDNA preparations from untreated cells and from cells treated with lindane were used to screen the DNA microarrays of about 6,000 genes. A total of 288 genes showed >2-fold induction in transcript levels, out of which 112 have not yet been characterized. The functional analysis of most known genes indicates that genes involved with mitochondrial dysfunction, oxidative stress, ionic homeostasis, mitochondrial organization, or biogenesis responded to lindane-mediated stress. In addition, several induced genes were shown to contribute to ER-mediated degradation and quality control. However, no significant changes in the transcript levels of ORFs related to DNA damage and repair were observed. Furthermore, the mRNA levels of some uncharacterized genes are significantly high, and the unveiling of these genes, along with that of known genes, might provide the opportunity to illustrate how yeast responds to environmental perturbation. This analysis will also facilitate the identification of some specific genes that could be used as biomarkers for a toxicity assay of lindane or other similar environmental pollutants.

Key Words: Bioassay, Environmental pollution, toxigenicity, DNA microarray, Lindane

Area of interest: Genome Wide Experimental Data Analyses

1. Introduction

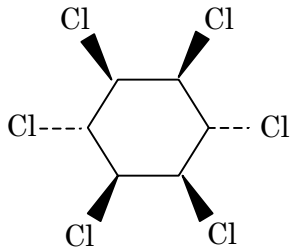
A large number of pesticides and insecticides are used in agriculture and by public health services without there being a complete appreciation of their potential damage to the environment. In addition, consumers are increasingly concerned about pesticide residues in agricultural crops, because of the potential risks of cancer and other diseases. Pesticides are potential chemical mutagens. A recent review, in which mutagenicity data on 100 commonly used pesticides were analyzed, revealed that 59%, 83%, and 71% of the tested chemicals were active in causing gene mutations, chromosomal damage, and DNA damage, respectively [1]. This calls for inexpensive, timely, and sensitive bio -monitoring of the health-associated risks of environmental pollutants.

Most of the existing bioassays are based primarily on a single end point, e.g., lethality or reproduction, and are inadequate in terms of the sensitivity, accuracy, duration, and expense of the test [2]. Alternative *in vitro* systems have been developed based upon biochemical alterations in response to environmental perturbation at the cellular level [2]. These methods allow the detection of macromolecules, namely, those either targeted by the chemicals or expressed in response to adverse conditions, such as different kinds of heat-shock proteins (Hsp) [2]. These molecules have become specific indicators of exposure to environmental pollution and their biochemical effects. However, some limitations, such as differences in response among species and variation in constitutive Hsp levels, have restricted the application of this bioassay method [2].

One alternative bioassay method could be the use of a model system to examine changes in gene expression as indicators of biochemical effects. Recently developed cDNA microarrays can be used effectively for this purpose [3]. It is quite understandable that minor changes in the environment might provoke a wide range of responses in a living cell. Such responses could induce a change in the expression of a specific gene or a group of genes that would enable a cell to adjust to the adverse conditions. A cDNA microarray technique has the potentiality to monitor the expression of macromolecules other than those expressed by common stress indicator genes, which could be used as biomarkers for a specific agent or group of chemical agents [4]. However, we have no standard method to understand toxicity from the numerous amount of information obtained by DNA microarray analysis.

In this study, we used yeast as a model organism to develop a bioassay based on DNA microarray analysis and a bioinformatics procedure to understand the toxicity of chemicals. As a eukaryotic organism, the cellular structure of yeast greatly mimics that of higher organisms, and yeast can easily adapt to altered environmental conditions. In addition, a yeast cDNA microarray of about 6,000 genes, which represents nearly the entire genome, is available. This prompted us to carry out a study to establish a yeast microarray-based bioassay to determine the toxicity and classification of environmental pollutants. We are also interested in developing a database of stress-responsive genes using already defined toxicants, as a means of developing standard methods to understand the toxicity from the data obtained by DNA microarray. As a part of this study, we tested the organochlorine pesticide lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), which is widely used as a pesticide in agriculture, in public health services, and as a wood preservative [5]. In agriculture, lindane is used as a soil insecticide as well as a seed dressing and for foliar application on rice, potato, tomato, onion, and other crops [6]. As a neuro-excitator, lindane produces hyperexcitability and convulsions in humans and other animals [7][8]. The TDL0 for humans is suggested to be 111-180 mg/kg, LD50 for rats is 35mg/kg, and LD50 for mice is 86mg/kg (http://w-chemdb.nies.go.jp/). Since it is a persistent chemical, its use as a pesticide causes the accumulation of lindane in the environment. Thus, lindane could be considered as an agricultural pollutant. It has

been reported to cause mitochondrial curing but not chromosomal mutation in *Saccharomyces cerevisiae* [9]; however, this study did not explain the underlying mechanisms of mitochondrial toxicity. In mammals, lindane toxicity has been shown to be associated with impaired male reproductive function and low steroidogenic activity [10][11][12]. Here, we want to suggest two methods to explain the global gene expression program employed by yeast in response to stress induced by lindane. One is classification of induced and repressed genes according to the functional gene category. The other is hierarchical clustering to characterize gene expression profiles by comparison with those of other chemicals and physical stress.



Lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane)

2. Materials and Methods

2.1 Strain, chemicals, and growth conditions

Saccharomyces cerevisiae strain S288C (α SUC2 mal gal2 CUP1) was used as an indicator strain for cDNA microarray analysis. It was grown at 25°C to a mid-log phase (A_{660}=1) in 200ml of YPD medium (2% polypeptone, 1% yeast extract, and 2% glucose). To optimise the culture conditions for lindane, different amounts of lindane were directly added to the exponentially growing cultures. Lindane was purchased from Sigma-Aldrich K.K. (Tokyo, Japan). Growth was measured from the optical density of the culture at different indicated time points. For transcriptional analysis, exponentially growing cells were given exposure to an optimised concentration of lindane (1.3 mM) dissolved in DMSO for 2h, and control cells were treated with DMSO alone. These experiments were carried out three times as independent culture experiments. After treatment, the cells were harvested by centrifugation, and the cell pellets were stored at -80°C until used for RNA extraction.

2.2 Preparation of mRNA and hybridizations

Total RNA was isolated by a hot-phenol method as described elsewhere [13]. Poly (A) +RNA was purified from total RNA with an Oligotex –dT30 mRNA purification kit (Takara, Kyoto, Japan). Fluorescently labeled cDNA was synthesized by oligo dT-primed polymerization using PowerScript™ reverse transcriptase (Clontech, CA, USA). The cDNA made from the poly (A)+RNA of the control cells was fluorescently labeled with Cy3, and that of the lindane-treated sample was labeled with Cy5. The reason for this choice of labelling was two fold. The first reason was based on experimental conditions; yeast cells were cultured with lindane and they grew slowly during the experimental period (Figure 1). A lower growth rate represses some genes, mainly those

related to protein synthesis [14], yet the repressed genes do not necessarily reflect a specific effect of the lindane treatment. This means that the information on repressed genes does not help us to understand the specific effect of the lindane treatment. The second reason for the labelling was based on the stability of the dyes during scanning. The scanning laser damages dyes, and this damage is more significant for Cy5; Cy5 breaks more quickly during scanning than does Cy3. Furthermore, breakage is significant in spots with low intensities. As a result, a control spot gave higher induced values when it was labelled with Cy5 than when it was labelled with Cy3. This is important for the actual induced genes, as the intensity of the control should be lower than that of the lindane-treated cells. The mean values of the Cy3-labeled control and the Cy5-labeled control had much greater margins of error than the values obtained for the control labelled with Cy3 and the sample labelled with Cy5 (data not shown). For each labelling, 2-4μg of poly (A)+RNA was used. The two labelled cDNA pools were mixed and hybridized with a yeast DNA chip (DNA Chip Research, Inc., Yokohama, Japan) for 24-36h at 65^{o}C. A DNA chip represents the ORFs of almost all of these genes of yeast (5884) printed as spots on a glass slide.

2.3 Microarray analysis

After hybridization, the labelled microarrays were washed and dried. Subsequently, labelled microarrays were scanned with a confocal laser ScanArray 4000 system (GSI Lumonics, Billerica, MA, USA). The resulting image data were quantified by using the QuantArray Quantitative Microarray Analysis application program (GSI Luminics, Billerica, MA, USA). The fluorescence intensity of each spot on an image was subtracted from each background by the fixed circle method for each image, and the ratio of intensity of Cy5/Cy3 was calculated and normalized with ACT1 (positive control) by using the GeneSpring software (Silicon Genetics, CA, USA). The background around the spot was subtracted from the fluorescence intensity of each spot and any signal arising from the TE buffer was subtracted using the value obtained from a spot of TE only in each block of 16 spots. Expression was calculated by the following method: expression ratio = (normalized cy5 intensity/normalized cy3 intensity). The details of the microarray procedure have been described previously [14]. The relative-fold changes in the ratios of fluorescent intensity represent the average change in gene expression affected by the lindane treatment. More than a two-fold increase was considered to be induced. Induced ORFs were categorized into different functional subcategories according to the Munich International Centre For Protein Sequence (MIPS, http://www.mipsbiochem.mpg.de/) classification systems. MIPS has developed a process for analysing and presenting the expression and functional data concerning yeast genes collected from different sources.

2.4 Hierarchical clustering

Hierarchical clustering was carried out by using Gene Spring [15][16]. The settings for the calculations were as follows: The similarity was measured by standard correlation, the separation ratio was 1.0, and the minimum distance was 0.001 [15][16]. For the calculation, 3874 genes were used. These genes were selected if they had ever been induced with above-average intensity under at least one condition. This selection was made to ignore low-intensity genes. Low intensity means a high possibility of getting erroneous results.

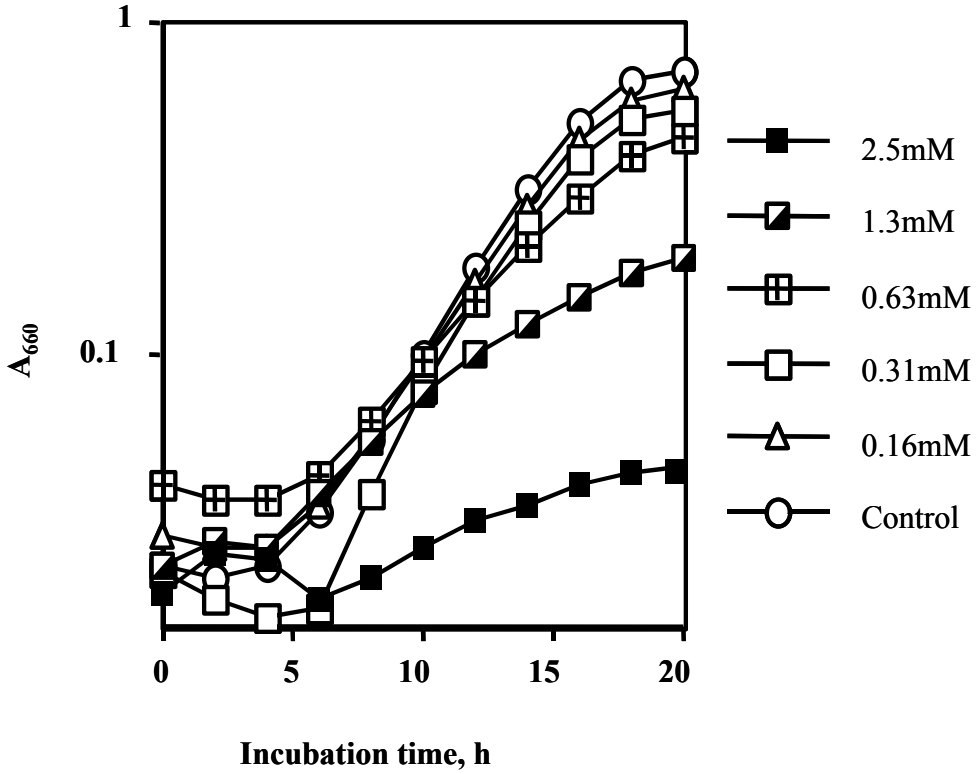


Figure 1. Growth profile of yeast cells treated by lindane within 2h. Lindane was added to the YPD medium at the concentration indicated in the figure, and growth was monitored at 660nm absorbance. Yeast cells were grown with 2.5 mM (■), 1.3 mM (◪), 0.63 mM (⊞), 0.31 mM (□), 0.16 mM (Δ), and control (O).

3. Results and discussion

3.1 Experimental conditions for lindane treatment

To obtain a maximum response to lindane treatment, we grew the yeast in different concentrations of lindane, ranging from 0 to 2.5 mM, for different lengths of time, as shown in Figure 1. We estimated that 1.3 mM of lindane could inhibit approximately 50% of the growth and induce detectable changes in the transcript levels. Our conditions may not reflect the direct effect of lindane on cells, because this concentration is much higher than those of the TDL0 for humans, LD50 for rats, and LD50 for mice (http://w-chemdb.nies.go.jp/). However, higher concentrations of lindane (2.5 mM) exerted severe inhibitory effects on growth, and we failed to isolate enough mRNA from the living cells. Similarly, a smaller amount of lindane failed to evoke any detectable response to induce gene expression. For the cDNA microarray analysis, exponentially growing cells (A_{660}= 1.0) were given a 2h treatment in 1.3 mM of lindane, and mRNA was collected for hybridization with the microarray. In a previous report, we showed that a 2h treatment was not always the best for the detection of all genes, but that it was sufficient for the detection of the induced genes [14].

3.2 Overview of gene expression

As a strong toxicant, lindane was reported to cause mitochondrial curing in yeast cells [9]. Therefore, presumably, a cell exposed to lindane will try to protect itself from the toxigenic effects of lindane by activating pathways to either replace the mitochondrial function or to somehow thwart the activity of lindane, e.g., by preventing its entry into the cell or by setting up a detoxification after cell entry. In this process, the gene expression profile will inevitably be altered, i.e., some genes would be up-regulated or some would be repressed. To monitor overall changes in the expression, 6,000 ORFs of *Saccharomyces cerevisiae* were screened by DNA microarray technology. After repeating three independent experimental sets (the control and lindane treatments), three sets of microarray data were obtained and the average ratios (Cy5 of the chemical treatment/Cy3 of the control) of hybridization intensity were used for further analysis. A ratio of hybridization intensity (Cy5/Cy3) of >2.0-fold was considered to indicate an up-regulated ORF. In a preliminary experiment, we found that more than 99.9% of genes showed less than 2.0 and 99% of genes showed more than 0.5 with the same source of mRNA (data not shown). In the lindane-treated cells, 288 ORFs (5%) were up-regulated. As the first methods, their biological characteristics were classified in the functional categories of MIPS, as shown in Figure 2. In the functional categories, the number of genes differ among the categories, thus the percentages of induced or repressed genes in the categories were shown in Figures 2 and 3. In response to lindane treatment, the number of induced genes was the highest in the categories of cellular organization (80 genes), followed by metabolism (72 ORFs); protein destination (32 ORFs); cell rescue, defense, cell death, and aging (28 ORFs); energy (28 ORFs); and ionic homeostasis (10 ORFs), as shown in Figure 2. In the categories of ionic homeostasis, we could see a significant induction of genes concerned with the homeostasis of cations (9 ORFs). Detoxification (13 ORFs) and stress-response (17 ORFs) genes were also observed in the categories of cell rescue, defense, cell death, and aging (28 ORFs). In case of metabolism categories, significant gene induction was observed with amino acid degradation (4 ORFs), amino acid transport (1 ORF), c-compound and carbohydrate utilization (36 ORFs), and lipid, fatty acid, and isoprenoid metabolism (10 ORFs). Perturbations in the ER and mitochondrial functions of treated cells were observed by augmented expression of different ORFs concerned with ER and mitochondrial function. Interestingly, the functions of a large set of induced genes (112 ORFs, about 40% of induced genes) are not yet known, although the mRNA levels of some of them are even higher than those of ORFs with known functions.

In addition, ratios of hybridization intensity below 0.5 were considered to indicate down-regulated ORFs. A total of 346 ORFs (6%) were found to be down-regulated, and they were mostly associated with protein synthesis (105 ORFs), cellular organization (195 ORFs), and metabolism (53 ORFs), as shown in Figure 3.

As the next step of classification, we tried to characterize toxicity by focusing on each functional category as shown below.

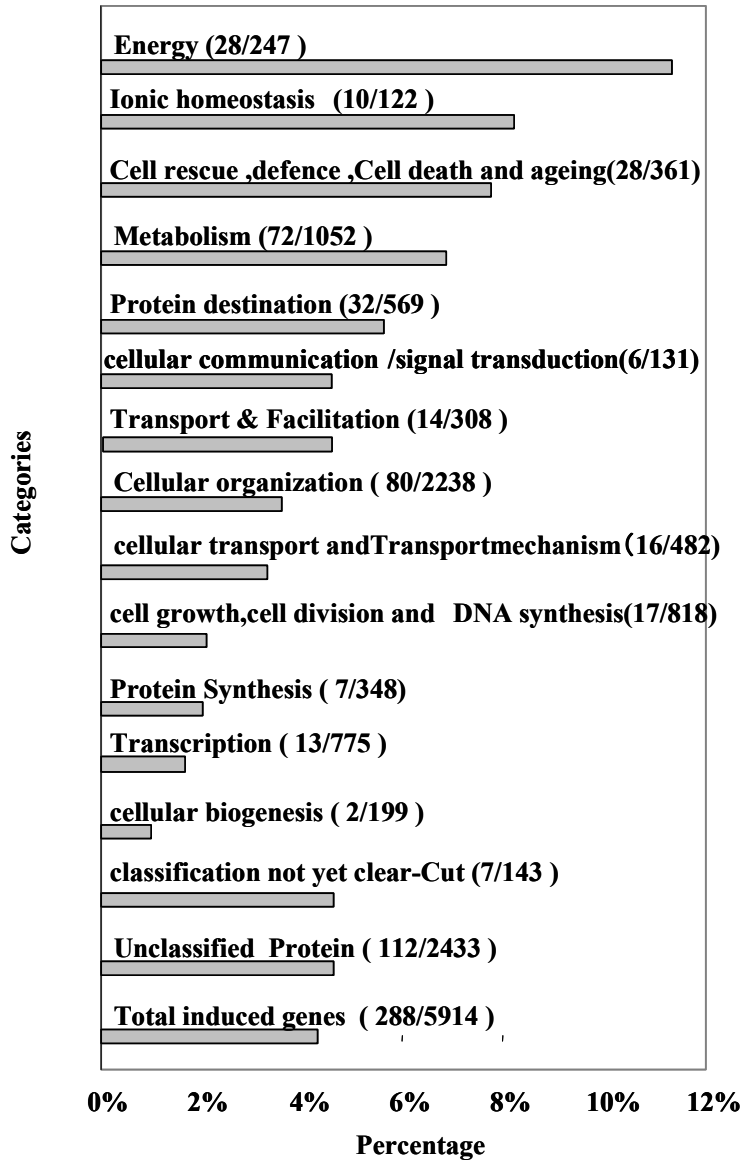


Figure 2. Classification of up-regulated genes into functional categories according to MIPS. The horizontal axis indicates the % of induced genes (induced ORFs/ total ORFs x100) belonging to different categories indicated by bar lines.

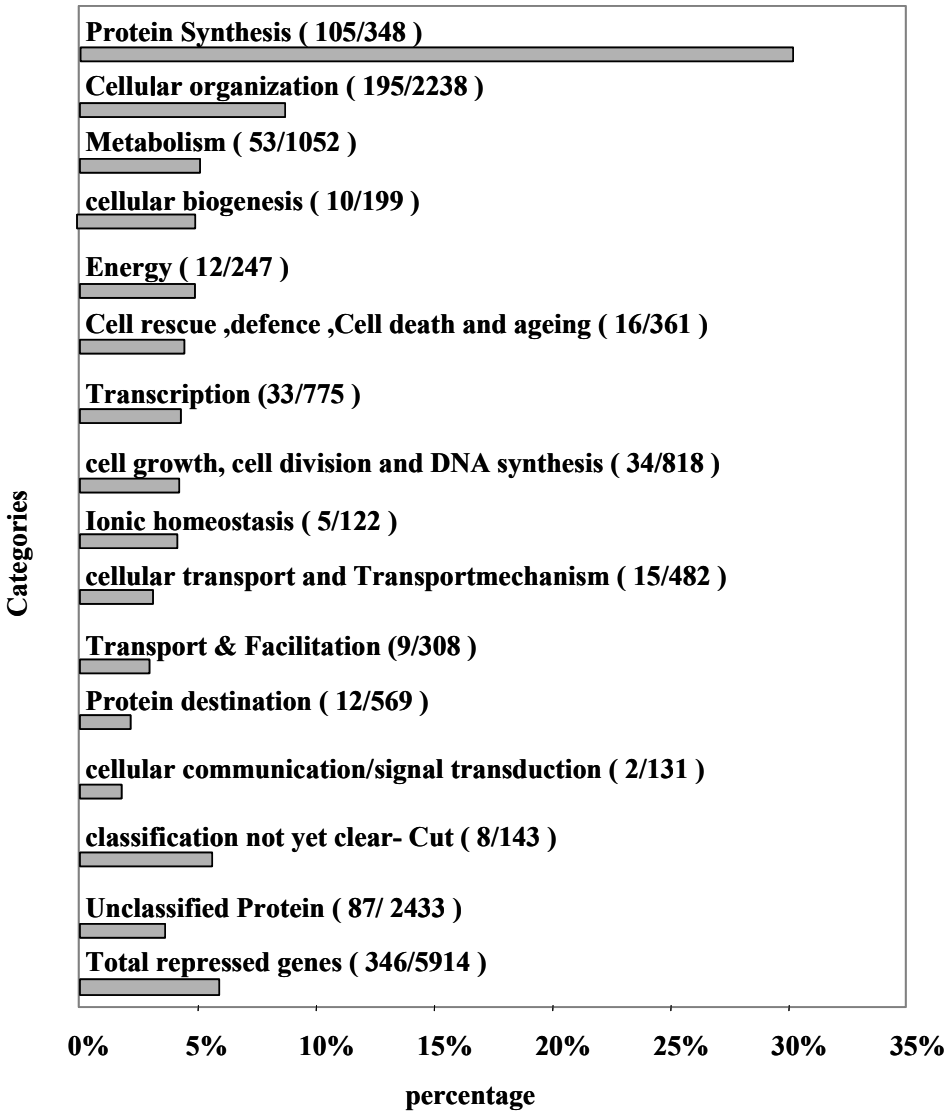


Figure 3. Classification of down- regulated genes into functional categories according to MIPS horizontal axis indicates the % of induced genes (induced ORF/ total ORF x 100) belonging to different categories indicated by bar lines.

3.3 Ionic homeostasis

Metal ions are essential for vital life processes, and their homeostasis is controlled through a strict regulation of uptake, storage, and secretion. For this purpose, cells have developed highly regulated control mechanisms, and any alteration in this system might severely affect the life processes [17]. Transcript profiles showed higher expressions of *MAC1*, which is a nuclear protein - reported to be induced in Cu-deficient cells. This protein serves as a transcription factor for some genes, such as *FET3* (multicopper oxidase), *CTR1* (Cu transporter), and *FRE1*, which are expressed in Cu-deprived cells under the tight control of *MAC1* [18][19]. Consistently with this finding, in lindane-treated cells, the mRNA levels of *CTR1, FET5*, and *FRE3* were elevated along with *MAC1*. This observation indicates that the lindane treatment might induce Cu deprivation in the cell.

Table 1. Transcription of ORFs (> 2 fold) related to ionic homeostasis, transcription and drug transporters

ORFs	SD	Fold induc.	Gene name	Description
Homeostasis of cations				
YNL259C	2.3	4.9	ATX1	Antioxidant protein and metal homeostasis factor, protects against
YOR381W	1.3	3.6	FRE3	strong similarity to ferric reductase Fre2p
YFL041W	0.6	3.4	FET5	multicopper oxidase, type 1 integral membrane protein
YPR124W	0.4	2.9	CTR1	High affinity copper transporter into the cell, probable integral
YOR153W	0.5	2.6	PDR5	multidrug resistance transporter
YKL190W	0.3	2.0	CNB1	Type 2B protein phosphatase\; regulatory B subunit of calcineurin
YLL051C	0.7	2.0	FRE6	strong similarity to ferric reductase Fre2p
mRNA transcription				
YMR021C	1.2	3.0	MAC1	metal-binding transcriptional activator
YDR259C	1.1	2.8	YAP6	transcription factor, of a fungal-specific family of bzip proteins
YOR344C	0.2	2.4	TYE7	TYE7, a 33 kDa serine-rich protein, is a potential member of the basic
YMR139W	0.4	2.2	RIM11	Serine/threonine protein kinase, phosphorylates the mitotic activator
YPL203W	0.5	2.1	PKA3	cAMP-dependent protein kinase catalytic subunit
YKL190W	0.3	2.0	CNB1	Type 2B protein phosphatase\; regulatory B subunit of calcineurin
YPR107C	0.4	2.0	YTH1	protein of the 3' processing complex
YER159C	0.5	2.0	BUR6	Transcriptional regulator which functions in modulating the activity of the general transcription machinery in vivo
Drug transporters				
YHL047C	9.7	16.9	TAF1	similarity to C.carbonum toxin pump
YEL065W	1.6	4.3	SIT1	probable multidrug resistance protein
YHL040C	1.6	4.0	ARN1	ferrichrome-type siderophore transporter
YBR052C	1.0	3.0		strong similarity to S.pombe brefeldin A resistance protein obr1

Cu is an essential prosthetic group in proteins, such as superoxide dismutase (SOD), a scavenger of highly toxic free-oxygen radicals in cells, which, thus, helps cells to suppress oxidative stress [20]. In addition to perturbation in Cu uptake, the presence of lindane in the medium also causes up-regulation of genes involved in iron homeostasis. From an expression profile, we could see that the genes *TAF1* (*ARN2*) and *SIT1 (ARN3*) were significantly up-regulated. These genes are members of the major facilitator super family and are expressed as a part of the *AFT1* regulon [21]. *SIT1* and *TAF1* were reported to be expressed in vesicles and to act as transporters for bacterial siderophore ferrioxamine B (FOB) and for fungal siderophore triacetylfusarinine C, respectively, in yeast cells. The *TAF1*-disrupted mutant *Saccharomyces cerevisiae* was reported to show the complete loss of iron uptake and utilization [22]. From our results given in Table 1, we could see that one of the highly up-regulated genes among the lindane-treated cells was *TAF1* (>16.7-fold). This suggests that lindane causes a change in iron homeostasis by an unknown mechanism.

3.4 Transcription of ORFs to relieve oxidative stress

As mentioned above, it is reasonable to argue that lindane treatment might exert oxidative stress in cells, presumably by inactivating the superoxide dismutase (SOD) system, which can eliminate highly toxic free radicals. An increase in the Cu^{2+} uptake by induction of the *MAC1* transcription factor helps the cellular SOD system to detoxify free radicals and thus protect DNA and proteins from oxidative damage. Consistently with this concept, the expression profile in Table 2 also showed that the antioxidant *ATX1* was dramatically increased among the lindane-treated cells. *ATX1*, a metallochaperone, was observed to reduce the free radicals stoichiometrically [23]. A recent study showed that *ATX1* acts as an intra-organellar transporter of Cu molecules, particularly to a secretory pathway through the Golgi complex [24][25][26]. Also increased among the lindane-treated cells was *ZWF1*, which encodes glucose phosphate dehydrogenase. This enzyme plays an

Table 2. Transcription of ORFs (> 2 fold) related to stress response and detoxification

ORFs	SD	Fold induc.	Gene name	Description
Stress response				
YBR072W	0.4	6.6	HSP26	heat shock protein 26
YFL014W	1.7	5.5	HSP12	12 kDa heat shock protein
YDR258C	0.5	3.9	HSP78	Mitochondrial heat shock protein 78 kDa
YNL241C	1.0	3.2	ZWF1	Glucose-6-phosphate dehydrogenase
YML070W	0.8	3.1	DAK1	putative dihydroxyacetone kinase
YMR021C	1.2	3.0	MAC1	metal-binding transcriptional activator
YLL026W	0.8	2.9	HSP104	heat shock protein 104
YCL035C	0.6	2.7	GRX1	glutaredoxin
YLR109W	0.7	2.6	AHP1	Alkyl hydroperoxide reductase
YBR169C	1.1	2.5	SSE2	HSP70 family member, highly homologous to Sse1p
YMR173W	0.4	2.4	DDR48	heat shock protein
YNL160W	0.7	2.4	YGP1	YGP1 encodes gp37, a glycoprotein synthesized in response to nutrient limitation which is homologous to the sporulation-specific
YFR052W	0.1	2.2	RPN12	cytoplasmic 32 - 34 kDa protein
YIR038C	0.3	2.0	GTT1	glutathione S-transferase
YLL060C	0.1	2.0	GTT2	glutathione S-transferase
Detoxificaton				
YHL047C	9.7	16.9	TAF1	similarity to C.carbonum toxin pump
YNL259C	2.3	4.9	ATX1	Antioxidant protein and metal homeostasis factor, protects against
YEL065W	1.6	4.3	SIT1	probable multidrug resistance protein
YHL040C	1.6	4.0	ARN1	ferrichrome-type siderophore transporter
YNL241C	1.0	3.2	ZWF1	Glucose-6-phosphate dehydrogenase
YCL035C	0.6	2.7	GRX1	glutaredoxin
YLR043C	0.9	2.6	TRX1	thioredoxin
YOR153W	0.5	2.6	PDR5	multidrug resistance transporter
YNL239W	0.3	2.1	LAP3	Aminopeptidase of cysteine protease family
YIR038C	0.3	2.0	GTT1	glutathione S-transferase
YLL060C	0.1	2.0	GTT2	glutathione S-transferase

important role in the pentose phosphate pathway to produce the cellular- reducing agent $NADPH^{+}$, thus providing protection against oxidative stress [27]. Furthermore, *TRX1* and *GRX1*, which have been implicated in the suppression of oxidative stress by maintaining a reduced state in the cell [28][29][30], were also increased among lindane-treated cells. These two genes are targeted by the transcriptional factor *YAP1*. *YAP1* is induced in response to oxidative or other stresses and activates many downstream genes to protect cells from stress [28][29][30]. We did not observe up-regulation of *YAP1*; however, *YAP6*, a member of the same family, was up- regulated in the lindane-treated cells (Table 1). A recent study on *MCR1*, whose expression was augmented in lindane-treated cells (Table 4), reported that it encodes mitochondrial NADH b_5 reductase and, by removing free radicals, protects cells from oxidative damage [31]. Therefore, from the information presented above, we conclude that lindane treatment induces oxidative stress in yeast cells, and that, in response to this assault, cells activate various detoxification mechanisms to reduce the oxidative stress.

Several heat-shock proteins, *HSP12, HSP26, HSP78,* and *HSP104,* were also significantly elevated in lindane-treated cells as shown in Table 2. *HSP78* is a mitochondrial protein that was found to be crucial for the maintenance of respiratory competence and the integrity of mitochondrial chromosomes under stress conditions [32]. In addition to mitochondrial *HSP78,* the cytosolic chaperone *HSP104* also helps to reactivate damaged proteins accumulated under stress conditions; however, the chaperone *HSP12* was induced in response to a wide range of stresses.

3.5 Induction of ORFs associated with ER-mediated quality control

Proteins of secretory pathways undergo folding and maturation in the ER lumen, and, thus, ER-resident proteins include many chaperonins, glycosylases, and disulfide isomerases [33]. However, proteins that fail to acquire their proper structure are accumulated in the ER lumen where they eventually induce stress. To cope with this problem, a cell activates two different but overlapping pathways: an ER-associated degradation (ERAD) pathway and an unfolded protein response (UPR) [34]. By activating the UPR pathway, cells try to rescue the unfolded or misfolded protein by inducing correctional enzymes; those misfolded proteins, that can not be corrected by UPR, are relocated to the cytoplasm by an ERAD pathway for proteasome-mediated degradation. Some of the genes shown in Table 3 that are working in UPR pathways [35], such as *LHS1, EUG1,* and *ERO1,* were found to be up- regulated in the lindane-treated cells.

Table 3. Transcription of ORFs (>2 fold) related to organization of ER

ORFs	SD	Fold induc.	Gene name	Description
YKL065C	1.0	3.8	YET1	Yeast endoplasmic reticulum 25 kDa transmembrane protein
YBR201W	2.7	3.5	DER1	involved in degradation proteins in the ER
YJR073C	0.6	2.9	OPI3	Methylene-fatty-acyl-phospholipid synthase (unsaturated phospholipid N-methyltransferase)
YML130C	0.4	2.9	ERO1	protein kinase which functions at the G(sub)2/M boundary// involved in regulation of DNA replication //degraded in G1 by an anaphase-promoting complex and Hct1p dependent mechanism
YDR518W	0.9	2.7	EUG1	Protein disulfide isomerase homolog
YER012W	0.8	2.4	PRE1	22.6 kDa proteasome subunit
YFR052W	0.1	2.2	RPN12	cytoplasmic 32 - 34 kDa protein
YDR086C	1.0	2.2	SSS1	endoplasmic reticulum protein that is part of the Sec61 trimeric complex and the Ssh1 trimeric complex
YKL073W	0.6	2.1	LHS1	novel member of the Hsp70 family of molecular chaperones that localizes to the lumen of the endoplasmic reticulum://chaperone of the
YGL048C	0.3	2.0	RPT6	26S proteasome regulatory subunit :ATPase
YOR288C	0.3	2.0	MPD1	Disulfide isomerase related protein

Lhs1p is an *HSP70*, as is ER chaperonin, and helps misfolded proteins to fold correctly [36]. Ero1p (oxidoreductase) is required for the maintenance of an ER redox-potential [37], which is essential for many enzymatic reactions. *EUG1* is a protein disulfide isomerase (PDI)-like enzyme that helps the folding and maturation by catalyzing disulfide bond formation in the ER [38]. Some of the up-regulated genes, such as Der1p, *SSS1, PRE1,* and *RPN12* in Table 3 and probably *UBC8,* work in the ERAD pathway. Der1p is an ER-membrane protein that helps to remove misfolded proteins from the ER lumen to the cytosol for degradation [39]. *SSS1,* a small subunit of Sec61p that constitutes the ER pore, also plays an important role in protein translocation [40]. *PRE1* and *RPN12* are small subunit 20S proteasomes that are associated with ubiquitinated protein degradation [41]. Although the function of *UBC8* is still unknown, as a ubiquitin ligase, it might catalyze the ubiquitination of misfolded proteins for degradation [42]. From these findings, we can argue that lindane treatment causes the accumulation of misfolded or unfolded proteins in the ER lumen that have activated both the UPR and ERAD pathways to rescue the ER from stress. It is difficult to understand how lindane induces the ERAD/UPR pathway. While it was shown that the ability of misfolded ER proteins to stimulate Ca^{2+} influx at the plasma membrane did not require Ire1p or Hac1p, and Ca^{2+} influx and signaling factors were not required for initial UPR signaling, activation of the Ca^{2+} channel, calmodulin,

calcineurin, and other factors was necessary for the long-term survival of cells undergoing ER stress [43]. As *CNB1* (regulator for calcineurin) was induced (Table 1) but *IRE1* and *HAC1* (involved in the signaling pathway for UPR pathway) were not affected (Data not shown) by the lindane treatment, the ERAD/UPR pathway may be activated by a Ca^{2+}mediated ER stress response.

3.6 Transcription of ORFs related to mitochondrial organization

Global gene expression analysis also revealed that a large number of genes involved in mitochondrial organization were up-regulated in lindane-treated cells as shown in Table 4. We could see that ORFs expressing the mitochondrial ribosomal proteins *MRP8, MRPL10,* and *MRPL36* were abundantly induced. Other ORFs include the components of the respiratory complex, the TCA cycle, the transport factor, and stabilization factors. It is interesting to see that, in response to lindane, *CYC2,* a gene associated with cytochrome c import into mitochondria, was up- regulated. It was reported that, in addition to cytochrome c import, this gene also increases the stability against ionic imbalance and, thus, maintains mitochondrial integrity [44]. Only one ORF expressing the mitochondrial heat-shock protein *HSP78* was found to be elevated in the mRNA level in response to the provocation of lindane. This mitochondrial chaperonin was reported to have reactivated the damaged protein in stressed cells when stress was relieved. While it is difficult to rationalize the cause of higher expression of ribosomal proteins in lindane-treated cells, one possibility is that an increased rate of protein synthesis allows damaged mitochondria to recover. It is well known that a large number of proteins in stressed cells remain inactive or unstable and that *de novo* protein synthesis might occur to overcome a shortage of protein. Our data also showed that, by inducing a stabilizer Phb2p [45], cells try to save newly synthesized protein from oxidative damage or unwanted degradation (Table 6).

Table 4. Genes concerned with mitochondrial organization induced by the lindane treatment. Gene induction level > 2.

ORFs	SD	Ratio*	Gene name	Description
YKL142W	4.1	6.9	MRP8	mitochondrial ribosomal protein
YDR258C	0.5	3.9	HSP78	Mitochondrial heat shock protein 78 kDa
YGR008C	0.5	3.7	STF2	ATPase stabilizing factor
YDL174C	0.3	3.6	DLD1	mitochondrial enzyme D-lactate ferricytochrome c
YOR136W	0.7	3.2	IDH2	isocitrate dehydrogenase (NAD+) subunit 2, mitochondrial
YIL111W	0.2	3.2	COX5B	Cytochrome-c oxidase chain Vb
YNL284C	0.6	2.8	MRPL10	Mitochondrial ribosomal protein MRPL10 (YmL10)
YOR037W	1.0	2.5	CYC2	cytochrome c mitochondrial import factor
YKL150W	0.3	2.4	MCR1	NADH-cytochrome b5 reductase
YPL224C	0.6	2.2	MMT2	mitochondrial iron transporter
YIL155C	1.0	2.2	GUT2	glycerol-3-phosphate dehydrogenase, mitochondrial
YKL194C	0.4	2.1	MST1	mitochondrial threonine-tRNA synthetase
YGR028W	0.5	2.1	MSP1	40 kDa putative membrane-spanning ATPase
YML110C	0.2	2.0	DBI56	TBP Associated Factor 65 KDa
YBR122C	0.2	2.0	MRPL36	Mitochondrial ribosomal protein MRPL36 (YmL36)

3.7 Mitochondrial dysfunction

Since lindane was reported to cause mitochondrial damage [9], we were interested in determining whether a transcript profile could produce some evidence of mitochondrial dysfunction. We could see that the expression of several TCA cycle genes, *CIT2, IDH2,* and *DLD1,* was elevated

in lindane-treated cells in Table 5. It is now well established that cells with defective mitochondrial function, as a consequence of nuclear mutations or the total absence of mitochondrial DNA (ρ^0) [46], activate some nuclear genes (retrograde regulation) to compromise for the altered situation [47][48]. The best example of such retrograde response is the expression of *CIT2* and *DLD1,* which are induced in response to a mutation that disrupts mitochondrial function and causes a loss of TCA cycle activity or a loss of mitochondrial DNA [49][50][51]. It could be assumed that lindane treatment inhibits mitochondrial respiration and induces the expression of the nuclear *CIT2* gene to facilitate a more efficient utilization of carbon via the transfer of metabolites of glyoxalate cycles (succinate) to TCA cycles [52]. Our data also support recent microarray data on mitochondrial dysfunction that showed the up-regulation of many ORFs that provide support to the cells to overcome the blockage of the TCA cycle in ρ^0 cells [53]. Additionally, in lindane-treated cells, the ORF *DIP5* was dramatically up-regulated (about 9-fold), similarly to the case of ρ^0 cells [53]. *DIP5* encodes dicarboxylic amino acid permease, which enhances the transport of amino acids in ρ^0cells, which lack the functional amino acid biosynthesis pathway.

Table 5. Transcription of ORFs (>2 fold) related to tricarboxylic-acid pathway, glyoxylate cycle, fermentation and aminoacid transport

ORFs	SD	Fold induc.	Gene name	Description
Tricarboxylic-acid pathway				
YGR244C	1.0	3.9	LSC2	Succinate-CoA Ligase (ADP-Forming)
YOR136W	0.7	3.2	IDH2	isocitrate dehydrogenase (NAD+) subunit 2, mitochondrial
Glyoxylate cycle				
YCR005C	2.9	2.9	CIT2	non-mitochondrial citrate synthase, peroxisomal
YPR006C	0.4	2.8	ICL2	Isocitrate lyase, may be nonfunctional
Fermentation				
YPL088W	0.9	3.7		similarity to aryl-alcohol dehydrogenases
YDL174C	0.3	3.6	DLD1	mitochondrial enzyme D-lactate ferricytochrome c oxidoreductase
YAL060W	1.5	3.3	FUN49	stereospecific (2R, 3R)-2,3-butanediol dehydrogenase
Amino-acid transport				
YPL265W	5.3	8.9	DIP5	dicarboxylic amino acid permease

3.8 Transcription of ORFs related to DNA repair and replication

In our previous study, lindane was found to cause mitochondrial curing, but no chromosomal mutation was observed [9]. Thus, we focused on the genes concerned with the DNA repair system. We listed the genes associated with DNA damage, repair, and or replication in Table 6. However, our results did not show any significant changes in the transcript levels of ORFs that corresponded to the DNA damage or repair category. Only *HSP12,* which has been implicated with DNA repair, was found to increase; however, *HSP12* is expressed ubiquitously under various kinds of stress. These results are strongly consistent with the biological results shown previously.

3.9 Comparison of genome wide expression profiles caused by lindane with those by other physical and chemical stresses.

DNA microarray technology informs us of the expression levels for each gene after the exposure of living cells to stress conditions. In addition, these expression profiles can be used for the comparison of stresses by the statistical methods of cluster analysis as shown in Figure 4. In our laboratory, we are accumulating the genome-wide mRNA expression profiles obtained after exposing yeast cells to physical and chemical stresses (http://kasumi.nibh.go.jp/~egenomix/). For

chemical stress, we have so far accumulated profiles concerned with cadmium chloride (0.3 mM), mercuric chloride (0.7 mM), lead chloride (2 mM), sodium arsenite (0.3 mM), sodium n-dodecyl

Table 6. Genes concerned with DNA synthesis and replication,recombination and DNA repair, and DNA repair. Gene induction level >1.5.

ORFs	SD	Ratios*	Gene name	Description
DNA synthesis and replication				
YGR231C	0.1	2.1	PHB2	mitochondrial protein, prohibitin homolog\; homolog of mammalian BAP37 and S. cerevisiae Phb1p
YGR132C	0.1	1.5	PHB1	mitochondrial protein, prohibitin homolog\; similar to S. cerevisiae
YDR068W	0.1	1.5	DOS2	involved in genome stability
YJR006W	0.3	1.5	HYS2	DNA-directed DNA polymerase delta, 55 KD subunit
Recombination and DNA repair				
YIL072W	0.5	1.7	HOP1	Meiosis-specific protein involved in homologous chromosome synapsis and chiasmata formation
YPL194W	0.3	1.5	DDC1	DNA damage checkpoint gene
YDR030C	0.4	1.5	RAD28	Protein involved in the same pathway as Rad26p, has beta-transducin (WD-40) repeats
DNA repair				
YFL014W	1.7	5.5	HSP12	12 kDa heat shock protein
YGR144W	0.2	1.9	THI4	involved in thiamine biosynthesis and DNA repair
YDL200C	0.8	1.7	MGT1	O6-methylguanine DNA repair methyltransferase
YKL145W	0.4	1.6	RPT1	putative ATPase, 26S protease subunit component
YPL194W	0.3	1.5	DDC1	DNA damage checkpoint protein
YGR258C	0.3	1.5	RAD2	homolog of xeroderma pigmentosum group G (XPG) protein, copufurifies with transcription factor, TFIIH, mRNA is cell cycle regulated and induced by DNA damage and by meiosis
YJR006W	0.3	1.5	HYS2	DNA-directed DNA polymerase delta, 55 KD subunit

* Ratios were averages of 3 experiments.

benzosulfonate (LAS) (0.02%), sodium lauryl sulfate (SDS) (0.01%), capsaicin (0.82 mM), 2,4,5-trichlorophenol (TCP) (16 μM), thiuram (75 μM), TPN (10 μM), zineb (2 ppm), maneb (2 ppm), roundup (1500 times dilution), gingerol (1.36 mM), acrolein (0.20 mM), and dimethylsulfoxide (DMSO) (10%, 1.41 M). As for physical stresses, we have accumulated data concerning freezing and thawing (-80^{o}C) and hydrostatic pressures of 180 MPa and 40 MPa. Using the expression profiles caused by those stresses, we carried out cluster analysis. For this cluster analysis we decreased the number of target genes from 6000 to 3875. The ORFs selected were those that had shown high intensity (more than average) under at least one stress condition. This selection was carried out because ORFs of low intensity are likely to yield erroneous results. As shown in Figure 4, expression profiles were between those of gingerol and DMSO. DMSO was used as the solvent for capsaicin, 2,4,5-trichlorophenol, thiuram, TPN, zineb, maneb, and gingerol and was added to the control, thus the similar expression profile of lindane with DMSO can be the character of lindane treated cells. In the cluster from capsaicin [54] to DMSO [16], these chemicals cause significant damage in membrane structure and cellular organelles, which may be why the induced genes concerned with the ER (Table 3) and mitochondria (Table 4) by the lindane treatment are clustered between those chemicals. Thus, for yeast cells, lindane can be the chemical that mainly causes damage to membrane structures. It was reviewed that lindane affects membranes [55], and our results agree with the reviewed results. It is interesting that the agricultural chemical and environmental pollutant lindane was not included in the group of other agricultural chemicals and pollutants such as thiuram, TPN, zineb, and maneb. These chemicals may cause oxidative stress and can be mutagenic [56]. Cluster analysis shows that lindane is not among the group of chemicals that

strongly cause oxidative stress. Thus, cluster analysis can be used not only for finding similar chemicals but also for different chemicals.

Figure 4. Hierarchical clustering of genome-wide expression profiles in response to lindane and other chemicals. Yeast cells were treated with different chemicals for two hours as follows: Lindane (1.3mM), cadmium chloride (Cd)(0.3 mM), mercuric chloride (Hg)(0.7 mM), lead chloride (Pb)(2 mM), sodium arsenite (As)(0.3 mM), sodium n-dodecyl benzosulfonate (LAS) (0.02%), sodium lauryl sulfate (SDS) (0.01%), capsaicin (0.82 mM), 2,4,5-trichlorophenol (TCP) (16 µM), thiuram (75 µM), TPN (10 µM), zineb (2 ppm), maneb (2 ppm), Roundup (1500 times dilution), gingerol (1.36 mM), acrolein (0.20 mM), and dimethylsulfoxide (DMSO) (10%, 1.41 M). Physical stresses were treatment with 180 MPa at 4^{o}C for 0 minute (180 MPa), 40 MPa at 4^{o}C overnight (40 MPa), and -80^{o}C storage for one week (Freeze 60 min), and these cells were then allowed to recover for one hour at 25^{o}C.

Hierarchical clustering was carried out as described in materials and methods. All the data can be obtained from the database (http://kasumi.nibh.go.jp/~egenomix/).

3.10 Conclusion

In this study, we used a yeast cDNA microarray to analyse the transcription levels of about 6,000 genes, making an almost complete genomic program of the yeast life cycle, in response to lindane-mediated toxicity. We employed two methods to understand the toxicity from the global gene expression data. Classification of induced and repressed genes according to functional categories of genes helps us to understand the mechanism of toxicity. Then, hierarchical clustering to characterize gene expression profiles by comparison with those of other chemicals and physical

stress helps us to find the chemicals that have similar toxicity. The later methods can be applied for the prediction of chemicals in the environment. We could also expect that some genes with specialized functions would be expressed in response to specific toxicants and that those genes could be used as biomarkers for a specific toxicant or group of similar toxicants.

Consistently with earlier reports, our results indicate that lindane toxigenicity affected mitochondrial function. Consequently, numerous genes associated with mitochondrial organization and biogenesis were abundantly expressed. Similarly, in mammals, lindane-induced low steroidogenesis and defective male reproductive function were thought result from the altered function of some mitochondrial enzymes [12]. In addition, genes involved with oxidative stress and ionic homeostasis, which are linked with mitochondrial dysfunction, were also significantly activated in lindane-treated cells. Interestingly, we also observed the up-regulation of ERAD and UPR components in response to lindane treatment. However, our study did not show significant evidence to implicate lindane as a potential DNA damaging agent. Nevertheless, we could not exclude the possibilities that lindane metabolites might play a role in chromosomal mutation or that our experimental conditions might not have been appropriate to induce DNA damage. Furthermore, the functions of many highly induced genes have not been clarified yet, and, obviously, characterization of these genes will shed light on the mechanisms of lindane-induced toxigenicity at the physiological level as well as contribute to developing an effective bioassay method. Meher Parveen is supported by New Energy and Industrial Technology Development Organization (NEDO).

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