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Mechanisms of Patulin Toxicity under Conditions That Inhibit Yeast Growth

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Patulin, 4-hydroxy-4*H*-furo[3,2*c*]pyran-2(6*H*)-one, is one of the best characterized and most widely disseminated mycotoxins found in agricultural products. Nonetheless, the mechanisms by which patulin causes toxicity are not well understood. Thus, the cytotoxicity of patulin was characterized by analysis of the yeast transcriptome upon challenge with patulin. Interestingly, patulin-induced yeast gene expression profiles were found to be similar to gene expression patterns obtained after treatment with the antifungal agricultural chemicals thiuram, maneb, and zineb. Moreover, patulin treatment was found to activate protein degradation, especially proteasome activities, sulfur amino acid metabolism, and the defense system for oxidative stress. Damage to DNA by alkylation was also suggested, and this seemed to be repaired by recombinational and excision repair mechanisms. Furthermore, the results provide potential biomarker genes for the detection of patulin in agricultural products. The results suggest the possibility of applying the yeast transcriptome system for the evaluation of chemicals, especially for natural chemicals that are difficult to get by organic synthesis.

KEYWORDS: Patulin; DNA microarry; yeast; mutagenesis; mycotoxin

INTRODUCTION

Patulin, 4-hydroxy-4*H*-furo[3,2*c*]pyran-2(6*H*)-one (**Figure 1**), is produced by many different molds and was first isolated as an antimicrobial active compound from *Penicillium griseofulvum* in the 1940s (*1*). However, during the 1950s and 1960s, it became apparent that, in addition to its antibacterial, antiviral, and antiprotozoal activities, patulin was toxic to both plants and animals, and, during the 1960s, patulin was reclassified as a mycotoxin (*1*). Thus, patulin is now considered to be a significant food contaminant, especially in fruit juices (*2*−*4*). Contamination in apple juice has been reported in some countries. For example, in one survey in Turkey, 60% of the apple juice samples were found to be contaminated at concentrations ranging from 19.1 to 732.8 μg/L (*2*). In Poland (*3*) and Japan (*4*), 22 and 19.7% of apple juice samples surveyed, respectively, were found to be contaminated with patulin. In many countries, the maximum allowable patulin concentration in apple juice is 50 μg/L.

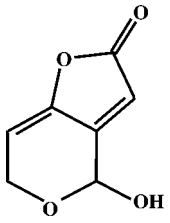

Figure 1. Structure of patulin.

The chemistry and biochemistry of patulin toxicity have been studied extensively. Fliege and Metzler (*5*) characterized the electrophilic properties of patulin using thiol metabolites of glutathione. They found three kinds of cyclic adduct arising from the nucleophilic activity of α-amino groups of the glutamic acid and cysteine residues (*5*). Glutathione was considered to be a scavenger of patulin as the addition of glutathione reduces the toxicity of patulin towards rumen fermentation (*6*), and patulin reduces glutathione levels in rat liver slices (*7*). The scavenging activity and mechanisms by which proteins interact with patulin were also characterized by Fliege and Metzler (*8*). They showed

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that patulin formed intra- and intermolecular protein cross-links through reaction with cysteine, lysine, and histidine side chains and α-amino groups (*8*). However, few papers have described the mechanism of patulin toxicity at the cellular level. Sabater-Vilar et al. (*9*) reported that patulin produced by *Aspergillus clavatus* in a sample of compacted fodder containing malt residue was consumed by cattle that subsequently suffered serious damage to the nervous system. Kim et al. (*10*) reported functional complementation by the *Aspergillus flavus* antioxidative stress gene, mitochondrial superoxide dismutase, in yeast. The toxic effects of patulin on thyroid and testis and on hormone levels in growing male rats (*11*) and on proliferation of porcine lymphocytes (*12*) have also been reported. With the exception of glutathione−patulin interactions, no studies have connected biochemical mechanisms to biological endpoints.

The availability of yeast DNA microarrays provides the possibility of monitoring gene expression levels as a function of toxin exposure and, consequently, as a means of determining mechanisms of toxicity (*13*−*17*). For example, cadmium treatment was found to induce yeast genes involved in sulfur amino acid metabolism, oxidative stress response, and heat shock response (*13*). This pattern of induction was found to be in agreement with many previous studies (*13*).

In this study, we applied DNA microarray analysis to patulin toxicity and observed the response in yeast after exposure to 50 ppm of patulin. We were able to confirm that the observed induction of genes involved in sulfur amino acid metabolism, protein metabolism, and oxidative stress response was consistent with the response expected on the basis of the chemical and biochemical mechanisms described by Fliege and Metzler (*5*, *8*). We also suggest the possibility of using the system (DNA microarray) to monitor patulin and related mycotoxins in food products. Our system is now open for the evaluation of restricted amounts of mycotoxin.

MATERIALS AND METHODS

Strains, Growth Conditions, and Patulin Treatment. *Saccharomyces cerevisiae* strain S288C (*Mat alpha SUC2 mal mel gal2 CUP1*) was grown in YPD medium (2% polypeptone, 1% yeast extract, 2% glucose) at 25 °C as a preculture for 2−3 days. This strain was used because the DNA microarray probes were produced using S288C DNA as the template for PCR. Patulin was purchased from MP Biochemicals (Irvine, CA) and was dissolved in DMSO. This stock solution was added directly to the YPD medium or the YPD medium containing yeast cells such that it was diluted >100-fold. The yeast cells were cultured at 25 °C until absorbance at 650 nm reached 1.0 and collected 2 h after the patulin was added to cell cultures.

DNA Microarray Analysis. DNA microarray analysis was carried out on three independent cultures as described (*13*−*17*). Total RNA was isolated according to the hot-phenol method. Poly(A)+ RNA was purified from total RNA with Oligotex-dT30 mRNA purification kits (Takara, Kyoto, Japan). Two to four micrograms of poly (A)+ RNA was used for each labeling experiment, and the same amount of each poly (A)+ RNA was used on each slide. The two labeled cDNA pools were mixed and hybridized with a yeast DNA chip (ver. 2.0, DNA Chip Research, Inc., Yokohama, Japan) for 24−36 h at 65 °C. On this microarray, ORFs of 200−8000 bp length (0.1−0.5 ng) had been spotted, such that 6037 genes could be analyzed under these conditions (*13*). Details of the microarray procedure and validation studies with our conditions have been described (*13*−*15*). Detected signals for each ORF were normalized by intensity dependent (LOWESS) methods (*18*). Genes classified as induced or repressed were those passing one sample *t* test (*p* value cutoff of 0.05) and additionally showing >2-fold higher or lower expression, respectively, compared to the control. These selected genes were characterized for function according to the functional categories established by MIPS (*19*) and the SGD (*20*). The data obtained in this experiment have been assigned accession number GSE2923 in the Gene Expression Omnibus Database (*21*).

Cluster Analysis of the mRNA Expression Profiles after Patulin Treatment. Hierarchical cluster analysis was performed using GeneSpring ver. 4.2.1 software (Silicon Genetics, Redwood City, CA) (*15*). The clustering algorithm arranges conditions according to their similarity in expression profiles across all conditions, such that conditions with similar patterns are clustered together as in a taxonomic tree. The setting for the calculation was as follows: The similarity was measured by Pearson correlation, the separation ratio was 1.0, the minimum distance was 0.001, and 3800 open reading frames (ORFs) were used for the calculation. These 3800 ORFs were selected on the basis of having previously exhibited higher than average intensities in another trial (*16*).

Reverse transcriptase Polymerase Chain Reaction (RT-PCR) was carried out to select the biomarker genes and to confirm the microarray results as described previously (*22*). The gene names (systematic names) and forward and reverse primer sequences (5′ to 3′) are presented in **Figure 2**.

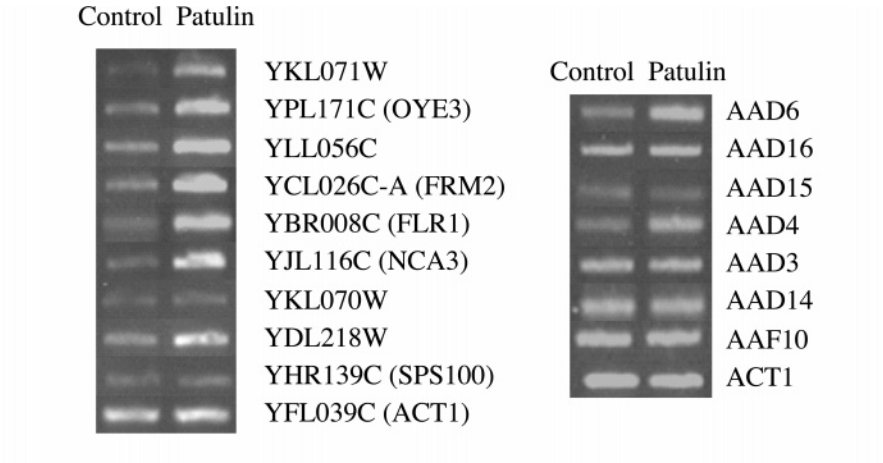
RT-PCR was performed using the One-Step RNA PCR kit (TaKaRa, Shiga, Japan). Temperature and cycle conditions were as follows: 70 °C for 3 min, 50 °C for 30 min, 92 °C for 2 min, 20−30 cycles of (94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s), and 72 °C for 10 min.

RESULTS AND DISCUSSION

Conditions for Patulin Treatment. As a first step, we characterized the effect of patulin treatment on yeast growth. Without biological or physiological characterization of the treatment, we cannot prove that the induction or repression of specific genes is due to the treatment. Lack of growth inhibition would merely show that the condition studied did not cause any cellular stresses and the results obtained did not necessarily reflect the stress. **Figure 3** shows yeast growth as a function of patulin concentrations. No growth was observed at concentrations >100 ppm, whereas inhibition could be seen at concentrations >13 ppm. On the basis of this dose−response analysis, 50 ppm of patulin was found to be inhibitory to nonlethal growth and, therefore, chosen as the test concentration in our experiments. The fatal dose of patulin in the experiment using yeast cells was very high, and differences were found in the metabolism of patulin in yeast cells and the digestive tracts of animals. It is also necessary to consider the influence of the medium on SH radicals.

Overview of Patulin-Induced Genes. Among the 5989 ORFs that exhibited intensities over the cutoff value and had *p* values of <0.05, 490 genes exhibited >2-fold higher intensities and 447 genes had <0.5-fold intensities following patulin treatment. The highly induced genes are listed in **Table 1**. The most highly induced genes were *AAD6* followed by *AAD16*, *OYE3*, *AAD4*, and YLL056C. Most of these genes encode dehydrogenases and were induced >50-fold. Although the *AAD*s are very similar to aryl-alcohol dehydrogenases, their functions have not yet been determined. Seven *AAD* genes have similar DNA sequences. *AAD6*'s sequence is partially similar to that of *AAD4* (86.9%), *AAD3* (80.7%), *AAD14* (84.5%), and *AAD10* (85.1%), and *AAD4*'s sequence is partially similar to that of *AAD16* (91.7%), *AAD15* (78.3%), *AAD3* (83.0%), *AAD14* (90.3%), and *AAD10* (85.5%); thus, it is possible that cross-hybridization of some has resulted in "false" induction of others (*23*).

The 490 induced genes were characterized using the functional categories of MIPS (**Table 2**). There were many genes induced in the categories of "protein fate", "metabolism", "cell rescue, defense, and virulence" and "binding protein (protein with binding function or cofactor requirement)" (**Table 2**). In addition, a high percentage of genes in these categories were



PCR- Primers		
Systematic (common)	Forward	Reverse
YKL071W	GCCAATATGGCCTTGATCATTT	AGGCAAAAGCAAAGTCACATCA
YPL171C (OYE3)	CCGAGGGTACTAACGATTTTGC	CATGATTTCAGTTCTTGTTCCAACC
YLL056C	GGCCTCTATCCCTGTTGATGAC	TGAGACGCATGAGTTGGGTATT
YCL026C-A (FRM2)	TCTTGCAGAGATGAGGCTTACG	TGCGAATTTCCTGTTCAGTGAT
YBR008C(FLR1)	TCGTCCTTAGTGGGGTTCCTAA	TCTTTGTCGACAGATGGACGAT
YJL116C (NCA3)	GGGTACTTCTGGTTCGGATGTC	AGGGGAATTGAGGGTATCAAAA
YKL070W	CATGTGGAAATGCTGGAAAGAG	ATAATCTGGGGCATCAGAAACG
YDL218W	CAATGTTAGGACCGGTGATGTC	TATGGCATTTGATTGACGTTCC
YHR139C (SPS100)	ATGGTTCAGGAATTGGGTTCAT	CGATCTTTCTTTCGTCCAGTCA
YFL056C (AAD6)	TCTCATTTTGGGAGGAATGTCA	CGTCGTATTTCTTATAGTCAGTGG
YFL057C (AAD16)	GAACAAACAGATGCAGAAATCA	ACGTCCGAAATAGATGAGGGAT
YOL165C (AAD15)	ATTAGCCAAGGTTGCTGAGGAA	GATGTCAAAAGGAACTACATTTTCC
YDL243C (AAD4)	GTACTGTTTCGGGTACTTCTAA	TCTGTTCTGGTGTCAGTTTGATAC
YCR107W (AAD3)	GGGTCCGCGTCCGACTCATCTA	GGCAGTGTTACTTTCACCTGC
YNL331C(AAD14)	AGAAGCTTTCACCCCTCACATC	ACACTCGGTGGATAAGGCAACT
YJR155W(AAD10)	GTTAAAAGTTGCGGAAGAACATGG	TGAGCGGCAAAGTTTTCTCTAA

Figure 2. Confirmation of gene induction by RT-PCR. The gene code or gene names are shown on the right side. The primers are shown in the box.

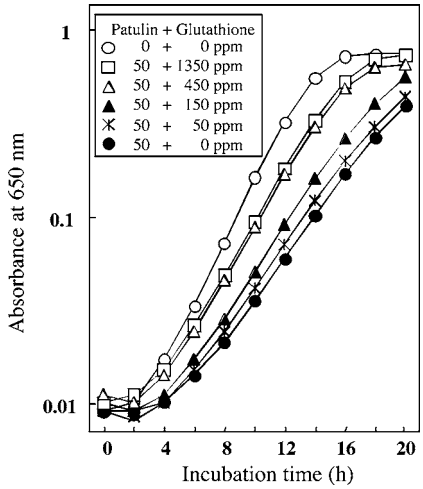


Figure 3. Effect of patulin on yeast growth. Patulin, dissolved in DMSO at a concentration of 2000 ppm, was added to YPD medium at the indicated concentrations.

found to have been induced (number of induced genes in the category/number of genes in the category, and number of induced genes in the category/490 induced genes) (**Table 2**). The 447 repressed genes were also categorized. The only significant shared characteristic among these genes indicated that "protein synthesis" was repressed (data not shown). A repression of protein synthesis is a common observation in yeast exposed to chemicals as chemical treatments are expected to result in lower growth rate and rates of protein synthesis.

To characterize the response to the patulin treatment, cluster analysis of the mRNA expression profile (*15*, *16*) was performed using Pearson correlation. The profile of the patulin treatment was found to cluster with that of maneb and belonged to the agricultural chemicals group. The chemicals in this group are used as fungicides, except for acrolein. Thiuram, maneb, and zineb are dithiocarbamate fungicides that have been shown to cause oxidative stress and protein denaturation in yeast (*15*).

Table 1. Genes Highly Induced by Patulin Treatment[a]

systematic no.	common name	-fold	description
YFL056C	AAD6	107	similarity to aryl-alcohol dehydrogenases
YFL057C	AAD16	103	aryl-alcohol dehydrogenase
YPL171C	OYE3	71	NAPDH dehydrogenase (old yellow enzyme)
YDL243C	AAD4	64	similarity to aryl-alcohol dehydrogenase
YLL056C		59	similarity to *Y. pseudotuberculosis* epimerase
YOL165C	AAD15	47	putative aryl-alcohol dehydrogenase
YCR107W	AAD3	46	similarity aryl-alcohol reductase
YNL331C	AAD14	42	similarity aryl-alcohol reductase
YCL026C-A	FRM2	42	involved in fatty acid regulation
YBR008C	FLR1	33	antiporter involved in multidrug resistance
YJR155W	AAD10	31	similarity to aryl-alcohol dehydrogenase
YJL116C	NCA3	25	regulation of synthesis of Atp6p and Atp8p
YDL218W		22	weak similarity to protein YNR061c
YKL070W		22	similarity to *B. subtilis* transcriptional protein
YKR076W	ECM4	16	involved in cell wall biogenesis and architecture
YER143W	DDI1	15	induced in response to DNA alkylation damage
YLL060C	GTT2	14	glutathione *S*-transferase
YHR139C	SPS100	14	sporulation-specific wall maturation protein
YLR460C		13	similarity to *C. carbonum* toxD protein
YLR303W	MET17	12	*O*-acetylhomoserine sulfhydrylase
YJL045W		11	strong similarity to flavoprotein
YCR102C		10	similarity to *C. carbonum* toxD gene

[a] *p* value in *t* test of listed below 0.002.

Table 2. Functional Categories of Induced Genes

	no. of ORFs		percentage of ORFs	
	in category	induced	in category	in 490 ORFs
metabolism	1066	118	11.1	24.0
energy	365	36	9.9	7.3
cell cycle and DNA processing	1001	60	6.0	12.2
transcription	1063	44	4.1	9.0
protein synthesis	476	5	1.1	1.0
protein fate	1137	138	12.1	28.2
binding protein	1034	105	10.2	21.4
protein activity regulation	238	18	7.6	3.7
cellular transport	1031	79	7.7	16.1
cellular communication	234	10	4.3	2.0
cell rescue, defense, and virulence	548	108	19.7	22.0
interaction with the cellular environment	458	32	7.0	6.5
interaction with environment	5	1	20.0	0.2
development	70	2	2.9	0.4
biogenesis of cellular components	854	52	6.1	10.6
cell type differentiation	449	32	7.1	6.5
unclassified proteins	2038	107	5.3	21.8

Table 3. Subcategories in Significantly Activated Categories

	no. of ORFs		
	in category	induced	%[a]
protein fate			
folding and stabilization	91	26	28.6
targeting, sorting, and translocation	277	18	6.5
modification	606	74	12.2
posttranslational modification of amino acids	24	2	8.3
processing (proteolytic)	88	31	35.2
assembly of protein complexes	196	19	9.7
degradation	250	70	28.0
binding protein			
protein binding	384	50	13.0
peptide binding	3	0	0.0
nucleic acid binding	340	17	5.0
motor protein	5	0	0.0
structural protein	56	3	5.4
lipid binding	17	3	17.6
amino acid binding	3	0	0.0
C-compound binding	9	1	11.1
metal binding	27	2	7.4
nucleotide binding	225	27	12.0
complex cofactor	57	11	19.3
metabolism			
amino acid	243	44	18.1
nitrogen and sulfur	96	23	24.0
nucleotide	227	11	4.8
phosphate	414	38	9.2
C-compound and carbohydrate	504	49	9.7
lipid, fatty acid, and isoprenoid	272	20	7.4
vitamins, cofactors, and prosthetic groups	163	25	15.3
secondary metabolism	77	10	13.0
cell rescue, defense, and virulence			
degradation of foreign compounds	1	0	0.0
detoxification	102	25	24.5
stress response	175	34	19.4
other cell rescue activities	9	3	33.3
oxidative stress	53	21	39.6

[a] Number of ORFs induced/number of ORFs (in category) × 100.

Patulin Activates Protein Degradation, Especially Proteasome Activities. Because many of the induced genes belong to the "protein fate" category, it is suggested that protein metabolism is activated. Thus, we further characterized the induced genes relative to protein metabolism. In **Table 3**, subcategories and sub-subcategories in the category of protein fate are listed with the number of induced genes. High numbers of genes were observed in the subcategories "modification", 74 ORFs, "processing (proteolytic)", 31 ORFs, and "degradation", 70 ORFs, with high proportions occurring in "processing (proteolytic)", 35.2%, "folding and stabilization", 28.6% and "degradation", 28.0% categories. The subcategory "processing (proteolytic)" is the sub-sub-subcategory in the "modification" subcategory, and "modification" and "degradation" share many of the same ORFs, such as those that contribute to protein degradation. It seems that genes involved in protein degradation contribute to the significant induction of genes in the category of protein fate. The significant induction of genes in the "binding protein" category may be related to molecular chaperone activity (**Table 3**), which further reflects an activation of protein metabolism.

We further listed the ORFs categorized in protein fate in **Table 4**. The most highly induced gene was *DDI1*, followed by *UBC5*, *UMP1*, *ATG1*, and *NLP4*. In addition, the *PRE* and *RPN* groups of genes encoding proteasome-related proteins were highly induced. Among the 32 ORFs involved in proteasome function, 25 were induced >2-fold, with the lowest induction being 1.6-fold (data not shown). Thus, we may conclude that the patulin treatment induced activities associated with protein degradation, particularly those involving the proteasome. It should be noted that the most strongly induced gene, *DDI1*, has been reported to encode a protein induced by DNA alkylation damage and has an ubiquitin-associated domain (*20*).

Patulin Treatment Activates Sulfur Amino Acid Metabolism and Oxidative Stress-Inducible or -Related Defense System. The patulin treatment also significantly induced genes in the "metabolism" and "cell rescue, defense, and virulence" categories. Thus, we further characterized these categories. **Table 3** lists the subcategories belonging to the categories of metabolism and cell rescue, defense, and virulence. The proportions of induced genes were high within the subcategories of nitrogen and sulfur amino acid. This suggests that sulfur amino acid metabolism is activated. The induction levels of the *MET* and *CYS* genes are listed in **Table 4**. Cysteine is synthesized from sulfate and a carbon skeleton by *MET3* (sulfate adenyl transferase), *MET14* (ATP) adenosine-5-phosphosulfate 3-phosphote transferase), *MET16* (3-phosphoadenyl sulfate reductase), *MET10* (sulfite reductase flavin-binding subunit), *MET17* (*O*-acetylhomoserine sulfhydrylase), *MET2* (homoserine-acetyltransferase), *CYS4* (cystathionine β-synthase), and *CYS3* (cystathionine γ-lyase), in this order according to the SGD. These genes were induced from 1.8- to 11.9-fold. These results clearly show that sulfur amino acid metabolism, and especially cysteine synthesis, was activated. It should be noted that *MET12*, *MET13*, *MET6*, and *MET7*, involved in methionine synthesis, were not induced (**Table 4**).

The category of cell rescue, defense, and virulence includes genes that play a role in stress response including heat shock proteins, oxidative stress scavengers, and transporters involved in multidrug resistance. A high proportion of induced genes were found in the subcategories of detoxification, stress response, and oxidative stress. The subcategory of detoxification includes oxidative stress scavengers and transporters, and the subcategory of stress response includes heat shock proteins and oxidative stress scavengers. Thus, it is clear that the significant activation of genes in the category of cell rescue, defense, and virulence is due to the induction of genes encoding heat shock proteins or involved in oxidative stress. The genes categorized in oxidative stress are listed in **Table 4**. Among them, *GTT2* (glutathione *S*-transferase), *TRX2* (thioredoxin II), *TSA2* (thiol peroxidase), *PRX1* (mitochondrial isoform of thioredoxin per-

Table 4. Induced Genes Involved in Protein Metabolism, Oxidative Stress, and Methionine Metabolism Subcategories

protein fate			oxidative stress			methionine metabolism		
systematic	-fold	common	systematic	-fold	common	systematic	-fold	common
YER143W	14.8	DDI1	YLL060C	14.2	GTT2	YLR303W	11.9	MET17
YDR059C	7.8	UBC5	YOL151W	9.3	GRE2	YPR167C	8.9	MET16
YBR173C	6.6	UMP1	YKL086W	5.3	SRX1	YJR010W	6.4	MET3
YBL078C	5.1	ATG8	YPL223C	4.7	GRE1	YIR017C	3.0	MET28
YBR170C	5.0	NPL4	YGR209C	4.4	TRX2	YFR030W	2.8	MET10
YMR174C	4.7	PAI3	YDR453C	4.0	TSA2	YNL277W	2.6	MET2
YKL103C	4.2	LAP4	YFL014W	3.7	HSP12	YKL001C	2.6	MET14
YLR387C	4.1	REH1	YLR109W	3.4	AHP1	YDR253C	2.6	MET32
YGL180W	4.1	ATG1	YBL064C	3.3	PRX1	YIL046W	2.1	MET30
YDR092W	4.0	UBC13	YDR011W	3.1	SNQ2	YBR213W	1.9	MET8
YGL141W	4.0	HUL5	YNR074C	3.0		YKR069W	1.9	MET1
YER012W	3.9	PRE1	YDR513W	3.0	TTR1	YNL103W	1.4	MET4
YFR052W	3.9	RPN12	YLR043C	3.0	TRX1	YOL064C	1.2	MET22
YHR138C	3.8		YER042W	2.6	MXR1	YPL023C	1.1	MET12
YHL030W	3.6	ECM29	YDR032C	2.3	PST2	YGL125W	1.1	MET13
YOL038W	3.5	PRE6	YHR008C	2.3	SOD2	YER091C	0.9	MET6
YKL145W	3.5	RPT1	YJR104C	2.2	SOD1	YOR241W	0.8	MET7
YDL007W	3.4	RPT2	YBR006W	2.2	UGA2	YPL038W	0.6	MET31
YML092C	3.3	PRE8	YML028W	2.1	TSA1	YIL128W	0.4	MET18
YGL240W	3.2	DOC1	YDR353W	2.1	TRR1			
YGR253C	3.1	PUP2	YIR037W	2.0	HYR1	YAL012W	5.5	CYS4
YHR027C	3.1	RPN1	YHR205W	2.0	SCH9	YGR155W	1.8	CYS3
YDL097C	3.0	RPN6	YGR097W	2.0	ASK10			
YJR117W	3.0	STE24	YPL196W	1.9	OXR1			

Table 5. Induced DNA Repair Genes

systematic no.	common name	-fold	description
YDL059C	RAD59	5.7	recombination and DNA repair protein
YGL163C	RAD54	5.3	DNA-dependent ATPase of the Snf2p family
YER142C	MAG1	3.9	3-methyladenine DNA glycosylase
YAR007C	RFA1	3.4	DNA replication factor A, 69 kDa subunit
YEL037C	RAD23	2.8	nucleotide excision repair protein (ubiquitin-like protein)
YJR052W	RAD7	2.6	nucleotide excision repair protein
YML032C	RAD52	2.3	recombination and DNA repair protein
YML088W	UFO1	2.3	involved in degradation of Ho protein
YMR201C	RAD14	2.1	nucleotide excision repair protein
YNL250W	RAD50	2.0	DNA repair protein
YKL054C	DEF1	1.9	repair and proteolysis in response to DNA damage
YER089C	PTC2	1.9	protein serine/threonine phosphatase of the PP2C family
YAL015C	NTG1	1.9	DNA repair protein
YER028C	MIG3	1.8	similarity to Mig1p
YBR114W	RAD16	1.8	nucleotide excision repair protein
YML058W	SML1	1.8	protein inhibitor of ribonucleotide reductase
YLR234W	TOP3	1.7	DNA topoisomerase III
YBR073W	RDH54	1.7	mitotic diploid-specific recombination and repair and meiosis
YDL200C	MGT1	1.7	O^6-methylguanine DNA repair methyltransferase
YLR427W	MAG2	1.6	weak similarity to human transcription regulator Staf-5

[a] p value in t test of listed below 0.03.

oxidase), *TTR1* (glutaredoxin), *TRX1* (thioredoxin I), *TSA1* (thiol-specific antioxidant, *TRR1* (thioredoxin reductase), and *HYR1* (glutathione peroxidase) are concerned not only with oxidative stress but also with the SH compounds according to the SGD. The significant activation of genes within the categories of metabolism and cell rescue, defense, and virulence suggests an activation of metabolism of SH-related compounds.

Mutagenicity of Patulin. Patulin is a mycotoxin that exhibits significant genotoxicity. Thus, we focused on the mutagenicity of patulin by listing induced genes contributing to DNA repair (**Table 5**). The most highly induced gene in this group was *RAD59*. This gene was grouped among genes that contribute to recombinational DNA repair by SGD. *RAD54*, *RFA1*, *RAD52*, *RAD50*, and *RDH54* were also shown to contribute to recombinational repair by SGD. According to SGD, *RAD59*, *RAD52*, *RAD50*, *RDH54*, *MRE11* (0.84-fold induction) and *XRS2* (1.2-fold induction) are involved in double-strand break repair via break-induced replication. On the other hand, nucleotide excision repair proteins were also induced. *RAD23*, *RAD7*, *RAD14*, and *RAD16* were grouped with genes contributing to nucleotide excision repair according to SGD. It is interesting that *MAG1* was listed as the third most highly induced gene in this category. *MAG1* has been shown to play a role in the repair of DNA alkylation damage (*25*−*27*). In addition, *MGT1* and *MAG2* also showed relatively high induction values and also contribute to the repair of alkylated DNA (*28*, *29*). To date, only these three genes have been listed by SGD as contributing to the repair of DNA alkylation damage. These results suggest that the patulin treatment damages DNA by alkylation and that the damage is repaired by recombinational and excision repair mechanisms. It should be noted that other repair genes such as for base excision repair (*NTG1*, 1.9-fold induction; *NTG2*, 1.2-fold induction; *APN*, 1.0-fold induction), mismatch repair (*MSH2*, 1.1-fold induction; *MSH3*, 0.6-fold induction), and translation synthesis (*30*) (*REV3*, 0.89-fold induction; *REV7*, 1.04-fold induction) were not induced to a significant extent.

Biomarker Genes for Patulin. DNA microarrays can be used to understand chemotoxicity, and this system can be also used for the selection of reporter genes indicative of such toxicity. The genes highly induced by the patulin treatment are candidates for reporter genes. To confirm the possibility of using these genes as reporters, we further characterized the induction level by RT-PCR. We selected YKL071W, YPL171C (*OYE3*), YLL056C, YCL026C-A (*FRM2*), YBR008C (*FLR1*), YJL116C (*NCA3*), YKL070W, YDL218W, and YHR139C (*SPS100*) as candidate reporters because their induction levels were significant (**Table 1**). Although YKL071W was not listed in **Table 1** because of faulty results in two of three experiments, this gene exhibited a 253-fold induction in the single experiment. All of the genes yielded more RT-PCR product compared to the control, especially YKL071W, YPL171C, YLL056C, YCL026C-

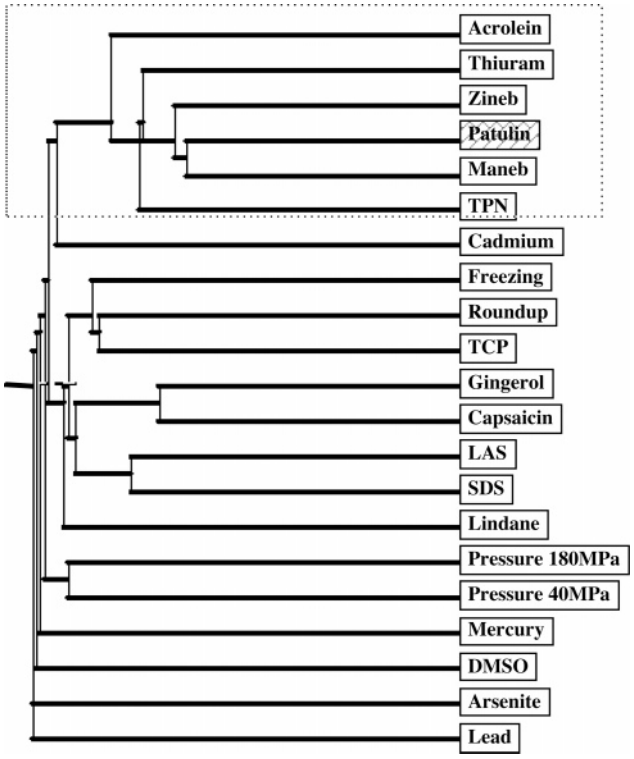


Figure 4. Cluster analysis of the mRNA expression profiles after patulin treatment. Hierarchical cluster analysis was performed using GeneSpring ver. 4.2.1 software with Pearson correlation for similarity as described in the text.

A, YBR008C, YJL116, and YDL218W (**Figure 4**). Thus, these genes may be used as reporters for the simple detection of patulin contamination. As described above, seven kinds of *AAD*s are highly induced, but this can be due to some cross-hybridization. Thus, we confirmed the induction levels by selecting primer sequences specific for each *AAD*. As expected, all of the *AAD*s were not induced. Only *AAD6* and *AAD4* were induced by the patulin treatment. This result is in agreement with the results of Delneli et al. (*23*), who analyzed several multiple *AAD* deletion mutants and suggested that only *AAD6* and *AAD4* were induced by oxidative stress (*23*). *AAD6* and *AAD4* may serve as useful reporter genes for detecting the presence of patulin. However, the specificity of the expression profile was not examined in this study, which is restricted to the response of *S. cereviseae* to patulin. Future studies will include similar experiments using other mycotoxins. The physico- or immunochemical method is easier or more specific for assaying food that has been contaminated by patulin. However, it is not possible to correlate the change in the landlord of the mold with the change in the global environment or to the pollution of the unknown.

Mycotoxins, spread in the environment and in food products, are natural products. This makes it difficult to evaluate these toxicities because pure products cannot be obtained easily. Thus, evaluation methods have to be developed using restricted amounts of mycotoxins. In the present study, we characterized the transcriptional response of yeast cells exposed to the mycotoxin patulin. Moreover, the yeast system is beneficial as it can be applied with 2−3 mL of culture.

The main responses to patulin exposure were the activation of proteolytic activity, sulfur amino acid metabolism, and antioxidant defense system. These responses are in excellent agreement with the chemical and biochemical characterization of patulin that indicates reactivity with thiol metabolites (*5*) and

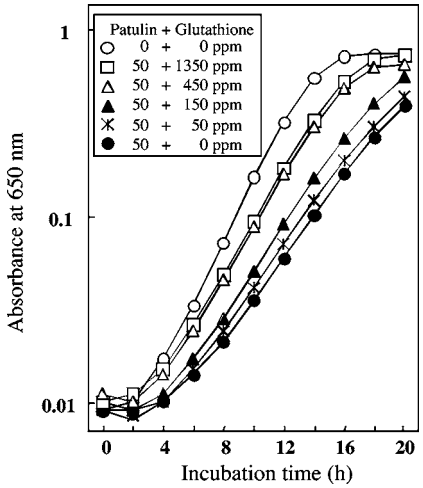


Figure 5. Glutathione restored growth inhibition caused by patulin. Yeast cells were grown in medium containing 50 ppm of patulin and the indicated amount of glutathione.

cysteine, lysine, and histidine side chains and α-amino groups in proteins (*8*). The induced proteolytic activity is required for the degradation of cross-linked and denatured proteins caused by reaction with patulin. The reaction of patulin with thiol metabolites results in starvation of sulfur amino acids, and this also decreases the scavenging activity of oxygen radicals by cysteine or glutathione. The expression profile after the patulin treatment was similar to those induced by exposure to the dithiocarbamate fungicides thiuram, maneb, and zineb. This similarity may be due to the shared reactivity of patulin and these fungicides to thiol compounds, the histidine side chain, and α-amino groups in proteins.

Morgavi et al. (*6*). showed that SH-containing reducing agents such as cysteine and glutathione prevent patulin toxicity toward the microbial fermentation that occurs in the rumen. We also confirmed that glutathione can modulate patulin toxicity. **Figure 4** shows the effect of glutathione on the growth inhibition caused by 50 ppm of patulin. Glutathione (50 ppm) slightly decreased the inhibitory effect by patulin, and this effect was increased with an increase in glutathione concentration. However, the growth rate of the yeast culture exposed to 450 ppm of glutathione (**Figure 5**) corresponded to the growth rate observed in the presence of 13 ppm of patulin (**Figure 4**). This suggests that the effect of glutathione is not so drastic compared to direct detection of patulin in the presence of glutathione (*6*). This implies that SH compounds do not always decrease the toxicity of patulin and that the patulin−glutathione adduct may also cause toxicity.

The damaging activity of patulin to DNA was also characterized. The induction of *MAG1*, *MGT1*, and *MAG2* (**Table 5**) is evidence of the alkylation of DNA (*26*−*29*). It has been suggested that yeast has four characterized pathways for the repair of damaged DNA bases, namely, base excision repair, nucleotide excision repair, recombination, and translation synthesis (*31*). The DNA alkylatuion induced by patulin seems to be repaired by recombinational and nucleotide excision mechanisms (**Table 5**). The specificity and possible cooperation between these two repair mechanisms are not well understood. However, it should be noted that recombination repair induces a high frequency of recombination, and this can be the reason for mutagenicity (*30*). Recombination repair is not a mechanism for removal of damaged DNA, but rather a mechanism for tolerating damage (*30*).

Again, the similarity of expression profiles between patulin and dithiocarbamate is very interesting, as thiuram, maneb, and zineb are also used as fungicides. These chemicals are artificial fungicides, whereas patulin has a biological origin. This suggests caution concerning the use of artificial fungicides. The microbial producers of natural fungicides must be resistant to their own products and, therefore, may also be resistant to artificial fungicide**s**. Thus, it is possible that the use of these artificial fungicides may increase populations of patulin-producing fungi.

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