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# Evaluation of toxicity of the mycotoxin citrinin using yeast ORF DNA microarray and Oligo DNA microarray

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## Abstract

**Background:** Mycotoxins are fungal secondary metabolites commonly present in feed and food, and are widely regarded as hazardous contaminants. Citrinin, one of the very well known mycotoxins that was first isolated from *Penicillium citrinum*, is produced by more than 10 kinds of fungi, and is possibly spread all over the world. However, the information on the action mechanism of the toxin is limited. Thus, we investigated the citrinin-induced genomic response for evaluating its toxicity.

**Results:** Citrinin inhibited growth of yeast cells at a concentration higher than 100 ppm. We monitored the citrinin-induced mRNA expression profiles in yeast using the ORF DNA microarray and Oligo DNA microarray, and the expression profiles were compared with those of the other stress-inducing agents. Results obtained from both microarray experiments clustered together, but were different from those of the mycotoxin patulin. The oxidative stress response genes – *AADs*, *FLR1*, *OYE3*, *GRE2*, and *MET17* – were significantly induced. In the functional category, expression of genes involved in "metabolism", "cell rescue, defense and virulence", and "energy" were significantly activated. In the category of "metabolism", genes involved in the glutathione synthesis pathway were activated, and in the category of "cell rescue, defense and virulence", the ABC transporter genes were induced. To alleviate the induced stress, these cells might pump out the citrinin after modification with glutathione. While, the citrinin treatment did not induce the genes involved in the DNA repair.

**Conclusion:** Results from both microarray studies suggest that citrinin treatment induced oxidative stress in yeast cells. The genotoxicity was less severe than the patulin, suggesting that citrinin is less toxic than patulin. The reproducibility of the expression profiles was much better with the Oligo DNA microarray. However, the Oligo DNA microarray did not completely overcome cross hybridization.

## Background

Mycotoxins are fungal secondary metabolites commonly present in the feed and food, and are widely considered as hazardous contaminants. However, the toxicity of these natural chemicals are not properly evaluated because of the difficulties in isolating these chemicals and also because of the lack of interests as they have no industrial applications. The costs for producing the pure mycotoxins are the biggest obstacle in their evaluation process. On the other hand, development of analytical methods are needed to identify new mycotoxins, to fight against the spreading toxins, and also to meet the growing demands for the toxicological studies.

Citrinin [518-75-2], 4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-crboxylic acid (Figure 1), which was first isolated from *Penicillium citrinum* [1], is produced by more than 10 kinds of fungi [1]. Citrinin is the one of the well-known mycotoxins, which is possibly spread all over the world. Although citrinin is one of the well-characterized mycotoxins, information on its mechanism of toxic action is limited. Clinically, citrinin was shown to cause renal disease in poultry, pigs, dogs and rats [2,3]. The electron transport system of the kidney and liver mitochondria were considered as the target of the toxic action of citrinin [4].

The availability of yeast DNA microarrays provides the possibility of monitoring gene expression levels as a function of toxin exposure, and consequently, provides a mean to determine the mechanism of toxicity [5,6]. The essential features of this yeast system are the small volume of yeast culture required for the analysis, high reproducibility of the expression profiles and availability of the massive functional information of genes on DNA microarray [7,8]. For example, cadmium treatment was found to induce yeast genes involved in the sulfur amino acid metabolism, oxidative stress response, and heat shock response [6]. This expression pattern of induced genes was in agreement with many previous studies [6]. We applied this system to evaluate the action mechanism of patulin, one of the most potent mycotoxins, and found that patulin targets proteins and possibly DNA [7]. Our results suggested that patulin probably acts as a mutagen [7].

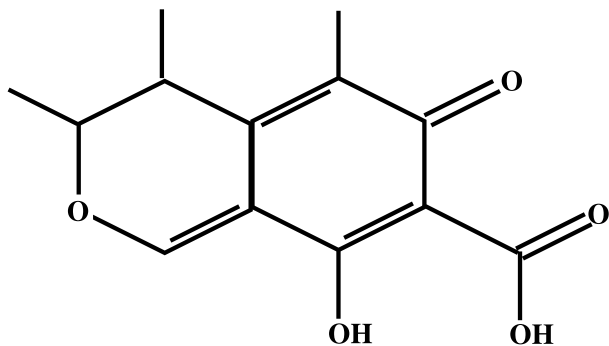


**Figure 1**
Chemical structure of citrinin.

In this report, we studied the toxicity of citrinin to yeast cells using the traditional ORF (Open Reading Frame) DNA microarray [6] and Oligo (Oligo-nucleotide) DNA microarray systems [9]. Results from both microarray studies suggested that the oxidative stress was the main cause for toxicity, but this oxidative stress did not lead to any DNA damage. This observation was different from what was found with another mycotoxin patulin [7]. To detoxify against the citrinin, the yeast cells mainly used glutathione modification and pumped out the toxin using transporters. We have also discussed how the two DNA microarrays were adapted for evaluating the mycotoxin action.

## Results

### *Conditions for the citrinin treatment*

As a first step, we characterized the effect of citrinin on yeast growth because without any biological or physiological characterization we will not be able to prove that the induction or repression of specific genes is due to the treatment. Lack of growth inhibition would merely indicate that the conditions used for the study did not cause any cellular stress. Figure 2 shows yeast growth as a function of different concentrations of citrinin. As shown, we observed growth inhibition at concentrations greater than 108 ppm, and at 970 ppm of citrinin there was no growth. Based on this dose-response analysis, 300 ppm of citrinin was chosen for subsequent experiments, as this concentration was found to be inhibitory to non-lethal growth (data not shown). This concentration citrinin is slightly higher than that was used for the patulin treatment [7], and citrinin may be less toxic to yeast cells.

### *Overview of citrinin induced and repressed genes through ORF DNA microarray and Oligo DNA microarray*

From three independent citrinin treatment experiments, we obtained 12 sheets of DNA microarray results. Three sheets (OR-1, OR-2, OR-3 in Figure 3) were from the ORF DNA microarray, one from each citrinin treatment. For the Oligo DNA microarray, we performed three hybridizations for each experiment and obtained 9 sheets of data (OL-1-1, OL-1-2, OL-1-3, OL-2-1, OL-2-2, OL-2-3, OL-3-1, OL-3-2, OL-3-3 in Figure 3), including dye swap for the OL-1-1, OL-1-2, and OL-1-3 sheets. From the microarray data (Figure 3) we calculated the correlation factors to determine the reproducibility between the different

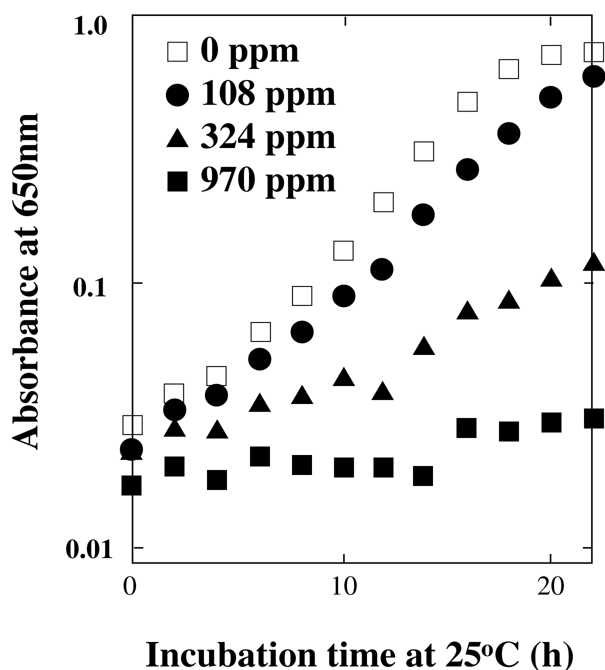


**Figure 2**
**Effect of citrinin on yeast growth**. Citrinin dissolved in DMSO at a concentration of 20000 ppm was added to the YPD medium to achieve the indicated concentration. The stock solution was added directly to the yeast cells grown for 2–3 days such that they were diluted more than 100-fold.

hybridization conditions (region A in Figure 3), citrinin treatment (region B of Figure 3), dye swap (region C of Figure 3), and DNA microarray (region D in Figure 3). The correlation factors for the ORF DNA microarray were from 0.83 to 0.88. For the Oligo DNA microarrays, the correlation factors were from 0.93 to 0.99 for 9 sheets, and from 0.96 to 0.99 for the same source of total RNA (Figure 3). The correlation factors between the ORF DNA microarray and Oligo DNA microarray showed relatively low correlation factors (0.67–0.73) than those among the same type of DNA microarray. These results suggest that the reproducibility of the Oligo DNA microarray is better than those of the ORF DNA microarray (Region B in Figure 3).

From the ORF DNA microarray, we obtained 5,928 ORFs exhibiting intensities over the cut-off value at least in one experiment. Among these ORFs, 155 ORFs showed more than two times higher intensity than that of the untreated control and having t-test P-value less than 0.05. In addition, 363 ORFs, having statistically different intensities from that of the control with the t-test P-value less than 0.01, were recognized as induced genes. On the other hand, 73 ORFs, having two times lower intensity than that of the untreated control and having t-test P-value less than 0.05, were recognized as repressed genes. Similarly, 471 ORFs having statistically different intensities from the control with the t-test P-value less than 0.01 were also recognized as repressed genes.

From the Oligo DNA microarray, we obtained 5,869 ORFs exhibiting intensities over the cut-off value at least in one experiment. Among these ORFs, 113 ORFs showed more than two times higher intensity than that of the untreated control and having t-test P-value less than 0.05. In addition, 801 ORFs, having statistically different intensities from the control with the t-test P-value less than 0.01, were recognized as induced genes. On the other hand, 41 ORFs, having two times lower intensity than that of the untreated control and having t-test P-value less than 0.05, were recognized as repressed genes. Similarly, 1123 ORFs were recognized as repressed genes whose intensities were statistically different from that of the control with the t-test P-value less than 0.01. Apparently, the number of induced and repressed genes ($P < 0.5$) were higher for the ORF DNA microarray and the number of statistically significant ($P < 0.01$) induced and repressed genes were higher for the Oligo DNA microarray. These differences might arise from the different numbers of data collected from the two microarrays.

Table 1 lists the highly induced genes according to their average induction values obtained from the ORF and Oligo DNA microarrays without any statistical selection. The most highly induced gene was *FRM2* followed by *AADs*, *FLR1*, *OYE3*, *GRE2*, and *MET17*. The most abundantly induced genes were *AADs*. Interestingly, *AADs*, *FLR1*, *OYE3*, *GRE2*, and *MET17* are the genes that are significantly induced by oxidative stress[10,11]. The strongly repressed genes were listed in Table 2. In contrast to the highly induced genes, there was a good agreement between the degree of repression of the repressed genes from both the ORF and Oligo DNA microarray analysis. The most strongly repressed gene was YPL095C followed by *ARO10*, *ZRT1*, *USV1*, *CWP1*, and *RPI1*.

To compare with the other stress factors, we carried out the cluster analysis of the ORF and Oligo DNA microarray expression data using the average value for each microarray. As shown in Figure 4, the expression profiles of the ORF microarray and Oligo microarray clustered together. The citrinin-induced response was very similar to that of the maneb. The citrinin-induced gene expression data did not cluster with those of the patulin, thiuram and acro-

lein. These results suggest that the citrinin treatment-induced response was not similar to that of the mycotoxin patulin. Thus, unlike patulin, which is known to target proteins [7,12], citrinin might not cause protein denaturation.

#### *Functional categogorization of citrinin induced genes*

To characterize the effect of citrinin to yeast cells, the induced genes were categorized using the functional categories of MIPS. As summarized in Table 3, there were significant number of induced genes in the categories of "metabolism", "cell rescue, defense and virulence", and "energy". In addition, a high percentage of genes in these categories were found to be induced ((number of induced genes in the category/number of genes in the category) × 100). In the category of "metabolism", the subcategories of "amino acid metabolism", "nitrogen and sulfur metabolism", "metabolism of vitamins", and "secondary metabolism" were significantly induced.

In the subcategories of "amino acid metabolism" and "nitrogen and sulfur metabolism", we found that the induced genes mainly belonged to the sulfur amino acid metabolism (Table 4). Among the 25 genes listed, 21 genes can be recognized as the induced genes in at least one of the DNA microarrays. These results strongly suggest that the citrinin-treated yeast cells require methionine or glutathione. In the subcategories of "metabolism of vitamins" and "secondary metabolism", there were no groups of genes specific for vitamins and secondary metabolism, but they merely overlapped with the genes for the sulfur amino acid metabolism.

Table 5 summarized the list of the induced genes belonging to the category of "cell rescue, defense and virulence". The significantly induced genes in this category were transporters, especially the ABC transporters. Several of these transporters – such as *FLR1*, *PDR5*, *SNQ2*, *ATR1*, and *YOR1* – are involved in multi-drug resistance, and are important for the tolerance against a broad range of organic anions [13-16]. It should be also noted that the *GTT2* gene, which encodes the glutathione-S-transferase protein, was highly induced and the *YCF1* gene, which codes for the vacuolar glutathione S-conjugate transporter, was also induced. The relatively significant induction of the genes in the "energy" category was due to the *AAD*s and the related genes, as these genes are categorized as the dehydrogenase (data not shown).

Citrinin was suggested to cause damages to the mitochondria. Table 6 lists the cellular localization of the induced gene products. It is clear that many of these gene products, which are localized in the mitochondria, were induced; however, the proportion of these induced genes among the total number of induced genes are not so high (Table 6, Impact). The degrees of impact values of induced genes in the mitochondria from both the microarrays were very similar to the degree of impact value of the total genes in the entries (Table 6). Although our results suggest that citrinin affected mitochondria, but we can not say that the citrinin toxicity is specific to mitochondria. In the list of highly induced genes (Table 1), the YLR346C, *GTT2*, *PDR5*, and YKL070W genes (shown in bold in Table 1) were counted as the gene products localized in the mitochondria. As these genes are also expressed in other

| | OR-1 | OR-2 | OR-3 | OL-1-1 | OL-1-2 | OL-1-3 | OL-2-1 | OL-2-2 | OL-2-3 | OL-3-1 | OL-3-2 | OL-3-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OR-1 | 1.00 | 0.83 | 0.88 | 0.73 | 0.72 | 0.73 | 0.71 | 0.69 | 0.72 | 0.72 | 0.73 | 0.69 |
| OR-2 | | 1.00 | 0.88 | 0.72 | 0.71 | 0.71 | 0.70 | 0.67 | 0.70 | 0.70 | 0.72 | 0.68 |
| OR-3 | B | | 1.00 | 0.72 | 0.71 | 0.72 | 0.69 | 0.67 | 0.70 | 0.71 | 0.72 | 0.67 |
| OL-1-1 | | | | 1.00 | 0.99 | 0.99 | 0.97 | 0.95 | 0.96 | 0.96 | 0.96 | 0.93 |
| OL-1-2 | | D | | | 1.00 | 0.99 | 0.97 | 0.96 | 0.96 | 0.96 | 0.96 | 0.94 |
| OL-1-3 | | | | A | | 1.00 | 0.97 | 0.96 | 0.97 | 0.96 | 0.97 | 0.93 |
| OL-2-1 | | | | | | | 1.00 | 0.98 | 0.98 | 0.98 | 0.98 | 0.96 |
| OL-2-2 | | | | | | C | | 1.00 | 0.97 | 0.97 | 0.96 | 0.94 |
| OL-2-3 | | | | | | | A | | 1.00 | 0.98 | 0.97 | 0.95 |
| OL-3-1 | | | | | | | | | | 1.00 | 0.98 | 0.96 |
| OL-3-2 | | | | | | | | B | | | 1.00 | 0.96 |
| OL-3-3 | | | | | | | | | | A | | 1.00 |

**Figure 3**
**Correlation factors among the different experiments (same conditions but different treatments)**. A, Different sheets of microarray. B, Different citrinin treatment. C. Different labeling (dye swap), D, Different types of microarray. Dye swap was carried out with the OL-1-1, OL-1-2 and OL-1-3 sheets.

**Table 1: List of highly induced genes by the citrinin treatment**

| | | | ORF-Array | | Oligo-Array | | |
|---|---|---|---|---|---|---|---|
| Systematic Name | Common Name | Average (Fold) | Fold | t-test P-Value | Fold | t-test P-Value | MIPS_Description |
| YCL026C-A | FRM2 | 104.0 | 162.4 | 0.002 | 45.7 | 0.000 | Involved in fatty acid regulation |
| YFL057C | AAD16 | 63.5 | 86.1 | 0.003 | 40.8 | 0.000 | Aryl-alcohol dehydrogenase |
| YFL056C | AAD6 | 47.0 | 39.8 | NA* | 54.2 | 0.000 | Putative aryl-alcohol dehydrogenase, |
| YDL243C | AAD4 | 46.3 | 53.4 | 0.000 | 39.2 | 0.000 | Aryl-Alcohol Dehydrogenase |
| YBR008C | FLR1 | 33.6 | 37.9 | 0.000 | 29.4 | 0.000 | Putative H+ antiporter involved in multidrug resistance |
| YPL171C | OYE3 | 29.9 | 31.9 | 0.001 | 27.8 | 0.000 | NAPDH dehydrogenase (old yellow enzyme), isoform 3 |
| YOL165C | AAD15 | 26.6 | 51.3 | 0.000 | 1.9 | 0.000 | Putative aryl alcohol dehydrogenase |
| YIR041W | PAU15 | 23.6 | 1.7 | 0.159 | 45.3 | 0.000 | Similarity to members of the Srp1p/Tip1p family |
| YJR155W | AAD10 | 22.3 | 43.7 | 0.000 | 1.0 | 0.858 | Putative aryl-alcohol dehydrogenase |
| YNL331C | AAD14 | 22.3 | 21.5 | 0.001 | 23.1 | 0.000 | Putative aryl-alcohol dehydrogenase |
| **YLR346C**** | | 22.3 | 22.9 | 0.002 | 21.7 | 0.000 | Protein of unknown function localised to mitochondria |
| YOL151W | GRE2 | 19.5 | 18.3 | 0.000 | 20.7 | 0.000 | Methylglyoxal reductase (NADPH-dependent) |
| YCR107W | AAD3 | 15.0 | 28.4 | 0.000 | 1.6 | 0.000 | Aryl-alcohol dehydrogenase |
| YLR303W | MET17 | 14.7 | 12.3 | 0.000 | 17.1 | 0.000 | O-acetylhomoserine sulfhydrylase |
| YLL056C | | 13.6 | 16.5 | 0.000 | 10.7 | 0.000 | Weak similarity to Y. pseudotuberculosis epimerase |
| **YLL060C**** | GTT2 | 13.2 | 13.1 | 0.000 | 13.3 | 0.000 | Glutathione S-transferase |
| **YOR153W*** | PDR5 | 12.5 | 16.3 | 0.000 | 8.8 | 0.000 | ABC transporter involved in multidrug resistance |
| YGR213C | RTA1 | 12.3 | 9.7 | 0.001 | 15.0 | 0.000 | Integral membrane protein |
| YOR049C | | 12.2 | 11.1 | 0.001 | 13.3 | 0.000 | Putative integral membrane transporter |
| YKR076W | ECM4 | 11.2 | 11.7 | 0.000 | 10.7 | 0.000 | Involved in cell wall biogenesis and architecture |
| YML131W | | 10.7 | 9.2 | 0.000 | 12.2 | 0.000 | Putative hydroxydehydrogenase |
| **YKL070W**** | | 10.7 | 9.1 | 0.020 | 12.3 | 0.000 | Similarity to B. subtilis transcriptional regulatory protein |
| YIL167W | | 9.3 | 9.0 | 0.005 | 9.5 | 0.000 | Serine dehydratase |

* NA, Not applicable (experiment was either performed less than three times or the data was not valuable
** Names indicated in bold means the genes whose products are localized in the mitochondria

organelles and are not specific to mitochondrial function, our results suggest that the effect of citrinin on mitochondria is true but not specific.

The functional categories of the repressed genes were also characterized (data not shown). As often seen with the stressed cells, the category of genes involved in "Protein synthesis" were significantly repressed but other significant character was not observed. The repression of the genes in the category of "Protein synthesis" can be the experimental marker, as this functional group is required for the actively growing cells, and not for the slowly growing or growth inhibited cells [17].

### *Confirmation of the significantly affected genes and evaluation of both DNA microarrays*

Except the *AAD15*, *AAD10*, *AAD3*, and *PAU15*, the highly induced genes were common between the ORF DNA microarray and Oligo DNA microarray. The *AAD* genes have strong similarity to each other and this caused cross hybridization in the ORF DNA microarray [18]. Some of the highly induced *AAD* genes could cross hybridize to the ORF DNA microarray spots corresponding to the *AAD15*, *AAD10*, and *AAD3*. To confirm which *AAD* gene was really induced, we performed RT-PCR analysis. As shown in Figure 5, citrinin treatment induced the *AAD4*, *AAD6*, and *AAD16* genes, but not the *AAD3*, *AAD10*, *AAD14*, and *AAD15* genes. Thus, the induction of the *AAD 4*, *AAD6*, and *AAD16* genes, as observed by both microarray analysis, were correct whereas the induction of the *AAD3*, *AAD10*, *AAD14*, and *AAD15* genes in ORF DNA microarray and the induction of the *AAD14* in Oligo DNA microarray were due to cross hybridization. We confirmed that the *AAD14* probe has only one mismatch to the *AAD4* ORF, and the apparent induction of the *AAD14* was due to the cross hybridiztion to the *AAD4*. In the Oligo DNA microarray, it seems that the cross hybridization has a limit of one miss match. The *PAU15* gene was also highly

**Table 2: List of strongly repressed genes by the citrinin treatment**

| Systematic Name | Common Name | Average (Fold) | ORF-Array | | Oligo-Array | | MIPS_Description |
|---|---|---|---|---|---|---|---|
| | | | Fold | t-test P-value | Fold | t-test P-value | |
| YPL095C | | 0.19 | 0.19 | 0.002 | 0.18 | 0.000 | Hypothetical ORF |
| YDR380W | ARO10 | 0.29 | 0.36 | 0.038 | 0.21 | 0.000 | Phenylpyruvate decarboxylase |
| YGL255W | ZRT1 | 0.33 | 0.36 | 0.015 | 0.30 | 0.000 | High-affinity zinc transporter |
| YKL096W | CWP1 | 0.35 | 0.38 | 0.000 | 0.32 | 0.000 | Cell wall mannoprotein |
| YIL119C | RPI1 | 0.37 | 0.28 | 0.028 | 0.46 | 0.000 | Putative transcriptional regulator |
| YHL028W | WSC4 | 0.39 | 0.51 | 0.033 | 0.28 | 0.000 | Cell wall integrity and stress response |
| YHR137W | ARO9 | 0.40 | 0.37 | 0.001 | 0.43 | 0.000 | Aromatic aminotransferase |
| YPR194C | OPT2 | 0.40 | 0.46 | 0.020 | 0.35 | 0.000 | Oligopeptide transporter |
| YMR120C | ADE17 | 0.41 | 0.41 | 0.007 | 0.41 | 0.000 | Enzyme of 'de novo' purine biosynthesis |
| YAR015W | ADE1 | 0.42 | 0.31 | 0.002 | 0.54 | 0.000 | SAICAR synthetase |
| YMR011W | HXT2 | 0.43 | 0.41 | 0.003 | 0.44 | 0.000 | High-affinity glucose transporter |
| YPR160W | GPH1 | 0.44 | 0.41 | 0.018 | 0.47 | 0.000 | Non-essential glycogen phosphorylase |
| YPL092W | SSU1 | 0.44 | 0.41 | 0.009 | 0.48 | 0.000 | Plasma membrane sulfite pump |
| YBL098W | | 0.45 | 0.42 | 0.002 | 0.47 | 0.000 | Kynurenine 3-mono oxygenase |
| YFR015C | GSY1 | 0.45 | 0.48 | 0.024 | 0.42 | 0.000 | Glycogen synthase |
| YOR315W | | 0.45 | 0.41 | 0.007 | 0.50 | 0.000 | Protein of unknown function, |
| YDL227C | HO | 0.45 | 0.49 | 0.048 | 0.42 | 0.000 | Site-specific endonuclease |

induced by citrinin treatment in Oligo DNA microarray. This gene has high similarity to other *PAU* genes, which were not induced. We, however, could not confirm the induction of the *PAU* genes by RT-PCR. Thus, the apparent induction of the *PAU15* was most likely due to the cross hybridization with some highly induced unknown gene.

## Discussion

Mycotoxins are fungal secondary metabolites that may be toxic to all kinds of organisms. So far, a few hundreds of mycotoxins are identified and this number can increase dramatically with the development of analytical equipment. Mycotoxins are naturally occurring chemicals. The large-scale production and industrial applications of these mycotoxins are limited, because the purification of these mycotoxins are costly and inadequate. Therefore, only a few mycotoxins were studied in detail. The DNA microarray technology provides an alternative evaluation tool to examine chemical toxicity in organisms. Particularly, the yeast DNA microarray is appropriate for evaluating the action of the mycotoxin because of the less amount of toxin required in this assay and good reproducibility of the expression profile.

Citrinin is the one of the well known mycotoxins produced by *Penicillium* and *Aspergillus* family and is possibly spread all over the world [1]. The yeast-based ORF DNA microarray and Oligo DNA microarray can provide information on the possible mechanisms of toxicity and detoxification effort by yeast cells. The list of highly induced genes in citrinin-treated yeast cells (Table 1) clearly shows that the *AADs*, *OYE3*, *MET17*, and *GRE2* genes, which are typical indicator genes for the oxidative stress [10,11], are highly induced. Thus, we can conclude that citrinin treatment causes oxidative stress. Previously, Delneli *et al.* [10] analyzed several *AAD* deletion mutants and suggested that only *AAD6* and *AAD4* were induced by oxidative stress. Our RT-PCR results however suggest the *AAD16* gene is induced. Except oxidative stress, we could not find any other cell repair response. It was suggested that citrinin causes damage to the mitochondria. However, we could not confirm that citrinin specifically affects mitochondria. Mitochondria can be the source of oxidative stress. Thus, it is possible that the oxidative stress caused by citrinin could enhance the self-induced oxidative damages in mitochondria. The mycotoxin patulin produced response in yeast cells that was similar to that of the citrinin, as the oxidative stress related genes were also

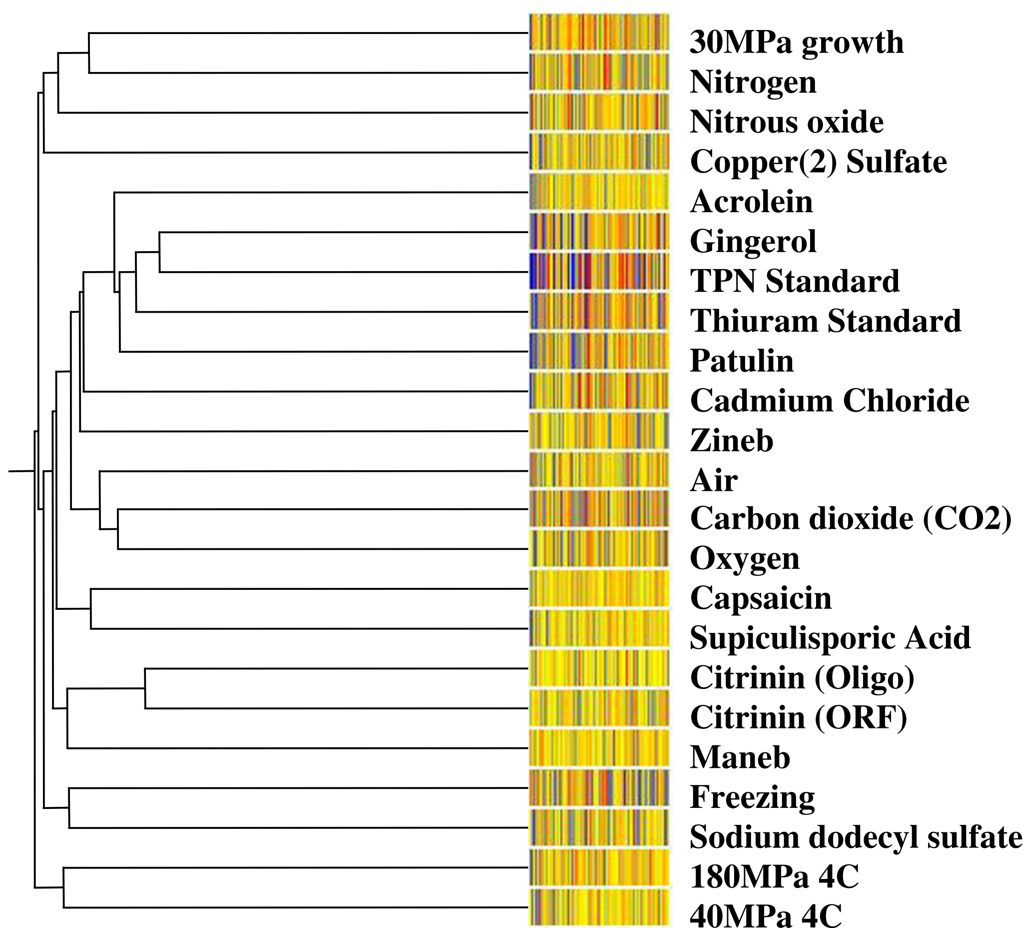


**Figure 4**
Cluster analysis of the mRNA expression profiles after the citrinin treatment. Hierarchical cluster analysis was performed using GeneSpring as described in the text.

induced by patulin treatment [7]. In addition, the patulin treatment strongly induced the genes contributing to the protein metabolism and DNA repair, and patulin was considered as a natural mutagenic chemical [7]. However, in comparison to the patulin treatment, the citrinin treatment did not induce the genes contributing to DNA repair (Table 7). Except the oxidative stress, citrinin did not show any significant toxicity to yeast cells. The less toxicity of citrinin than the patulin was also reported in other organisms [19].

Contrast to the information concerning the mechanism of citrinin-induced toxicity, information on the detoxification mechanism was clear. The activation of the methionine and glutathione metabolisms (Table 4) strongly suggest the contribution of glutathione in the detoxification process. Moreover, strong induction of the *DTT2* gene implies direct transfer of glutathione to citrinin. As the *PDR*s were also strongly induced (Table 5), it may be possible that the ABC transporters were involved in pumping out the citrinin-glutathione complex. Pumping out the toxin after glutathione modification is one of the main detoxification pathway used by many organism [19].

During the process of evaluating the citrinin toxicity, we also compared reproducibility of the ORF DNA microarray and Oligo DNA microarray. The Oligo DNA microarray showed higher correlation factor than the ORF DNA microarray (region B in Figure 2). This may have resulted from the cross hybridization exampled by *AAD*s. The apparent induction of the *AAD*s in the ORF DNA microarray was due to cross hybridization [7]. The Oligo DNA microarray showed less cross hybridization, as the expression levels of most of the *AAD*s obtained from this assay agreed with the RT-PCR results. However, the Oligo DNA microarray may have limits in terms of specificity, as the *AAD14* gene, which has one mismatch with the *AAD4*

**Table 3: Contribution of induced genes to functional categories**

| | | ORF DNA microarray | | | | OligoDNA microarray | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | F > 2 & P < 0.05* | | P < 0.01* | | F > 2 & P < 0.05* | | P < 0.01* | |
| Category subcategory | Total number in category | Number | %* | Number | % | Number | % | Number | % |
| Metabolism | 1521 | 54 | 3.6 | 103 | 6.8 | 51 | 2.4 | 266 | 17.0 |
| amino acid metabolism | 243 | 20 | 8.2 | 33 | 13.6 | 25 | 10.3 | 81 | 33.3 |
| nitrogen and sulfur metabolism | 96 | 9 | 9.4 | 15 | 15.6 | 11 | 11.5 | 39 | 40.6 |
| nucleotide metabolism | 227 | 6 | 2.6 | 12 | 5.3 | 2 | 0.9 | 22 | 9.7 |
| phosphate metabolism | 414 | 7 | 1.7 | 15 | 3.6 | 6 | 1.4 | 55 | 13.3 |
| C-compound and carbohydrate metabolism | 504 | 18 | 3.6 | 36 | 7.1 | 19 | 3.8 | 86 | 17.1 |
| lipid, fatty acid and isoprenoid metabolism | 272 | 7 | 2.6 | 20 | 7.4 | 5 | 1.8 | 30 | 11.0 |
| metabolism of vitamins, | 163 | 11 | 6.7 | 16 | 9.8 | 9 | 5.5 | 40 | 24.5 |
| secondary metabolism | 77 | 4 | 5.2 | 7 | 9.1 | 4 | 5.2 | 19 | 24.7 |
| ENERGY | 365 | 17 | 4.7 | 34 | 9.3 | 15 | 4.1 | 59 | 16.2 |
| CELL CYCLE AND DNA PROCESSING | 1001 | 9 | 0.9 | 37 | 3.7 | 4 | 0.4 | 119 | 11.9 |
| TRANSCRIPTION | 1063 | 10 | 0.9 | 39 | 3.7 | 9 | 0.8 | 87 | 8.2 |
| PROTEIN SYNTHESIS | 476 | 3 | 0.6 | 20 | 4.2 | 1 | 0.2 | 8 | 1.7 |
| PROTEIN FATE (folding, modification, destination) | 1137 | 23 | 2.0 | 65 | 5.7 | 8 | 0.7 | 159 | 14.0 |
| PROTEIN WITH BINDING FUNCTION | 1034 | 22 | 2.1 | 54 | 5.2 | 15 | 1.5 | 134 | 13.0 |
| PROTEIN ACTIVITY REGULATION | 238 | 2 | 0.8 | 6 | 2.5 | 1 | 0.4 | 23 | 9.7 |
| CELLULAR TRANSPORT | 1031 | 33 | 3.2 | 74 | 7.2 | 18 | 1.7 | 131 | 12.7 |
| CELLULAR COMMUNICATION | 234 | 1 | 0.4 | 4 | 1.7 | 1 | 0.4 | 28 | 12.0 |
| CELL RESCUE, DEFENSE AND VIRULENCE | 548 | 31 | 5.7 | 47 | 8.6 | 28 | 5.1 | 118 | 21.5 |
| INTERACTION WITH THE CELLULAR ENVIRONMEN | 458 | 16 | 3.5 | 28 | 6.1 | 9 | 2.0 | 71 | 15.5 |
| INTERACTION WITH THE ENVIRONMENT | 5 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 20.0 |
| TRANSPOSABLE ELEMENTS | 124 | 1 | 0.8 | 1 | 0.8 | 0 | 0.0 | 5 | 4.0 |
| DEVELOPMENT (Systemic) | 70 | 1 | 1.4 | 4 | 5.7 | 0 | 0.0 | 7 | 10.0 |
| BIOGENESIS OF CELLULAR COMPONENTS | 854 | 11 | 1.3 | 31 | 3.6 | 6 | 0.7 | 95 | 11.1 |
| CELL TYPE DIFFERENTIATION | 449 | 3 | 0.7 | 18 | 4.0 | 2 | 0.4 | 48 | 10.7 |
| UNCLASSIFIED PROTEINS | 2038 | 37 | 1.8 | 74 | 3.6 | 23 | 1.1 | 163 | 8.0 |
| Total | | 155 | | 363 | | 113 | | 801 | |

**Table 4: Glutathione and methionine metabolism related genes are induced by the citrinin treatment**

| Systematic Name | Common Name | Average (Fold) | ORF-Array | | Oligo-Array | | Description |
|---|---|---|---|---|---|---|---|
| | | | Fold | t-test P-value | Fold | t-test P-value | |
| YKR069W | MET1 | 2.6 | 2.3 | 0.002 | 2.9 | 0.000 | siroheme synthase |
| YFR030W | MET10 | 3.2 | 1.8 | 0.025 | 4.6 | 0.000 | sulfite reductase flavin-binding subunit |
| YKL001C | MET14 | 5.2 | 5.3 | 0.000 | 5.1 | 0.000 | ATP adenosine-5^-phosphosulfate 3^-phosphotransferase |
| YPR167C | MET16 | 5.1 | 5.6 | 0.001 | 4.6 | 0.000 | 3^-phosphoadenylylsulfate reductase |
| YLR303W | MET17 | 14.7 | 12.3 | 0.000 | 17.1 | 0.000 | O-acetylhomoserine sulfhydrylase |
| YNL277W | MET2 | 2.9 | 2.1 | 0.022 | 3.8 | 0.000 | homoserine O-acetyltransferase |
| YOL064C | MET22 | 1.7 | 1.8 | 0.000 | 1.7 | 0.000 | protein ser/thr phosphatase |
| YIR017C | MET28 | 4.7 | 2.0 | 0.198 | 7.5 | 0.000 | transcriptional activator of sulfur amino acid metabolism |
| YJR010W | MET3 | 8.9 | 6.0 | 0.000 | 11.8 | 0.000 | sulfate adenylyltransferase |
| YIL046W | MET30 | 1.1 | 1.2 | 0.203 | 1.1 | 0.147 | involved in regulation of sulfur assimilation genes |
| YPL038W | MET31 | 1.0 | 1.2 | 0.151 | 0.9 | 0.004 | transcriptional regulator of sulfur amino acid metabolism |
| YDR253C | MET32 | 2.7 | 2.2 | 0.004 | 3.3 | 0.000 | transcriptional regulator of sulfur amino acid metabolism |
| YNL103W | MET4 | 1.0 | 0.7 | 0.102 | 1.2 | 0.001 | transcriptional activator of sulfur metabolism |
| YER091C | MET6 | 2.7 | 2.6 | 0.001 | 2.7 | 0.000 | homocysteine methyltransferase |
| YBR213W | MET8 | 1.4 | 1.2 | 0.460 | 1.6 | 0.000 | siroheme synthase |
| YAL012W | CYS3 | 2.0 | 1.9 | 0.009 | 2.0 | 0.000 | cystathionine gamma-lyase |
| YGR155W | CYS4 | 1.9 | 2.4 | 0.062 | 1.5 | 0.000 | cystathionine beta-synthase |
| YJL101C | GSH1 | 2.4 | 2.2 | 0.000 | 2.7 | 0.000 | glutamate – cysteine ligase |
| YOL049W | GSH2 | 1.1 | 0.9 | 0.064 | 1.2 | 0.000 | Glutathione synthetase |
| YLR180W | SAM1 | 1.4 | 1.6 | 0.023 | 1.3 | 0.000 | S-adenosylmethionine synthetase 1 |
| YDR502C | SAM2 | 1.6 | 1.5 | 0.000 | 1.6 | 0.000 | S-adenosylmethionine synthetase 2 |
| YPL274W | SAM3 | 1.2 | 1.3 | 0.015 | 1.1 | 0.124 | S-adenosylmethionine permease |
| YPL273W | SAM4 | 0.9 | 0.8 | 0.010 | 1.0 | 0.008 | AdoMet-homocysteine methyltransferase |
| YJR130C | STR2 | 1.4 | 1.2 | NA | 1.6 | 0.000 | Cystathionine gamma-synthase |
| YGL184C | STR3 | 2.1 | 1.4 | 0.367 | 2.9 | 0.001 | cystathionine beta-lyase |

* NA, Not applicable (experiment was either performed less than three times or the data was not valuable

gene, was recognized as the induced gene. On the other hand, the *PAU15* gene was not recognized as the induced gene by the ORF DNA microarray and RT-PCR, but was recognized as induced gene by the Oligo DNA microarray. If the RT-PCR results were correct, these results suggest that the high specificity may not always produce correct results. Although the Oligo DNA microarray did not completely overcome the cross hybridization in the case of single mismatch, it is still a useful tool for detecting gene expression differences between similar genes.

## Conclusion

Citrinin caused growth inhibition in yeast cells at a concentration more than 100 ppm. Under this condition, we monitored the citrinin treatment-induced response using the ORF DNA microarray and Oligo DNA microarray. Results obtained from these microarray experiments suggest that citrinin induced oxidative stress in the yeast cells. The citrinin-induced genotoxicity was less severe than that of the patulin. Thus, citrinin is a less toxic substance than patulin. The expression profiles obtained from both types of DNA microarrays were essentially similar. The reproducibility of the expression profiles were much better and the cross hybridization was less with the Oligo DNA microarray.

## Methods

### *Strain, growth conditions, and citrinin 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 pre-culture for 2–3 days. This strain was used because the ORF DNA microarray probes were produced using the S288C DNA as the template for PCR [6] and because Oligo DNA microarray probes were designed based on the DNA sequence of this strain [20]. Citrinin was purchased from MP Biochemicals (Irvine, CA, USA) and was dissolved in DMSO (Dimethyl sulfoxide) to prepare a stock solution of 20000 ppm. To monitor the dose response of citrinin to yeast cells, the stock solution was added directly to the YPD medium containing the yeast cells such that they were diluted more than 100-fold. For the DNA microarray analysis, yeast cultures in YPD were diluted and grown overnight to an optical density (OD660) of 1.0. The citrinin stock solution was added to the cultures and yeast cells were allowed to grow for an additional 2 h. For the control cells, the same volume of DMSO was

**Table 5: List of highly induced genes in the category of "CELL RESCUE, DEFENSE AND VIRULENCE"**

| Systematic Name | Common Name | Average (Fold) | ORF-Array | | Oligo-Array | | Description |
|---|---|---|---|---|---|---|---|
| | | | Fold | t-test P-value | Fold | t-test P-value | |
| YBR008C | FLR1 | 33.6 | 37.9 | 0.000 | 29.4 | 0.000 | Plasma membrane multidrug **transporter** |
| YOL151W | GRE2 | 19.5 | 18.3 | 0.000 | 20.7 | 0.000 | NADPH-dependent methylglyoxal reductase |
| YLL060C | GTT2 | 13.2 | 13.1 | 0.000 | 13.3 | 0.000 | Glutathione S-transferase |
| YOR153W | PDR5 | 12.5 | 16.3 | 0.000 | 8.8 | 0.000 | Short-lived membrane ABC **transporter** |
| YGR213C | RTA1 | 12.3 | 9.7 | 0.001 | 15.0 | 0.000 | involved in 7-aminocholesterol resistance |
| YHR048W | | 5.8 | 3.4 | 0.003 | 8.2 | 0.000 | Hypothetical ORF |
| YDR011W | SNQ2 | 5.2 | 6.6 | 0.000 | 3.8 | 0.000 | ABC **transporter** |
| YML116W | ATR1 | 5.2 | 5.5 | 0.000 | 4.8 | 0.000 | Multidrug efflux pump of the major facilitator superfamily |
| YGR281W | YOR1 | 4.6 | 4.7 | 0.000 | 4.5 | 0.000 | ABC **transporter** |
| YNL231C | PDR16 | 3.8 | 3.4 | 0.000 | 4.3 | 0.000 | Phosphatidylinositol transfer protein |
| YHL040C | ARN1 | 3.8 | 3.1 | 0.002 | 4.5 | 0.000 | Member of the ARN family of **transporters** |
| YNL160W | YGP1 | 3.2 | 2.9 | 0.000 | 3.5 | 0.000 | May be involved in cellular adaptations prior to stationary pha |
| YMR038C | LYS7 | 3.1 | 3.3 | 0.000 | 3.0 | 0.000 | Copper chaperone for superoxide dismutase Sod1p |
| YGR209C | TRX2 | 3.0 | 3.1 | 0.025 | 3.0 | 0.000 | Thioredoxin |
| YMR173W | DDR48 | 2.9 | 3.0 | 0.003 | 2.8 | 0.000 | DNA damage-responsive protein |
| YHR136C | SPL2 | 2.8 | 3.5 | 0.000 | 2.2 | 0.000 | Protein with similarity to cyclin-dependent kinase inhibitors |
| YDR533C | | 2.8 | 3.0 | 0.000 | 2.6 | 0.000 | Possible chaperone and cysteine protease |
| YER042W | MXR1 | 2.6 | 2.2 | 0.001 | 2.9 | 0.000 | Peptide methionine sulfoxide reductase |
| YBL064C | | 2.5 | 3.0 | 0.000 | 2.1 | 0.000 | Mitochondrial peroxiredoxin with thioredoxin peroxidase |
| YER185W | | 2.5 | 2.8 | 0.003 | 2.3 | 0.000 | Hypothetical ORF |
| YDR135C | **YCF1** | 2.5 | ND * | | 2.5 | 0.000 | Vacuolar glutathione S-conjugate **transporter** |
| YDR032C | PST2 | 2.5 | 2.6 | 0.005 | 2.4 | 0.000 | Similarity to members of a family of flavodoxin-like proteins |
| YJL101C | GSH1 | 2.4 | 2.2 | 0.000 | 2.7 | 0.000 | Gamma glutamylcysteine synthetase |

* ND, Not determined

added to the yeast culture and this was incubated for 2 h. Cells were harvested by centrifugation and stored at -80°C until used.

### *DNA microarray analysis*

DNA microarray analysis was carried out on three independent cultures and total RNA was isolated by the hot-phenol method as described previously [21].

For the ORF type DNA microarray, yeast DNA microarray Ver. 2.0 (DNA Chip Research, Inc., Yokohama, Japan) was used and the hybridization was performed using the dual color method. The Cy3- or Cy5-labeled cDNA pools were synthesized by CyScribe First-Strand cDNA Labeling Kit (GE Healthcare UK Ltd., Buckinghamshire, England). On this microarray, a total of 6,037 kinds of amplified ORFs with 200–8,000 bp length (0.1–0.5 ng) were spotted. The Cy3- or Cy5-labeled aRNA mixed pools were hybridized for 24–36 h at 65°C. The details of our conditions for the microarray procedure and validation studies were previously described [6-8,21,22].

For the Oligo DNA microarray, 3D-Gene Yeast Oligo Chip 6K (Toray Industries Inc., Tokyo, Japan/DNA Chip Research, Inc., Yokohama, Japan) was used. For efficient hybridization, this microarray has 3-dimensions that is constructed with a well as the space between the probes and cylinder-stems with 30-mer oligonucleotide probes on the top. Total RNA was labeled with Cy3- or Cy5- using the Amino Allyl MessageAMP II aRNA Amplificatin Kit (Applied Biosystems, CA, U.S.A.). The Cy3- or Cy5-labeled aRNA pools and hybridization buffer containing micro beads were mixed, and hybridized for 16 h. The hybridization was performed using the supplier's protocols.

### *Data analysis*

Detected signals for each ORF were normalized by the intensity dependent (LOWESS) methods [23]. The cutoff values were the intensity of the background average plus 2SD. Genes were characterized for function according to the functional categories established by MIPS [24] and the SGD [25]. The data obtained in this experiment have been assigned accession number GSE6118 in the Gene Expression Omnibus Database [26].

Hierarchical cluster analysis was performed using the GeneSpring ver. 7.3.1 software (Silicon Genetics, CA, USA). The clustering algorithm arranges conditions according to their similarity in the expression profiles

**Table 6: Localization of the citrinin-induced gene products**

| | Entries | | ORF DNA microarray | | | | OligoDNA microarray | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | F > 2 & P < 0.05 | | P < 0.01 | | F > 2 & P < 0.05 | | P < 0.01 | |
| Localization | Number | Impact* | Number | Impact | Number | Impact | Number | Impact | Number | Impact |
| extracellular | 54 | 1.0 | 2 | 1.4 | 6 | 1.8 | 4 | 3.9 | 10 | 1.4 |
| bud | 149 | 2.9 | 3 | 2.2 | 5 | 1.5 | 0 | 0.0 | 13 | 1.8 |
| cell wall | 42 | 0.8 | 1 | 0.7 | 4 | 1.2 | 3 | 2.9 | 7 | 1.0 |
| cell periphery | 216 | 4.1 | 11 | 8.0 | 20 | 5.9 | 8 | 7.8 | 30 | 4.2 |
| plasma membrane | 186 | 3.6 | 8 | 5.8 | 18 | 5.3 | 5 | 4.9 | 29 | 4.1 |
| integral membrane/endomembranes | 176 | 3.4 | 10 | 7.2 | 14 | 4.2 | 7 | 6.9 | 23 | 3.2 |
| cytoplasm | 2906 | 55.8 | 94 | 68.1 | 191 | 56.7 | 76 | 74.5 | 449 | 63.2 |
| cytoskeleton | 204 | 3.9 | 3 | 2.2 | 5 | 1.5 | 2 | 2.0 | 25 | 3.5 |
| ER | 557 | 10.7 | 13 | 9.4 | 57 | 16.9 | 8 | 7.8 | 92 | 13.0 |
| golgi | 132 | 2.5 | 2 | 1.4 | 8 | 2.4 | 1 | 1.0 | 16 | 2.3 |
| transport vesicles | 139 | 2.7 | 2 | 1.4 | 6 | 1.8 | 0 | 0.0 | 13 | 1.8 |
| nucleus | 2157 | 41.4 | 49 | 35.5 | 129 | 38.3 | 35 | 34.3 | 304 | 42.8 |
| mitochondria | 1056 | 20.3 | 33 | 23.9 | 77 | 22.8 | 21 | 20.6 | 149 | 21.0 |
| peroxisome | 52 | 1.0 | 2 | 1.4 | 3 | 0.9 | 0 | 0.0 | 5 | 0.7 |
| endosome | 57 | 1.1 | 1 | 0.7 | 5 | 1.5 | 1 | 1.0 | 10 | 1.4 |
| vacuole | 280 | 5.4 | 14 | 10.1 | 27 | 8.0 | 8 | 7.8 | 47 | 6.6 |
| microsomes | 5 | 0.1 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 0.1 |
| lipid particles | 27 | 0.5 | 2 | 1.4 | 4 | 1.2 | 1 | 1.0 | 3 | 0.4 |
| punctate composite | 141 | 2.7 | 5 | 3.6 | 9 | 2.7 | 4 | 3.9 | 15 | 2.1 |
| ambiguous | 237 | 4.5 | 6 | 4.3 | 18 | 5.3 | 4 | 3.9 | 37 | 5.2 |
| KNOWN LOCALIZATION | 5209 | 100 | 138 | 100 | 337 | 100 | 102 | 100 | 710 | 100 |
| UNKNOWN LOCALIZATION | 1516 | | 17 | | 26 | | 11 | | 91 | |

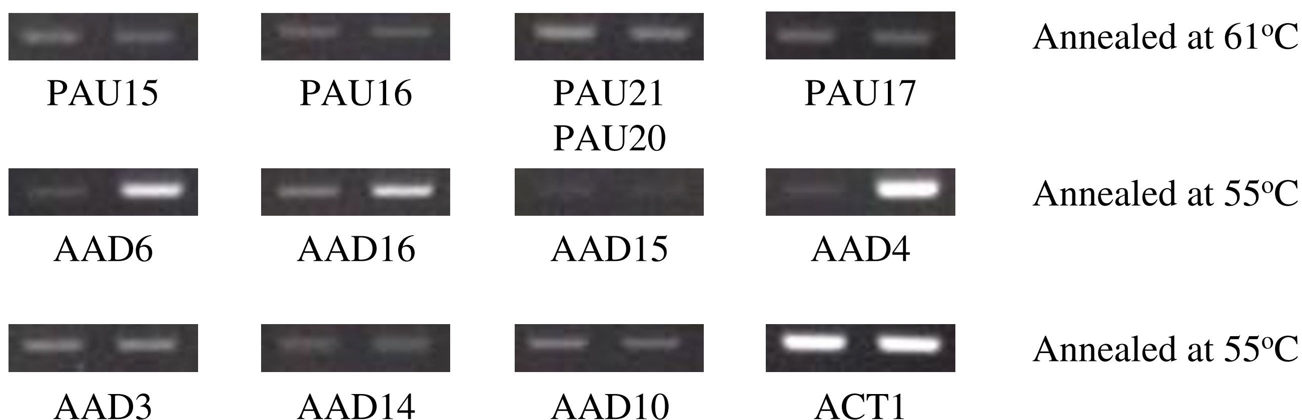


**Figure 5**
**Confirmation of gene induction by RT-PCR**. The RT-PCR analysis was performed using the primers described in Methods. Names of the genes are shown below the images.

**Table 7: Comparison of the patulin – and citrinin-induced genes contributing to DNA repair**

| | Fold Induction | | | |
|---|---|---|---|---|
| Systematic Name | Patulin | Citrinin | Common Name | MIPS_Description |
| YDL059C | 5.7 | 1.8 | RAD59 | Recombination and DNA repair protein |
| YGL163C | 5.3 | 1.0 | RAD54 | DNA-dependent ATPase of the Snf2p family |
| YGR209C | 4.4 | 3.0 | TRX2 | Thioredoxin II |
| YDR092W | 4.0 | 1.2 | UBC13 | E2 ubiquitin-conjugating enzyme |
| YER142C | 3.9 | 1.5 | MAG1 | 3-methyladenine DNA glycosylase |
| YHL024W | 3.7 | 1.1 | RIM4 | No sporulation |
| YFL014W | 3.7 | 0.9 | HSP12 | Heat shock protein |
| YPR193C | 3.7 | 1.2 | HPA2 | Histone and other Protein Acetyltransferase |
| YKL145W | 3.5 | 1.3 | RPT1 | 26S proteasome regulatory subunit |
| YMR173W | 3.4 | 2.9 | DDR48 | Heat shock protein |
| YAR007C | 3.4 | 1.0 | RFA1 | DNA replication factor A, 69 KD subunit |
| YPL194W | 3.1 | 1.1 | DDC1 | DNA damage checkpoint protein |
| YLR043C | 3.0 | 1.4 | TRX1 | Thioredoxin I |
| YOR023C | 2.9 | 1.0 | AHC1 | Component of the ADA histone acetyltransferase comple |
| YEL037C | 2.8 | 1.1 | RAD23 | Nucleotide excision repair protein (ubiquitin-like protein) |
| YMR302C | 2.8 | 0.9 | PRP12 | Involved in early maturation of pre-rRNA |
| YNL312W | 2.7 | 1.2 | RFA2 | DNA replication factor A, 36 kDa subunit |
| YIL143C | 2.7 | 1.1 | SSL2 | DNA helicase |
| YJR052W | 2.6 | 1.1 | RAD7 | Nucleotide excision repair protein |
| YOL068C | 2.4 | 0.7 | HST1 | Silencing protein |
| YGR231C | 2.4 | 1.0 | PHB2 | Prohibitin |
| YPR023C | 2.3 | 1.2 | EAF3 | Esa1p-associated factor |
| YML032C | 2.3 | 1.1 | RAD52 | Recombination and DNA repair protein |
| YIR025W | 2.2 | 1.2 | MND2 | Subunit of anaphase-promoting complex |
| YGL201C | 2.1 | 1.0 | MCM6 | Involved in replication |
| YMR201C | 2.1 | 0.9 | RAD14 | Nucleotide excision repair protein |
| YNL250W | 2.0 | 1.2 | RAD50 | DNA repair protein |
| YCR086W | 2.0 | 1.5 | CSM1 | Involved in nuclear migration |

across all conditions, such that conditions with similar patterns are clustered together as in a taxonomic tree. Data from 3874 genes were used for the calculation. These 3874 genes were selected on the basis of having previously exhibited higher than average intensities in another trial [21].

### *RT-PCR*

A reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to confirm the result of the microarray experiments for the genes showing different patterns of expression between the ORF type microarray and the oligo probe microarray. The primers for the *AADs* were described previously [7]. The primers for the *PAU*s are:

*PAU15* (YIR041W),

CTTGTTTCAAGCAGCTCATCCAAGT and ATGGAATCTCATTCGTAAAGGCATG; *PAU16*(YKL224C),

CTTGTTTCAAGCAGCTCATCCAAGT and CATATTCATAAAATGCTTCACG; *PAU21/22* (YOR394W, YPL282C),

TACCAGATTGAGACCGGCTATC and TACTCCACAAACACTGTTATTG; and

*PAU17* (YLL025W),

GAGCTCATTTGGCTGAATACTATATG and TGCAGATAGAGCGCTGGAGATG. Total RNA prepared for the microarray analysis was used as template for the RT-PCR experiments. Reverse transcriptase reaction was performed using the StrataScript First-Strand Synthesis System (STRATAGENE, CA, USA). The cDNA mixture was diluted 20 times, and 2 μl of the diluted solution was used for a 20 μl PCR reaction using the TaKaRa Ex Taq HS (TaKaRa, Shiga, Japan). Annealing temperature was originally set at 55°C. However, the *PAU*s showed multiple bands at 55°C and annealing temperature was increased to 61°C. Each amplification reaction was resolved on a

2% agarose gel and the DNA bands were visualized with EtBr staining.

## Abbreviations

ORF: open reading frame

Oligo: oligo-nucleotide

MIPS: Munich Information Center for Protein Sequences

SGD: Yeast Genome Database

DMSO: Dimethyl sulfoxide

RT-PCR : reverse transcriptase-polymerase chain reaction

## Authors' contributions

HI planned and designed the study and wrote the main draft of the manuscript. EK analyzed the DNA microarray results and performed the RT-PCR experiments. YS performed the ORF DNA microarray experiments. YU and YI performed the Oligo DNA microarray experiments. HN and YK analyzed the Oligo DNA microarray results and contribute on the cross hybiridization search based on the ligo nucleotide on the microarray. HH contributed the selection of mycotoxin and planned the experiments. YI has the responsibility for the budget supporting the most part of this work and planned and performed the mycotoxin experiments. All authors read and approved the final manuscript.

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25. **Yeast Genome Database** [http://www.yeastgenome.org/]
26. **Gene Expression Omnibus Database** [http://www.ncbi.nlm.nih.gov/geo/]