JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Comprehensive Gene Expression Analysis of Type B Trichothecenes

Tadahiro Suzuki and Yumiko Iwahashi*

Applied Microbiology Division, National Food Research Institute, 2-1-12 Kan-nondai, Tsukuba, Ibaraki 305-8642, Japan

Supporting Information

ABSTRACT: Type B trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), are secondary metabolites of *Fusarium* species and are major pollutants in food and feed products. Recently, the production trend of their derivatives, 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), and 4-acetylnivalenol (4-AcNIV or fusarenon-X), has been changing in various regions worldwide. Although *in vivo* behavior has been reported, it is necessary to acquire more detailed information about these derivatives. Here, the yeast *PDR5* mutant was used for toxicity evaluation, and the growth test revealed that DON, 15-AcDON, and 4-AcNIV had higher toxicity compared to 3-AcDON and NIV. 15-AcDON exerted the most significant gene expression changes, and cellular localization clustering exhibited repression of mitochondrial ribosomal genes. This study suggests that the toxicity trends of both DON products (DON and its derivatives) and NIV products (NIV and its derivatives) are similar to those observed in mammalian cells, with a notable toxic response to 15-AcDON.

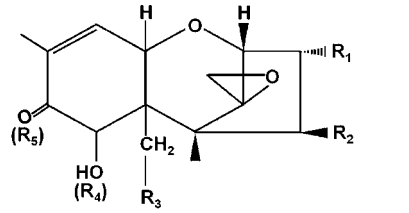
KEYWORDS: *Type B trichothecene, DNA microarray, yeast, PDR5*

INTRODUCTION

Fusarium head blight is a serious plant pathogen worldwide. This is mainly caused by *Fusarium* species, such as *Fusarium graminearum*. These fungi attack wheat and other food plants, and it is thought that, as a result, worldwide food and feed production suffers a considerable degree of economic damage every year.[1] In addition, these fungi occasionally produce toxic secondary metabolites, called trichothecene mycotoxins. Trichothecene mycotoxins are a common group of compounds associated with fusarium head blight. There are several end products and derivatives that are formed by combining several sets of functional moieties. Types A and B are prevalent groups in all types of trichothecenes. The type A trichothecene group contains highly toxic mycotoxins, such as T-2 and HT-2 toxins, while the type B group includes deoxynivalenol (DON) and nivalenol (NIV), and their derivatives, 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), and 4-acetylnivalenol (4-AcNIV or fusarenon-X) (Figure 1). DON and NIV have been recognized as major food-contaminating toxins, and a regulatory value for DON has been established in a number of countries.[2]

Type B trichothecenes are not as toxic as the T-2 and HT-2 toxins of the type A group, but the physical disorders observed in livestock are frequently caused by the type B group.[3,4] The toxicity to livestock is mainly caused by translation inhibition, attributed to the binding to the 60S ribosome, resulting in protein synthesis inhibition.[5,6] These toxins cause oxidative stress in mitochondria, G2/M cell cycle arrest, apoptosis, and immunotoxic effects.[7−10] In combination, these effects result in the delay of repair, cell death, intestine inflammation, and anorexia or diarrhea.

The distribution of these trichothecenes seems to be dependent upon environmental conditions. To date, a regulatory value has only been set for DON. However, attention should be paid to the derivatives as well because the synthesis of DON and NIV proceeds through the derivatives. Indeed, the detection of the derivatives 3-AcDON, 15-AcDON, and 4-AcNIV has been reported, and the quantities of DON derivatives are occasionally higher than T-2 and HT-2 toxins.[11] The synthesis pathway of DON and NIV is initially the same, but DON passes through the 3- or 15-acetylation process before undergoing deacetylation, while NIV passes through the 4-acetylation process that generates 4-AcNIV. The route to specific end products is divided by genetic and/or environmental conditions.[12,13] The synthesis pathway and acetylation processes that take priority are defined by locally characterized fungi.[14,15] These chemically phenotyped strains are called chemotypes, and the chemotype distribution has recently been changing. In North America and the Russian Far East, the 3-AcDON chemotype has been increasing and substituting the 15-AcDON type.[16−18] Therefore, it is necessary to pay attention to the changes in the detected derivatives.



Mycotoxins	R_1	R_2	R_3	R_4	R_5
DON	OH	H	OH	OH	=O
3-acetyl-DON	OAc*	H	OH	OH	=O
15-acetyl-DON	OH	H	OAc	OH	=O
NIV	OH	OH	OH	OH	=O
4-acetyl-NIV	OH	OAc	OH	OH	=O

OAc*: $OCOCH_3$

Figure 1. Structure of type B trichothecenes.

Received: May 15, 2012
Revised: August 16, 2012
Accepted: August 16, 2012
Published: August 16, 2012

dx.doi.org/10.1021/jf3020975 | J. Agric. Food Chem. 2012, 60, 9519−9527

However, the evaluation of the derivatives has not been sufficiently addressed, because only a small amount of these products are usually found in comparison to the end products. In addition, the evaluation of derivatives has not been given high priority, because it has been pointed out that the acetylation of the derivatives is a countermeasure to decrease mycotoxicity.[19] Because acetylated products are also detoxified rapidly *in vivo*, these derivatives seem to have the same toxicity as their parental toxins.[20] Furthermore, the accurate detection of these derivatives is difficult with simple assays, such as the enzyme-linked immunosorbent assay (ELISA). However, if the derivatives have different toxic characteristics or higher toxicity compared to the end products, then any change in chemotype distribution becomes important. Therefore, in this study, we aimed to compare the end products to their derivatives using the yeast experimental system. The yeast, *Saccharomyces cerevisiae*, is an appropriate eukaryote model, which has previously been used to evaluate trichothecene.[10,21−25] In these studies, the type B trichothecenes, DON and NIV, were mainly targeted, and the results were similar to studies with mammalian cells. Furthermore, it has been reported that the mutation in the *PDR5* gene, which codes the multidrug resistance ATP-binding cassette (ABC) transporter, increases the sensitivity to trichothecenes; hence, we used the *PDR5* mutant (*pdr5*).[22,26] The *PDR5* mutant has been used to study the function of *TRI* genes derived from *Fusarium* species;[27,28] thus, *pdr5* cells are thought to be suitable for investigating trichothecenes. Here, we investigated the characteristics of the type B trichothecenes, DON and NIV, and their derivatives, 3-AcDON, 15-AcDON, and 4-AcNIV, using culture assays and comprehensive gene expression profile analysis.

MATERIALS AND METHODS

Yeast Strains and Chemicals. In this study, we used the yeast (*S. cerevisiae*) strain BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) and the derivative *pdr1*, *pdr3*, and *pdr5* mutants (Thermo Fisher Scientific, Waltham, MA) with the loss of multidrug resistance transporter function. The frozen (−80 °C) glycerol stocks of yeast cells were transferred by an inoculating needle into 10 mL of YPD media (1% yeast extract, 2% peptone, and 2% glucose) in glass tubes and incubated on a rotary shaker at 150 rpm and 25 °C for 2 days. The mycotoxins DON, 3-AcDON, 15-AcDON, NIV, and 4-AcDON (fusarenon-X) were dissolved in dimethyl sulfoxide (DMSO), and 20 000 ppm stock solutions were prepared.

Growth Tests on Plate and in Liquid. Mycotoxin stock solutions were added to autoclaved YPD media with 1.5% agar (final concentration of 25 ppm), which was then poured into Petri dishes. A YPD agar solution with the same volume of DMSO was prepared as a control. Pre-incubated yeast cells were transferred to fresh YPD media and were incubated on a rotary shaker at 150 rpm and 25 °C for several hours until absorbance at 650 nm (A_{650}) = 1.0. A 10 times dilution series of each yeast mutant culture was prepared in YPD medium, and 2 μL of dilution samples were spotted on the test plate. After 2 days of incubation at 25 °C, test plates were examined. For the liquid culture test, 20−50 ppm mycotoxin solutions were prepared for each mycotoxin and DMSO was used as a control. A total of 5 μL of yeast cells (A_{650} = 1.0) were dispensed into a 96-well test plate with 200 μL of conditioned YPD solutions. The test plates were incubated at 25 °C, and the absorbance (A_{650}) was measured by a plate reader (Biomek2000, Beckman Coulter, Brea, CA) every 2 h.

RNA Preparation for DNA Microarray Analysis. For RNA extraction and purification, triplicate yeast cultures (A_{650} = 1.0, 10 mL) were grown at 25 °C in YPD media containing 25 ppm DON, 3-AcDON, 15-AcDON, NIV, or 4-AcNIV. The same volume of DMSO was added to the control culture. Culture solutions were incubated in a shaker at 150 rpm and 25 °C for 2 h. Cell pellets were obtained by centrifugation at 3000 rpm for 5 min, and total RNA was extracted using a commercial kit (FastRNA Pro Red kit, MP Biomedicals, Irvine, CA), following the instructions of the manufacturer. To avoid contamination of genomic DNA, samples were treated with DNase (DNase I, Takara, Shiga, Japan) before reverse transcription. The quality of total RNA was evaluated by a nucleic acid analyzer (Experion, Bio-Rad, Hercules, CA). RNA samples were used for synthesizing labeled RNA (amplified RNA) with 3′IVT Express kit (Affymetrix, Santa Clara, CA), and DNA microarrays (GeneChip Yeast Genome 2.0 array, Affymetrix) were processed according to the instructions of the manufacturer. Array data sets were transferred into GeneSpring analysis software (version 10, Agilent Technologies, Santa Clara, CA), and cluster analyses were performed for each treatment. After using the MAS5 algorithm to obtain summarized probe-level expression data, the average expression of three replicates was normalized to the control. An analysis of variance (ANOVA) was used for statistical analysis, and differences in gene expression with p value < 0.05 were considered significant. To avoid the detection of false positives, a multiple testing correction (Benjamini-Hochberg FDR) was applied to obtain corrected p values. Selected genes were categorized according to the Munich Information Center for Protein Sequences (MIPS[29]) and the *Saccharomyces* Genome Database (SGD[30]) annotation. Several clusters were visualized with spreadsheet software (Excel 2007, Microsoft, Redmond, WA). The microarray data set has been assigned the accession number GSE36954 in the Gene Expression Omnibus Database (GEO[31]).

Quantitative Polymerase Chain Reaction (PCR). cDNA templates were synthesized from the total RNA samples prepared for the DNA microarray analysis, using a commercial reverse transcription kit (Transcriptor First Strand cDNA Synthesis Kit, Roche, Basel, Switzerland). Sequence information of the target genes (*HXT9*, *HXT11*, *HXT12*, *PDR1*, and *PDR3*) was obtained from SGD, and each primer set that corresponded to the position around the 3′-end sequence was manually designed (Figure 7). *ACT1* was used as an internal control. The *ACT1* primer set (forward, 5′-ATTGCCGAAA-GAATGCAAAAGG-3′; reverse, 5′-CGCACAAAAGCAGAGATTA-GAAACA-3′), which has the same sequence as in a previous report,[32] was prepared. The *PDR1* primer set (forward, 5′-ACAATATTAA-CAACAACAACAGTAACAA-3′; reverse, 5′-GGAAGGAAGTTTTT-GAGAACTTTTA-3′) and the *PDR3* primer set (forward, 5′-CAACAGACAAAAAGACAACATTCTG-3′; reverse, 5′-CCATT-TACTATGGTTATGCTCTGCT-3′) were designed by Primer3.[33] For quantification, positive-control templates for constructing standard curves were prepared using standard PCR. In this study, 20 μL of reaction solutions [1 μL of each 10 μM primer, 4 μL of 5× buffer, 1.4 μL of 25 mM $MgCl_2$, 0.6 μL of 10 mM dNTPs, 1 μL of DNA template, 11 μL of distilled water, and 0.1 μL of taq polymerase (KAPA taq Extra, Kapa Biosystems, Woburn, MA)] were prepared and subjected to 95 °C for 2 min, followed by 33 cycles of 95 °C for 25 s, 55 °C for 15 s, and 72 °C for 45 s, in a thermal cycler (C1000, Bio-Rad). For each probe set, a template dilution series was prepared from these PCR products and 1 μL of the samples was dispensed into a 96-well PCR plate. The same volume of mycotoxin-treated sample templates was also dispensed into the plate. A total of 19 μL of reaction mix [0.4 μL of each 10 μM primer, 8.2 μL of distilled water, and 10 μL of 2× Master mix (KAPA SYBR FAST qPCR kit, Kapa Biosystems)] was added to the PCR plate. The sample plate was subjected to 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 20 s, in a thermal cycler (MX3000P, Agilent Technologies). The amplified *ACT1* product was used as an internal control, and triplicates were averaged.

RESULTS

Comparison of Sensitivity to Trichothecenes. It has been reported that the *PDR5* mutant (*pdr5*) is sensitive to trichothecenes, such as DON and T-2 toxin.[21,23,34] *PDR5* expression is regulated by a number of transcription factors, including dimers that consist of both Pdr1 and Pdr3, and these

dimers regulate a number of multidrug transporters.[35−37] However, it was not clear whether the transcription factor complex is more important than the presence of Pdr5. Thus, using the growth rate test, the sensitivity of the *PDR1* and *PDR3* mutants (*pdr1* and *pdr3*) was compared to *pdr5* cells. The plate growth test with 25 ppm of mycotoxins demonstrated that most of the toxins did not cause any growth inhibition to the transcription factor gene mutants (*pdr1* and *pdr3*), except for a slight repression by 15-AcDON (Figure 2a).

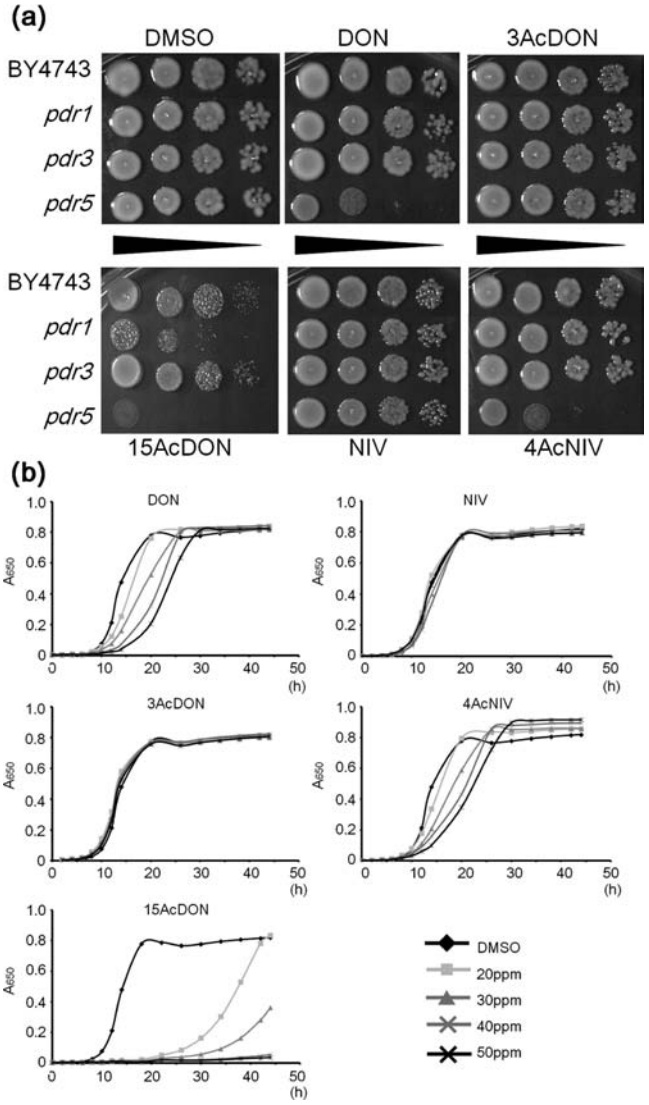


Figure 2. Growth inhibition by mycotoxins. (a) A 10-fold dilution series of yeast cells (parental strain BY4743 and the derivatives, *pdr1*, *pdr3*, and *pdr5*) was spotted onto YPD agar plates with 25 ppm of mycotoxins. (b) In the YPD liquid culture media, *pdr5* cells were exposed to 20−50 ppm of each mycotoxin. DMSO was used as a control. (◆) DMSO, (■) 20 ppm, (▲) 30 ppm, (gray ×) 40 ppm, and (black ×) 50 ppm. $n = 3$.

In contrast, *pdr5* showed high sensitivity to DON, 15-AcDON, and 4-AcNIV, resulting in a significantly different growth pattern. As for 3-AcDON and NIV, none of the mutant strains showed any growth inhibition. Because of the weak sensitivity to 3-AcDON and NIV, we examined the effect of different concentrations (20−50 ppm) of the mycotoxins on the growth of *pdr5* cells in liquid media. This assay showed the same sensitivity profile, and no differences were observed under both 3-AcDON and NIV treatments (Figure 2b). Furthermore, the most significant growth inhibition was observed with 15-AcDON.

Gene Expression Changes Observed by the DNA Microarray. Because *pdr5* cells showed different sensitivities to the different mycotoxins, we continued to examine their effect on the gene expression of these cells. *pdr5* cultures were incubated for 2 h with 25 ppm of each mycotoxin. RNA samples were obtained and used for DNA microarray analysis, and the data set was analyzed by ANOVA. A total of 2252 genes that showed an expression change of more than 2-fold at any condition were extracted. Approximately 40% of the genes mounted on the microarray chip was extracted in this study. In comparison to the extracted genes as a whole, the gene expression analysis more or less corresponded with the growth tests. According to the hierarchical clustering, the expression patterns of both 3-AcDON- and NIV-treated samples were very similar to the DMSO (control) sample. However, the expression patterns of DON-, 15-AcDON- and 4-AcNIV-treated samples were clearly different (Figure 3). The DNA

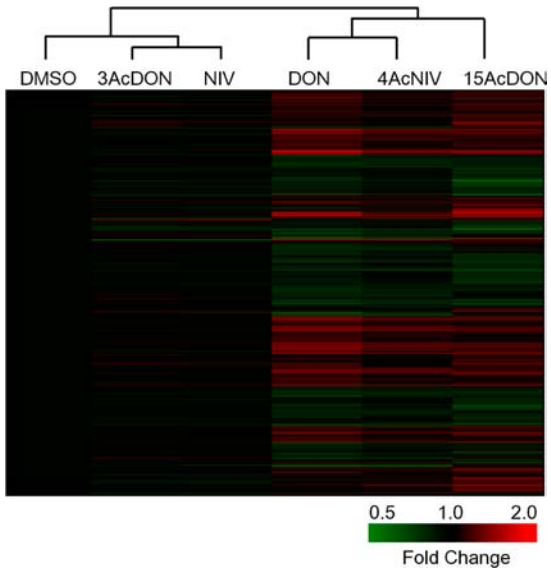


Figure 3. Hierarchical clustering map. ANOVA analysis was performed for each mycotoxin treatment, and genes that showed an expression change of more than 2-fold were extracted. Extracted genes were visualized and hierarchically clustered. DMSO was used as a control. Unchanged genes are shown in black.

microarray results were confirmed by quantitative PCR of *PDR1*, *PDR3*, and *ACT1*. *ACT1*, which codes the actin protein, was used as an internal control (see Figure S1 of the Supporting Information).

The extracted genes were divided into functional categories based on the MIPS database[29] (Table 1). When examining the total detection ratio, metabolism, energy, transcription, stress response, and cell differentiation functions showed a higher level than average (total extraction ratio of 40%). Conversely, protein activity regulation and protein fate showed a low detection ratio. Individual extraction numbers derived from each mycotoxin are listed on the right side of the tables. A large number of genes were extracted at all functional categories from the DON and 15-AcDON treatments, while a small number of genes were extracted from the 3-AcDON and NIV treatments.

dx.doi.org/10.1021/jf3020975 | J. Agric. Food Chem. 2012, 60, 9519−9527

Table 1. Functional Classifications of Detected Genes

gene category	probe set number[b]	detection number	detection ratio (%)[c]	piecemeal detection number[a]				
				15-AcDON	DON	4-AcNIV	3-AcDON	NIV
metabolism								
amino acid	171	101	59.1	61	69	35	10	8
nitrogen and sulfur	93	46	49.5	34	32	17	6	8
nucleotide	221	103	46.6	73	63	48	11	10
phosphate	418	174	41.6	123	99	60	20	12
C compound and carbohydrate	495	256	51.7	153	167	103	27	20
lipid, fatty acid, and isoprenoid	287	114	39.7	75	60	30	12	13
vitamins	158	72	45.6	41	52	27	8	6
secondary metabolite	76	50	65.8	28	34	21	5	5
energy	339	199	58.7	116	139	78	15	14
cell cycle and DNA processing	969	397	41.0	293	234	154	54	30
transcription	1012	488	48.2	331	378	273	29	24
protein synthesis	469	200	42.6	157	174	126	2	7
protein fate	1133	401	35.4	270	248	146	21	18
protein with binding function	1026	428	41.7	298	285	202	37	37
protein activity regulation	245	80	32.7	59	53	34	11	7
cellular transport	1010	396	39.2	280	226	127	37	31
cellular communication	231	88	38.1	65	52	29	13	9
cell rescue and defense response	521	240	46.1	175	149	89	32	18
cellular interaction	445	195	43.8	132	117	55	27	20
development	67	36	53.7	31	22	12	5	2
biogenesis of cellular components	844	347	41.1	240	220	135	31	22
cell type differentiation	449	209	46.5	147	122	83	25	25
unclassified proteins	879	410	46.6	282	256	174	38	41

[a]Each categorized gene was divided into toxic conditions; however, the sum of the piecemeal detection number at each category is not equal to the total detection number, because there are multiple counted genes. [b]Probe set number indicates the array chip mounted gene number. [c]Detection ratio indicates the ratio of the detection number divided by the probe set number. Because of the existence of multifunctional genes, the sum of the detection number is not equal to the total detection number of ANOVA.

Under the 4-AcNIV treatment, the number of extracted genes was intermediate. In comparison to the ratio, which was obtained by dividing the smallest number by the largest number in each functional category, protein synthesis demonstrated the most dramatic change. Transcription and protein fate demonstrated a similar trend (Figure 4).

Significant Expression Patterns in Particular Cellular Components. Every gene has an intracellular localization site; thus, the extracted genes were categorized on the basis of the cellular localization listed on MIPS[29] (Table 2). The total detection ratio in each category indicated that cell wall protein coding genes changed the most and endosome genes changed the least. However, the number of genes localized in the mitochondria, nucleus, or cytoplasm was much larger than cell wall or endosome genes. Moreover, the significant rate of change, which was obtained by dividing the smallest number observed with NIV by the largest number observed with DON, was observed in the mitochondria. DON induces translation inhibition by binding to the 60S ribosome; hence, it was thought that the expression patterns of ribosomal or translation system genes change because of abnormal translation and feedback regulation. Ribosomal and translation regulation genes are shown in Figure 5. The difference in expression patterns between cytoplasm and mitochondria is quite obvious. DON, 15-AcDON, and 4-AcNIV induced ribosomal and translation genes localized in the cytoplasm or nucleus, while they repressed the genes in the mitochondria.

Regulation of Multidrug Transporters under Mycotoxic Stress. The Pdr1−Pdr3 complex regulates many transporter genes, including *PDR5*. In addition, Pdr8 and

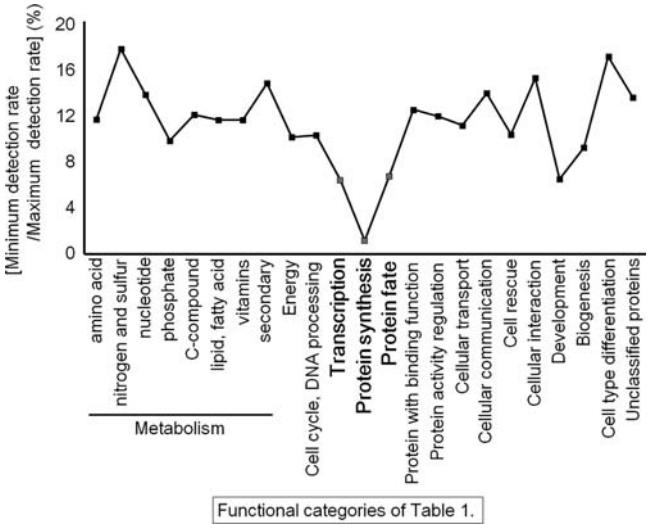


Figure 4. Changes in detection ratios in all functional categories. Each ratio was obtained by the division of the piecemeal detection number in Table 1. The smallest number of genes in the piecemeal detection of each functional category was divided by the largest number. Significantly changed categories are highlighted.

Yrr1 regulate similar types of genes.[38] Because these regulator genes were detected in this study, we analyzed them further (panels a and b of Figure 6). The Yrr1-regulated genes, *PLB1*, *YLR179C*, *YLR046C*, *SFK1*, *AZR1*, *SNG1*, and *YLL056C*, correlated well with the *YRR1* expression. The expression of *SNQ2*, *FLR1*, *YPL088W*, and *YMR102C*, which are regulated by

dx.doi.org/10.1021/jf3020975 | J. Agric. Food Chem. 2012, 60, 9519−9527

Table 2. Localization of Detected Genes

localization	probe set number[b]	detection number	detection ratio (%)[c]	piecemeal detection number[a]				
				15-AcDON	DON	4-AcNIV	3-AcDON	NIV
cell wall	40	25	62.5	16	15	16	7	5
peroxisome	52	28	53.8	18	13	8	0	1
plasma membrane	181	97	53.6	61	49	38	22	17
extracellular	49	26	53.1	21	18	14	6	5
cell periphery	211	103	48.8	64	57	35	20	15
integral membrane	158	77	48.7	62	44	27	13	10
mitochondria	993	476	47.9	325	340	171	24	19
nucleus	2036	941	46.2	662	659	458	81	61
bud	145	66	45.5	45	29	16	13	9
cytoplasm	2645	1122	42.4	734	742	466	92	88
cytoskeleton	202	83	41.1	64	36	17	9	5
ambiguous	225	88	39.1	63	51	30	8	9
endoplasmic reticulum	521	196	37.6	133	113	59	15	15
vacuole	269	98	36.4	69	52	39	18	15
punctate composite	131	46	35.1	23	25	13	1	0
transport vesicle	137	41	29.9	28	25	8	0	2
golgi membrane	94	25	26.6	17	11	4	2	2
golgi	154	39	25.3	28	18	7	3	3
endosome	54	13	24.1	10	7	2	0	0

[a]Each categorized gene was divided into toxic conditions; however, the sum of the piecemeal detection number at each category is not equal to the total detection number, because there are multiple counted genes. [b]Probe set number indicates the array chip mounted gene number. [c]Detection ratio indicates the ratio of the detection number divided by the probe set number. Because of the existence of multifunctional genes, the sum of the detection number is not equal to the total detection number of ANOVA.

both Pdr1−Pdr3 and Yrr1, correlated with the expression of *PDR1* and *PDR3*. Both Pdr1−Pdr3 and Pdr8 are transcription regulators of *PDR15*, but the expression pattern of that gene did not demonstrate any correlation with the expression of *PDR1*, *PDR3* or *PDR8*. Except for several genes and conditions, the expression of the Pdr1−Pdr3-regulated genes, *PDR10*, *IPT1*, *RTA1*, *YLR346C*, *PUG1*, *RSB1*, *TPO1*, *TPO2*, *TPO4*, *AQR1*, *DTR1*, and *YHK8*, largely correlated with the expression of *PDR1* and *PDR3*. A correlation with *PDR8* was not observed, because only a few genes that are regulated by this protein were extracted in this study.

Relationship between the Induction of *HXT9* and *HXT11* and Toxicity. The Pdr1−Pdr3 complex regulates the expression of *HXT9* and *HXT11*.[39,40] In this study, changes in the expression of these genes were detected but it was hard to interpret the relationship between the expression patterns of *PDR1* and *PDR3* and *HXT9* and *HXT11*. It has been suggested that Hxt9 and Hxt11 have a feedback regulation relationship with Pdr5; thus, we focused on these genes. However, the microarray probes for *HXT9*, *HXT11*, and *HXT12* were so similar that it was hard to distinguish between them. Therefore, we constructed individual primer sets for these genes (Figure 7) and measured their expression in *pdr5* cells. The expression patterns of *HXT9* and *HXT11* roughly correlated with the toxicity of the different mycotoxins. 15-AcDON induced the most significant change, except for *HXT12*, which is thought to be a pseudo-gene. The result indicating that DON induced a lower expression level compared to 4-AcNIV was confirmed.

DISCUSSION

In this study, we performed an evaluation of type B trichothecenes using the yeast experimental system. Plate and liquid culture growth assays revealed that the *PDR5* mutant is the most sensitive strain to type B trichothecenes and, hence, suitable for evaluation (Figure 2). The Pdr1−Pdr3 transcription regulation system may not play a critical role in the resistance to trichothecenes. Dysfunction of *PDR1* or *PDR3* resulted in normal cell growth, despite the loss of control of many multidrug transporter genes; therefore, it was thought that trichothecene resistance or efflux function is not inherent in the Pdr1−Pdr3-regulated proteins. *PDR5* is also regulated by Pdr1−Pdr3; however, it is thought that *PDR5* expression is normally regulated by other transcription regulators, such as Yap1, Stp5, and Rdr1.[41−43] The sensitivities to 3-AcDON and NIV were not observed in the growth test, and the DNA microarray analysis only showed minor changes under these conditions. According to our previous study and other reports, there is no doubt that Pdr5 contributes greatly to the efflux of trichothecenes;[27,28,34] however, in this study, there were some exceptions, such as 3-AcDON and NIV. This trend of 3-AcDON corresponds to a previous study using *Pichia pastoris*, in which 3-AcDON was less toxic compared to DON and its derivatives.[32] Meanwhile, NIV showed high toxicity in mammalian cell lines,[44−46] although it had no significant effect in this study. However, in mouse 3T3 cells, the toxicity trend of DON, 3-AcDON, 15-AcDON, NIV, and 4-AcNIV was similar to our results.[44] Therefore, these mycotoxins seem to have a toxic trend in yeast that is consistent with the trend in mammalian cells, even though the toxic mechanisms in yeast and mammalian cells do not always correspond. Additionally, Pestka[5] reported that 3-AcDON has slightly lower toxicity compared to DON and 15-AcDON. Taken together with our results, yeast is a good model for investigating trichothecenes. The great differences in toxicities between 3-AcDON, NIV, and other trichothecenes may be attributed to the yeast stress resistance mechanism, which may be a useful characteristic for clearly distinguishing between toxicities.

Examining gene expression, DNA microarray analysis demonstrated significant changes in the functional category of protein synthesis (Table 1), which is the major toxicity of type

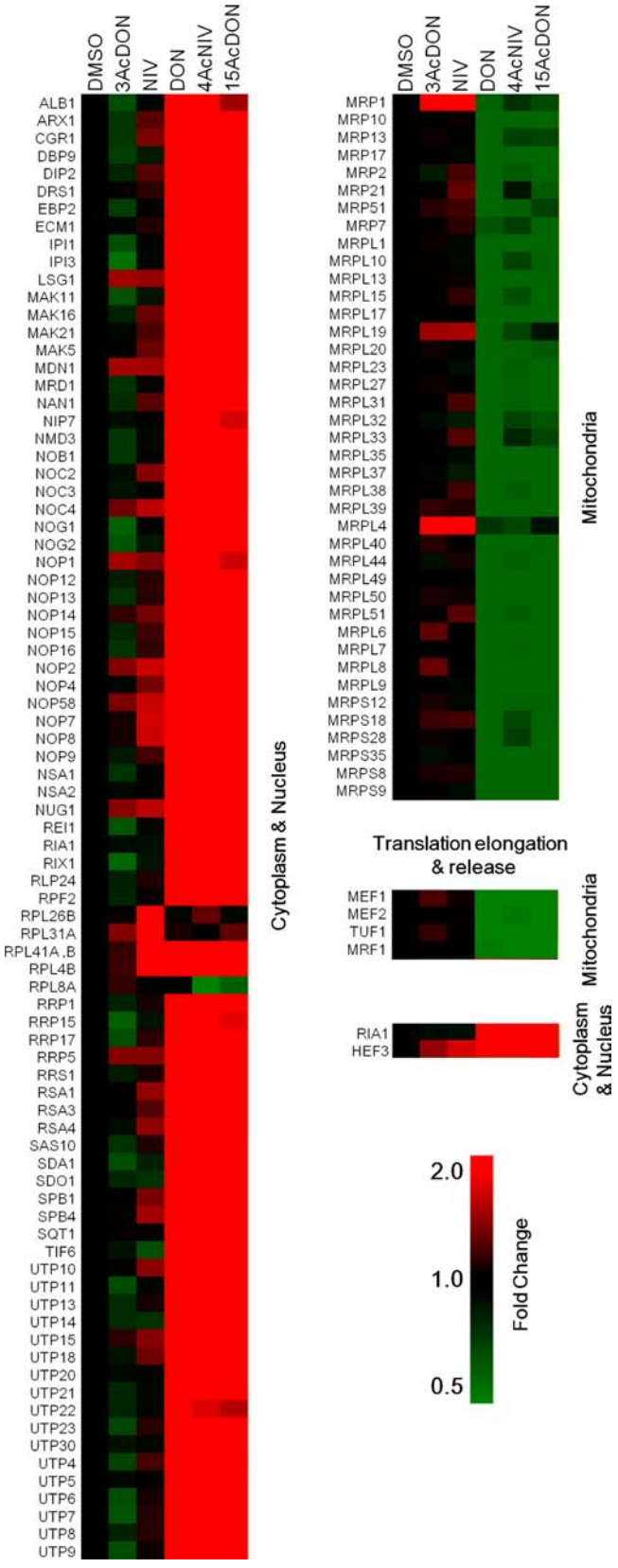


Figure 5. Expression changes of ribosomal proteins. Ribosomal and translation factor genes were categorized by localization. DMSO was used as a control. Unchanged genes are shown in black.

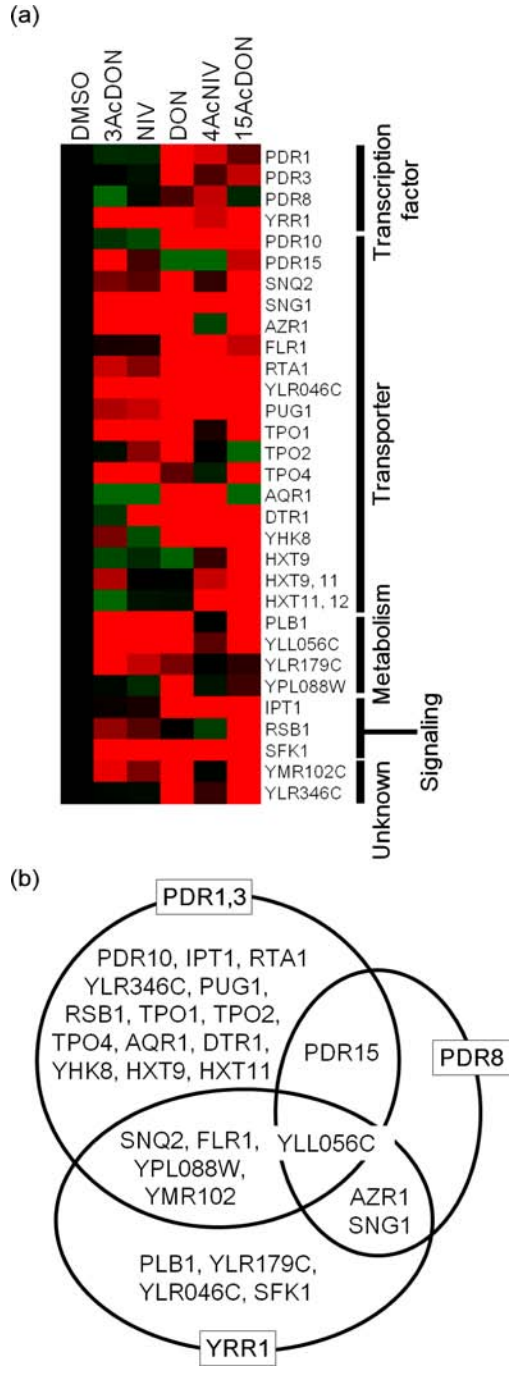


Figure 6. Expression changes of multidrug resistance genes. (a) The pleiotropic drug resistance transporter regulator genes and the regulated genes were extracted from the detected genes that were visualized in Figure 3. (b) Venn diagram showing the categories with the regulation relationships.

B trichothecenes. The total detection ratios of both protein synthesis and activity regulation genes were lower than the other categories. These results suggested that protein regulation genes are sensitive and appropriate markers for trichothecenes, even though the detection number is limited.

Pdr1−Pdr3 regulates various genes, and a number of the regulated genes roughly showed the corresponding expression patterns (Figure 6). Yrr1-regulated genes were detected in this study, but the number of transporter genes was much lower than Pdr1−Pdr3-regulated genes. The multidrug transporter capable of trichothecene efflux,[22] Snq2, which is regulated by both Pdr1−Pdr3 and Yrr1, was also detected in this study.

dx.doi.org/10.1021/jf3020975 | J. Agric. Food Chem. 2012, 60, 9519−9527

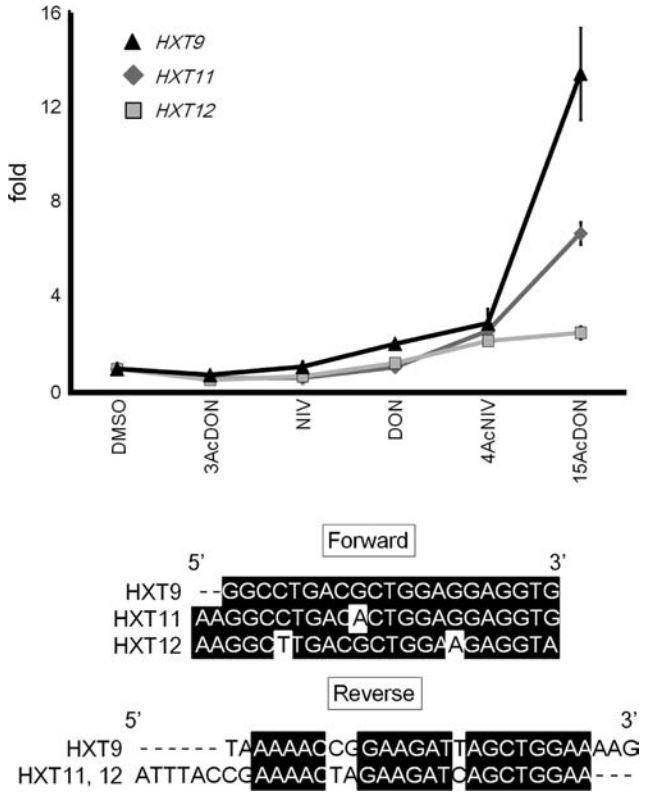


Figure 7. Quantitative PCR of hexose transporters. Real-time PCR-based quantitative estimation was executed using *ACT1* expression as an internal control. (▲) *HXT9*, (◆) *HXT11*, and (■) *HXT12* gene expression changes. Each primer set was designed manually. Inverted sites mean identical sequences. cDNA templates were prepared from the same RNA samples used for the DNA microarray analysis. Bars = standard error (SE); $n = 3$.

According to our study, *SNQ2* approximately corresponded to the Pdr1−Pdr3 expression pattern. Unfortunately, except for Pdr5, most multidrug transporters do not possess the resistance ability to trichothecenes; however, these data suggest that Pdr1−Pdr3 is a more influential transcription regulation system than Yrr1 under the toxic conditions caused by mycotoxins. Thus, we asked whether Pdr1−Pdr3 regulation affects these transporters. Many multidrug transporters are localized on the plasma membrane. Looking at the gene expression changes that influence transporter distribution on the membrane, the transporter expressions corresponded to Pdr1−Pdr3, suggesting that the influence of Pdr1−Pdr3 is comprehensive. *HXT9* and *HXT11*, which encode hexose transporters, were also detected in our study. The transcription of these genes is also regulated by Pdr1−Pdr3, and they are categorized as multidrug resistance genes.[39,40] The function of these transporters does not correspond with other multidrug transporters; however, Hxt9 and Hxt11 are involved in a feedback regulation of Pdr5 via the competing distribution on the membrane. Additionally, the loss of *HXT9* and *HXT11* induces tolerance to cycloheximide, and the overexpression of these genes induces high sensitivity to this compound. Interestingly, the loss of the Pdr5 function increases the expression of *HXT9* and *HXT11*;[39] thus, their expression levels in the *PDR5* mutant are thought to be somewhat higher than in the BY4743 parental strain. In this study, the expression of these genes was further induced by exposure to mycotoxins (Figure 7), with the highest change observed with 15-AcDON. *HXT12*, which is thought to be a pseudo-gene,[40,47] did not demonstrate significant changes compared to *HXT9* and *HXT11*, suggesting that Hxt12 does not play an important role in the feedback regulation. The fact that the expression of *HXT9* and *HXT11* was significantly higher under the 15-AcDON treatment compared to the other mycotoxins implies that 15-AcDON was the most toxic mycotoxin in our experimental system.

The cellular localization clustering results (Table 2) showed that the expression of a large number of genes was changed in the mitochondria, nucleus, and cytoplasm. Using the yeast experimental system, it has been reported that type A and B trichothecenes, such as T-2 toxin and DON, induce oxidative stress in mitochondria, as well as translation inhibition, which is independent of cytoplasmic reactions.[25] Furthermore, McLaughlin et al.[10] reported that a large number of mitochondrial gene mutant strains demonstrated sensitivity to trichothecin, which is a trichothecene compound,[48] and nearly half of the mutant strains encode mitochondrial ribosomal genes. In our study, it is clear that the gene expression of mitochondrial ribosomal genes was different from the nucleic ribosomal genes (Figure 5) and these mitochondrial genes were significantly repressed by highly toxic conditions. Apoptosis is a general reaction in cells, which is caused by mitochondrial disorder. It has been reported that the exposure of human cervical cancer cells to T-2 toxin induces the release of cytochrome *c* from mitochondria to the cytosol.[49] Moreover, the DON exposure experiment of human colon carcinoma cells reported the opening of permeability transition pore (PTP), loss of transmembrane potential, and caspase induction.[50] These effects cause the loss of respiratory activity, and the mitochondrial activity is thought to be dispersed. However, Bensassi et al.[50] reported that DON exposure does not directly induce PTP permeabilization. In our study, mitochondrial gene repression, which represents the loss of mitochondrial activity, and the induction of cytosolic genes were observed. This comprehensive information will provide new insight into the toxicities of trichothecene mycotoxins. Furthermore, it will make it possible to evaluate the toxicity of trichothecene mycotoxins simply by observing ribosomal protein genes with specific localizations.

Trichothecin was used as a representative compound to evaluate trichothecene toxicity;[10] however, a few differences in the structure result in a large difference in sensitivity. In fact, 3-AcDON and NIV induced distinctly different results compared to 15-AcDON and 4-AcNIV. This suggests that the toxicity evaluation of individual compounds is important to fully understand their toxic characteristics. In addition, a large number of ABC transporters, which function like Pdr5, are also localized in plant and mammalian cells. Consequently, further research focused on individual mycotoxins and transporters is required to understand their characteristics, because the structure of different transporters is not identical.

Both DON and NIV are generated through several pathways, including a constitution process of trichothecene derivatives in fungi. Except for the end products, it is thought that the toxicity of the derivatives is usually reduced to avoid an autotoxic effect; however, this does not necessarily mean that the derivatives have low toxicity against animals and plants. The high toxicity of 15-AcDON observed in this study has not been reported in mammalian cell studies,[5,44] and most studies have not used multidrug transporter mutants. Thereby, studying efflux function using mammalian or plant transporters may help to understand the difference in intracellular behavior between

mycotoxin end products and their derivatives. In the future, it might be necessary to carefully monitor the fungal products.

■ ASSOCIATED CONTENT

Supporting Information

Confirmation of the DNA microarray by quantitative PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-29-838-8103. Fax: +81-29-838-7996. E-mail: yumiko@affrc.go.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Katsuhide Fujita [National Institute of Advanced Industrial Science and Technology (AIST)] and Makiko Fukuda [Technology Research Association for Single Wall Carbon Nanotubes (TASC)] for their support in the preparation of the DNA microarray analysis data.

■ ABBREVIATIONS USED

A_{650}, absorbance at 650 nm; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; DON, deoxynivalenol; 3-AcDON, 3-acetyl-deoxynivalenol; 15-AcDON, 15-acetyl-deoxynivalenol; NIV, nivalenol; 4-AcNIV, 4-acetyl-nivalenol or fusarenon-X

■ REFERENCES

(1) Council for Agricultural Science and Technology (CAST). *Mycotoxins: Risks in Plant, Animal, And Human Systems*, Task Force Report; CAST: Ames, IA, 2003; Vol. *139*.

(2) Food and Agriculture Organization (FAO) of the United Nations. *Worldwide Regulations for Mycotoxins in Food and Feed in 2003*; FAO: Rome, Italy, 2004; Vol. *81*.

(3) Chowdhury, S. R.; Smith, T. K.; Boermans, H. J.; Sefton, A. E.; Downey, R.; Woodward, B. Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on performance, metabolism, hematology, and immunocompetence of ducklings. *Poult. Sci.* **2005**, *84*, 1179−1185.

(4) Girish, C.; Smith, T. Effects of feeding blends of grains naturally contaminated with fusarium mycotoxins on small intestinal morphology of turkeys. *Poult. Sci.* **2008**, *87*, 1075−1082.

(5) Pestka, J. J. Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Anim. Feed Sci. Technol.* **2007**, *137*, 283−298.

(6) Pestka, J. J. Mechanisms of deoxynivalenol-induced gene expression and apoptosis. *Food Addit. Contam.* **2008**, 1−13.

(7) Rotter, B. A.; Prelusky, D. B.; Pestka, J. J. Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health* **1996**, *48*, 1−34.

(8) Li, J.; Shimizu, T. Course of apoptotic changes in the rat gastric mucosa caused by oral administration of fusarenon-X. *J. Vet. Med. Sci.* **1997**, *59*, 191−199.

(9) Yang, H.; Chung, D. H.; Kim, Y. B.; Choi, Y. H.; Moon, Y. Ribotoxic mycotoxin deoxynivalenol induces G_2/M cell cycle arrest via $P21^{Cip/WAF1}$ mRNA stabilization in human epithelial cells. *Toxicology* **2008**, *243*, 145−154.

(10) McLaughlin, J. E.; Bin-Umer, M. A.; Tortora, A.; Mendez, N.; McCormick, S.; Tumer, N. E. A genome-wide screen in *Saccharomyces cerevisiae* reveals a critical role for the mitochondria in the toxicity of a trichothecene mycotoxin. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 21883−21888.

(11) Monbaliu, S.; Van Poucke, C.; Detavernier, C.; Dumoulin, F.; Van De Velde, M.; Schoeters, E.; Van Dyck, S.; Averkieva, O.; Van Peteghem, C.; De Saeger, S. Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC−MS/MS method. *J. Agric. Food Chem.* **2010**, *58*, 66−71.

(12) Bräse, S.; Encinas, A.; Keck, J.; Nising, C. F. Chemistry and biology of mycotoxins and related fungal metabolites. *Chem. Rev.* **2009**, *109*, 3903−3990.

(13) Desjardins, A. E. From yellow rain to green wheat: 25 years of trichothecene biosynthesis research. *J. Agric. Food Chem.* **2009**, *57*, 4478−4484.

(14) Yang, L.; van der Lee, T.; Yang, X.; Yu, D.; Waalwijk, C. *Fusarium* populations on Chinese barley show a dramatic gradient in mycotoxin profiles. *Phytopathology* **2008**, *98*, 719−727.

(15) Amarasinghe, C.; Wang, J. H.; Liao, Y. C.; Fernando, W. G. Difference in *TRI13* gene sequences between the 3-acetyldeoxynivalenol producing *Fusarium graminearum* chemotypes from Canada and China. *Int. J. Mol. Sci.* **2011**, *12*, 6164−6175.

(16) Puri, K. D.; Zhong, S. The 3ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15ADON population in spring wheat. *Phytopathology* **2010**, *100*, 1007−1014.

(17) Yli-Mattila, T. Ecology and evolution of toxigenic *Fusarium* species in cereals in northern Europe and Asia. *J. Plant Pathol.* **2010**, *92*, 12.

(18) Ward, T. J.; Clear, R. M.; Rooney, A. P.; O'Donnell, K.; Gaba, D.; Patrick, S.; Starkey, D. E.; Gilbert, J.; Geiser, D. M.; Nowicki, T. W. An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. *Fungal Genet. Biol.* **2008**, *45*, 473−484.

(19) Kimura, M.; Kaneko, I.; Komiyama, M.; Takatsuki, A.; Koshino, H.; Yoneyama, K.; Yamaguchi, I. Trichothecene 3-*O*-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. *J. Biol. Chem.* **1998**, *273*, 1654−1661.

(20) International Programme on Chemical Safety (IPCS). http://apps.who.int/ipsc/database/evaluations/chemical.aspx?chemID=2947.

(21) Mitterbauer, R.; Adam, G. *Saccharomyces cerevisae* and *Arabidopsis thaliana*: Useful model systems for the identification of molecular mechanisms involved in resistance of plants to toxins. *Eur. J. Plant Pathol.* **2002**, *108*, 699−703.

(22) Mitterbauer, R.; Weindorfer, H.; Safaie, N.; Krska, R.; Lemmens, M.; Ruckenbauer, P.; Kuchler, K.; Adam, G. A sensitive and inexpensive yeast bioassay for the mycotoxin zearalenone and other compounds with estrogenic activity. *Appl. Environ. Microbiol.* **2003**, *69*, 805−811.

(23) Abolinaal, S.; Mitterbauer, R.; Spadiut, O.; Peruci, M.; Weindorfer, H.; Lucyshyn, D.; Ellersdorfer, G.; LemmenS, M.; Moll, W. D.; Adarn, G. Engineered bakers yeast as a sensitive bioassay indicator organism for the trichothecene toxin deoxynivalenol. *J. Microbiol. Methods* **2008**, *72*, 306−312.

(24) Doyle, P. J.; Saeed, H.; Hermans, A.; Gleddie, S. C.; Hussack, G.; Arbabi-Ghahroudi, M.; Seguin, C.; Savard, M. E.; Mackenzie, C. R.; Hall, J. C. Intracellular expression of a single domain antibody reduces cytotoxicity of 15-acetyldeoxynivalenol in yeast. *J. Biol. Chem.* **2009**, *284*, 35029−35039.

(25) Bin-Umer, M. A.; McLaughlin, J. E.; Basu, D.; McCormick, S.; Tumer, N. E. Trichothecene mycotoxins inhibit mitochondrial translation—Implication for the mechanism of toxicity. *Toxins* **2011**, *3*, 1484−1501.

(26) Ghaffari, M. R.; Mardi, M.; Ehya, F.; Farsad, L. K.; Hosseini, S.; Ghareyazie, B. Mapping and expression analysis of a Fusarium head blight resistance gene candidate pleiotropic drug resistance 5 (PDR5) in wheat. *Iran J. Biotechnol.* **2010**, *8*, 112−116.

(27) McCormick, S. P.; Alexander, N. J.; Trapp, S. E.; Hohn, T. M. Disruption of *TRI101*, the gene encoding trichothecene 3-*O*-acetyltransferase, from *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* **1999**, *65*, 5252−5256.

(28) Muhitch, M. J.; McCormick, S. P.; Alexander, N. J.; Hohn, T. M. Transgenic expression of the *TRI101* or *PDR5* gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. *Plant Sci.* **2000**, *157*, 201−207.

(29) Munich Information Center for Protein Sequences (MIPS). http://mips.gsf.de/.
(30) *Saccharomyces* Genome Database (SGD). http://www.yeastgenome.org/.
(31) Gene Expression Omnibus Database (GEO). http://www.ncbi.nlm.nih.gov/geo/.
(32) Iwahashi, H.; Odani, M.; Ishidou, E.; Kitagawa, E. Adaptation of *Saccharomyces cerevisiae* to high hydrostatic pressure causing growth inhibition. *FEBS Lett.* **2005**, *579*, 2847−2852.
(33) *Primer3*; http://frodo.wi.mit.edu/.
(34) Suzuki, T.; Sirisattha, S.; Mori, K.; Iwahashi, Y. Mycotoxin toxicity in *Saccharomyces cerevisiae* differs depending on gene mutations. *Food Sci. Technol. Res.* **2009**, *15*, 453−8.
(35) Balzi, E.; Wang, M.; Leterme, S.; Van Dyck, L.; Goffeau, A. PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. *J. Biol. Chem.* **1994**, *269*, 2206−2214.
(36) Mamnun, Y. M.; Pandjaitan, R.; Mahé, Y.; Delahodde, A.; Kuchler, K. The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. *Mol. Microbiol.* **2002**, *46*, 1429−1440.
(37) Jungwirth, H.; Kuchler, K. Yeast ABC transporters—A tale of sex, stress, drugs and aging. *FEBS Lett.* **2006**, *580*, 1131−1138.
(38) Hikkel, I.; Lucau-Danila, A.; Delaveau, T.; Marc, P.; Devaux, F.; Jacq, C. A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. *J. Biol. Chem.* **2003**, *278*, 11427−11432.
(39) Nourani, A.; Wesolowski-Louvel, M.; Delaveau, T.; Jacq, C.; Delahodde, A. Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: Involvement of two hexose transporters. *Mol. Cell. Biol.* **1997**, *17*, 5453−5460.
(40) Ozcan, S.; Johnston, M. Function and regulation of yeast hexose transporters. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 554−569.
(41) Miyahara, K.; Hirata, D.; Miyakawa, T. yAP-1- and yAP-2-mediated, heat shock-induced transcriptional activation of the multidrug resistance ABC transporter genes in *Saccharomyces cerevisiae*. *Curr. Genet.* **1996**, *29*, 103−105.
(42) Akache, B.; Turcotte, B. New regulators of drug sensitivity in the family of yeast zinc cluster proteins. *J. Biol. Chem.* **2002**, *277*, 21254−21260.
(43) Hellauer, K.; Akache, B.; MacPherson, S.; Sirard, E.; Turcotte, B. Zinc cluster protein Rdr1p is a transcriptional repressor of the *PDR5* gene encoding a multidrug transporter. *J. Biol. Chem.* **2002**, *277*, 17671−17676.
(44) Sundstøl Eriksen, G.; Pettersson, H.; Lundh, T. Comparative cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. *Food Chem. Toxicol.* **2004**, *42*, 619−624.
(45) Pestka, J. J.; Uzarski, R. L.; Islam, Z. Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: Role of mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. *Toxicology* **2005**, *206*, 207−219.
(46) Severino, L.; Luongo, D.; Bergamo, P.; Lucisano, A.; Rossi, M. Mycotoxins nivalenol and deoxynivalenol differentially modulate cytokine mRNA expression in Jurkat T cells. *Cytokine* **2006**, *36*, 75−82.
(47) Wieczorke, R.; Krampe, S.; Weierstall, T.; Freidel, K.; Hollenberg, C. P.; Boles, E. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett.* **1999**, *464*, 123−128.
(48) Combrinck, S.; Gelderblom, W. C.; Spies, H. S.; Burger, B. V.; Thiel, P. G.; Marasas, W. F. Isolation and characterization of trichothecin from corn cultures of *Fusarium graminearum* MRC 1125. *Appl. Environ. Microbiol.* **1988**, *54*, 1700−1702.
(49) Chaudhari, M.; Jayaraj, R.; Bhaskar, A. S.; Lakshmana Rao, P. V. Oxidative stress induction by T-2 toxin causes DNA damage and triggers apoptosis via caspase pathway in human cervical cancer cells. *Toxicology* **2009**, *262*, 153−161.
(50) Bensassi, F.; Gallerne, C.; Sharaf El Dein, O.; Lemaire, C.; Hajlaoui, M. R.; Bacha, H. Involvement of mitochondria-mediated apoptosis in deoxynivalenol cytotoxicity. *Food Chem. Toxicol.* **2012**, *50*, 1680−1689.