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# Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis

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#### Abstract

Caffeic acid (3,4-dihydroxycinnamic acid, 12 mM) added to a fat-based growth medium reduces >95% of aflatoxin production by Aspergillus flavus NRRL 3357, without affecting fungal growth. Microarray analysis of caffeic acid-treated A. flavus indicated expression of almost all genes in the aflatoxin biosynthetic cluster were down-regulated, ranging from a  $\log_2$  ratio of caffeic acid treated and untreated of -1.12 (medium) to -3.13 (high). The only exceptions were genes *norB* and the aflatoxin pathway regulator-gene, *aflJ*, which showed low expression levels in both treated and control fungi. The secondary metabolism regulator-gene, *laeA*, also showed little change in expression levels between the fungal cohorts. Alternatively, expression of genes in metabolic pathways (i.e., amino acid biosynthesis, metabolism of aromatic compounds, etc.) increased ( $\log_2$  ratio > 1.5). The most notable up-regulation of A. flavus expression occurred in four genes that are orthologs of the Saccharomyces cerevisiae AHP1 family of genes. These genes encode alkyl hydroperoxide reductases that detoxify organic peroxides. These increases ranged from a log<sub>2</sub> ratio of 1.08 to 2.65 (moderate to high), according to real-time quantitative reverse transcription-PCR (qRT-PCR) assays. Based on responses of S. cerevisiae gene deletion mutants involved in oxidative stress response, caffeic, chlorogenic, gallic and ascorbic acids were potent antioxidants under oxidative stress induced by organic peroxides, tert-butyl and cumene hydroperoxides. Differential hypersensitivity to these peroxides and hydrogen peroxide occurred among different mutants in addition to their ability to recover with different antioxidants. These findings suggest antioxidants may trigger induction of genes encoding alkyl hydroperoxide reductases in A. flavus. The possibilities that induction of these genes protects the fungus from oxidizing agents (e.g., lipoperoxides, reactive oxygen species, etc.) produced during host-plant infection and this detoxification attenuates upstream signals triggering aflatoxigenesis are discussed. © 2007 Elsevier B.V. All rights reserved.

Keywords: Phenolic compounds; Aspergillus; Saccharomyces cerevisiae; Chemogenomics; Alkyl hydroperoxide reductase; Peroxiredoxin

## 1. Introduction

Aspergillus flavus and A. parasiticus are filamentous fungi capable of producing hepato-carcinogenic aflatoxins. Very low quantities (parts per billion) of aflatoxin contamination can have a significantly negative impact on food safety and economic value of a number of agricultural products (Campbell et al., 2003). Oxidative stress has been shown to stimulate aflatoxin biosynthesis in *A. parasiticus* (Jayashree and Subramanyam, 2000; Reverberi et al., 2005). Natural oxidative stress can result from production of or exposure to toxic reactive oxygen species (ROS), partially reduced forms of oxygen such as superoxide  $(O_2^-)$ , hydrogen peroxide  $(H_2O_2)$  and hydroxyl radicals (OH&z. rad;), or organic peroxides. Such molecules can be generated as by-products of respiration from the electron transport system, or by various metabolic activities, including host-response to infecting organisms (Moye-Rowley, 2003; Adam-Vizi, 2005 and

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references therein). ROS can cause potentially lethal injury to cells, such as lipid peroxidation, protein denaturation, and DNA damage. Levels of ROS can also increase under certain types of abiotic environmental stresses, such as drought and UV radiation (Torres and Dangl, 2005 and references therein). In view that aflatoxin biosynthesis is initiated by a polyketide synthase, and intermediates in its synthesis are highly oxygenated, it has been suggested that aflatoxigenesis is a potential fungal response to, and means of protection from, oxidative stress (Kim et al., 2005).

A short-chain analog of lipid peroxide, tert-butyl hydroperoxide (t-BuOOH), has been used as a model organic peroxide to induce oxidative stress in animal systems (Lin et al., 2000). In the microsomal cytochrome P450, t-BuOOH is metabolized to free radical intermediates (Minotti et al., 1986; Davies, 1989), causing lipid peroxidation (Masaki et al., 1989) and depletion of reduced glutathione (GSH) in cells (Martin et al., 2001). Cytotoxicity to t-BuOOH-treated hepatocytes was reversed when the cells were treated with compounds extracted from strawberries (Yau et al., 2002 and references therein). This provided some indication that various phytochemicals can attenuate cytotoxicity of organic peroxides. Cumene hydroperoxide, another organic peroxide, can also induce cellular oxidative stress. Cells treated with cumene hydroperoxide show reduced protein synthesis and have damaged membranes resulting in cytolysis (Ayala et al., 1996 and references therein).

A number of cellular systems have been discovered that respond to oxidative stress-based toxicity, some specifically to peroxides. Peroxiredoxins, peroxidases of AhpC/TSA family, reduce and detoxify hydroperoxides by donation of electrons from NADPH through thioredoxin/thiol-containing substances (Lee et al., 1999). In the yeast *Saccharomyces cerevisiae*, two transcription factors, Yap1p and Skn1p, regulate the antioxidative stress response of the cell (Brombacher et al., 2006). The Yap1p regulates antioxidative glutathione biosynthesis or GSH/ GSSG (oxidized glutathione) balance (Wu and Moye-Rowley, 1994; Grant et al., 1996). Skn1p regulates the thioredoxin system (Morgan et al., 1997), maintaining homeostasis in cellular thioredoxin/thiol-containing substances.

To date, there is no clear understanding how peroxide based oxidative stress induces aflatoxin biosynthesis. However, it has been shown that certain antioxidants inhibit aflatoxin biosynthesis. Hydrolysable tannins of walnut significantly inhibit aflatoxin production, with one of the anti-aflatoxigenic constituents being gallic acid (Mahoney and Molyneux, 2004). In the absence of the availability of a completely annotated genome for A. flavus, yeast deletion mutants can be useful models for examining target genes of antifungal/ antioxidative compounds (Parsons et al., 2004). The genome of S. cerevisiae has been fully sequenced and annotated, as has its oxidative stress response pathways (Winzeler et al., 1999). Using yeast deletion mutants, a number of fungal genes were identified that play a role in oxidative stress responses. These studies show that gallic acid acts as an antioxidant, reducing H<sub>2</sub>O<sub>2</sub>-based oxidative stress (Kim et al., 2005). Hydrolysable tannins, or gallic acid, were also shown to have antioxidant activities in mammalian cells, with gallotannins (gallic acid

esterified tannins) preventing neuronal cell death under oxidative stress (Ying and Swanson, 2000; Ying et al., 2001).

Aflatoxigenic aspergilli grow on fatty acid/lipid-rich plant materials (i.e., peanuts, tree nuts, corn, cotton seeds, etc.). Organic peroxides generated during fungal growth on such substances are a possible source of oxidative stress to these fungi. The anti-aflatoxigenic activity of natural antioxidants, such as gallic acid, may be associated with their ability to attenuate fungal oxidative stress responses up-stream of the aflatoxin production in *A. parasiticus* is achieved through activation of an *hsf2*-like transcription factor. This factor regulates genes that produce enzymes capable of counteracting oxidative stress (Reverberi et al., 2005).

The purpose of this study was to gain potential insight on the functional genomic processes associated with antioxidant-based inhibition of aflatoxin biosynthesis. Prior to this study, we were able to indirectly identify a number of candidate antioxidative stress response genes of *A. flavus* based on deletion mutant studies of *S. cerevisiae* (Kim et al., 2005) and orthologs found in an *A. flavus* Expressed Sequence Tag (EST) database (Yu et al., 2004). For the current study, we had access to microarrays of the *A. flavus* genome. We conducted microarray hybridization analyses to identify trends in gene expression associated with lowered aflatoxin biosynthesis resulting from treatment of *A. flavus* with the natural antioxidant, caffeic acid. By identifying such genes, methods can be developed to target these genes in order to reduce or prevent aflatoxin contamination of food products.

### 2. Materials and methods

### 2.1. Microorganisms and chemicals

A. flavus NRRL 3357 (ATCC 20026) was cultured on fatbased medium at 30 °C. The medium was prepared as follows: Commercial raw, organic, shelled pistachios were ground in a blender with dry ice. The ground pistachio kernels were passed through a sieve with a 1 mm screen and lyophilized. The pistachio kernel medium, consisting of 5% (w/v) ground pistachio kernels and 1.5% (w/v) Select agar (Sigma, St. Louis, MO) in distilled water, was autoclaved and 10 ml of the preparation was poured per 60 mm Petri dish. Spores from A. flavus cultured for 7 days on PDA were collected with a sterile cotton-tipped swab and suspended in 0.05% Tween 80. Using a Neubauer counting chamber, a spore suspension was prepared to inoculate Petri dishes in a single point with 200 spores in 5 µl 0.05% Tween 80 in the center of membrane filters (Poretics polycarbonate membrane filters, 0.2 µm pore size, 50 mm diameter; GE Osmonics, Minnetonka, MN) placed on top of the medium. Preweighed membrane filters with fungal mats were removed from the media, dried at 50 °C for 48 h, and weighed after being cooled in a desiccator for 24 h to determine cell mass on days 4, 5, and 6. Fungal mats and media were both extracted to determine total aflatoxin production on days 4, 5, and 6. Total RNA was isolated from the harvested fungi on day 4 for microarray analyses.

For yeast studies, wild type (BY4741; *Mat* a *his3* $\Delta 1$  *leu2* $\Delta 0$  *met15* $\Delta 0$  *ura3* $\Delta 0$ ) and gene deletion mutants of *S. cerevisiae* were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows: *yap1* $\Delta$  (transcription factor mutant), *gsh1* $\Delta$  ( $\gamma$ -glutamylcysteine synthetase mutant), *gsh2* $\Delta$  (glutathione synthetase mutant), *glr1* $\Delta$  (glutathione reductase mutant), *ahp1* $\Delta$  (alkyl hydroperoxide reductase mutant) (see below for culture conditions).

All chemicals used in this study, caffeic (3,4-dihydroxycinnamic), chlorogenic [3-(3,4-dihydroxycinnamic)], gallic (3,4,5trihydroxybenzoic), ascorbic acids, organic peroxides [*tert*butyl hydroperoxide (*t*-BuOOH) and cumene hydroperoxide] and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were purchased from Sigma (St. Louis, MO). Chemicals were dissolved in dimethyl sulfoxide (DMSO; <20 µl/ml media) for incorporation into media (except H<sub>2</sub>O<sub>2</sub> which was dissolved in distilled water, and caffeic acid which was added directly into the aflatoxin analysis medium).

### 2.2. Microarrays

The *A. flavus* NRRL 3357 genomic DNA amplicon microarray containing 5031 predicted genes, including those in the aflatoxin pathway cluster, was used to profile gene expression under treatment with caffeic acid, in comparison to non-treatment controls. The PCR amplicon fragments (approx. 530 bp on average) were generated from genomic DNA using specific primers designed based on *A. flavus* EST sequences (Yu et al., 2004). PCR products were purified using Millipore 96 well size exclusion vacuum filter plates, then re-suspended in water and diluted 1:1 with DMSO before printing. The microarrays were printed at The Institute for Genomic Research (TIGR, Rockville, MD) using an Intelligent Automation Systems spotting robot. The amplicon fragments were spotted at high density on Telechem Superamine aminosilane-coated microscope slides in triplicate for a total of 17,991 spots.

### 2.2.1. RNA isolation

Fungal mycelia harvested from membrane filters were ground to a fine powder using a pre-chilled mortar and pestle. Total RNA, from the same 4-day caffeic acid-treated and control samples, was purified from the fungal mycelia using a Qiagen RNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA).

#### 2.2.2. Hybridization

RNA labeling, pre-hybridization and hybridization were conducted according to the TIGR standard operating procedures (TIGR SOPs) found at <www.tigr.org/tdb/microarray/ protocolsTIGR.shtml>. To avoid labeling bias of the Cy5 and Cy3 dyes, each treatment was labeled with each dye (dye flip). Hybridized slides were scanned using the Axon GenePix<sup>TM</sup> 4000B microarray scanner. The independent TIFF images generated from each channel were analyzed using TIGR Spotfinder software <www.tigr.org/software> to acquire image intensity for relative transcript levels (Saeed et al., 2003). Data from TIGR Spotfinder analyses were stored in MAD, a relational database designed to effectively capture and store microarray data.

### 2.2.3. Data normalization and analysis

Signal intensities of microarray data were normalized using an intensity-dependent local regression technique, LOWESS (*LO*cally *WE*ighted Scatterplot Smoothing), implemented in the MIDAS software tool www.tigr.org/software, TIGR (Saeed et al., 2003). The resulting data were averaged over at least three (3) duplicate genes on each array (some genes were spotted in 6 duplicates) and over two (2) duplicate arrays (dye flip) for each experiment. All calculated gene expression ratios were log<sub>2</sub> transformed.

### 2.2.4. Validation

To validate expression data from microarray analyses a sensitive and accurate SYBR<sup>®</sup> Green real-time qRT-PCR was performed for genes of interest, including antioxidative enzymes. Primers for these selected genes were designed based on respective exon sequences (Table 1). Two replicates per gene, comprising three biological replicates in each reaction, were examined. Experiments were done according to the protocols of The Institute for Genomic Research (TIGR; Rockville, MD; www.tigr.org/tdb/microarray/protocolsTIGR. shtml; http://pga.tigr.org/sop/RT-PCR.pdf). The gene expression levels, in fold-inductions from qRT-PCR, were transformed into log<sub>2</sub> ratios for comparison with microarray results. Gene expression levels at each time point were normalized ( $\Delta\Delta$ CT analysis) to expression levels for the  $\beta$ -actin gene.

### 2.3. Analysis of aflatoxin

Aflatoxin B<sub>1</sub> was quantitated as described previously (Mahoney and Rodriguez, 1996), with each treatment in triplicate. Fungal mats, including spores and media, were removed from membranes and extracted with MeOH, and the aliquot dried under N<sub>2</sub>. The residue was derivatized with trifluoroacetic acid. Aflatoxin B<sub>1</sub> was quantified using reversed-phase HPLC on a 4.6 × 250 mm Vydak 218TP54 (C18) column (Grace Vydak, Hesperia, CA). Aflatoxin B<sub>2</sub> was detected in some samples at levels insignificant relative to B<sub>1</sub> (<0.1%) and was not quantitated.

### 2.4. Antioxidant bioassays

In vitro bioassays of S. cerevisiae deletion mutants, to examine antioxidative effects of test compounds, were performed as described by Kim et al. (2005). For yeast assays,  $1 \times 10^6$  cells (measured at OD 600 nm) cultured in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) were serially diluted from 10-fold to  $10^5$ -fold in SG (0.67% Yeast nitrogen base w/o amino acids, 2% glucose with appropriate supplements: 0.02 mg/ml uracil, 0.03 mg/ml amino acids). Cells from each serial dilution were spotted adjacently on SG agar medium incorporated with each compound to be tested. Each assay was performed in duplicate. Cells were grown at 30 °C for 7 days. Numerical scoring of bioassay results was as follows: 6=colonies were visible in all dilutions, 0=no colonies were visible in any dilution, 1=only the undiluted colony was visible, 2=the undiluted and 10-fold diluted colonies were visible, etc.

Table 1 Description of genes and primers chosen for qRT-PCR<sup>a</sup>

Primer name	Primer sequence (5' to 3')	(Putative) Function of the gene
NAFCN83TV-F NAFCN83TV-R	AGGGGACGTACCTGACAACA ACCCATCAGTCACCATGGCC	AhpC/Tsa family [antioxidation]
NAGAD20TV-F NAGAD20TV-R	GTCTTGGCTCACCTGTGATT CCCAGCATATCGAGAGCGAA	AhpC/Tsa family [antioxidation]
NAFDD05TV-F NAFDD05TV-R	CACCTGTGATTATTCTCGGA CCTGTAGTACTAGGCCCAGC	AhpC/Tsa family [antioxidation]
NAFAC53TV-F NAFAC53TV-R	TCGTCATTGACCACGGCAAG GTCCTTCTTCCTGTTGACGT	AhpC/Tsa family [antioxidation]
NAFDK26TV-F NAFDK26TV-R	TGGAACAGTCGGTAAGGCAA ATTCGCCAGGACCAGACACC	Copper/zinc superoxide dismutase [antioxidation]
NAGAG06TV-F NAGAG06TV-R	GTCTTGGTTTCGGATGCCGA GCACAGGCAAACCGTTCACT	Hydroxyquinol-1,2-dioxygenase [metabolism for aromatic compounds/molecules]
NAFFB03TV-F NAFFB03TV-R	AATCAACGACGTCAAAACGG GCCCAGTGCTGCATCTGTAG	Imidazoleglycerol phosphate synthase, cyclase subunit [l-histidine biosynthesis]
NAGAD56TV-F NAGAD56TV-R	CGAGGATGACTAGGCCCGCT GTATGGTGTCCCGCCATTAC	Dihydrodipicolinate synthase [1-lysine biosynthesis]
laeA-F laeA-R	GCTGGTACAATTTGGCTGTC CGCCTCCGACTTGACTTCTG	Aflatoxin pathway regulator
omtB-F omtB-R	AAGCAGATCATCCCAGTGAT CGAGCGAGTTCATCATGAGC	<i>O</i> -Methyltransferase I or <i>O</i> -methyltransferase B
TC 11908-F TC 11908-R	GTCCATGAAGGTCAAGATCA CGTCGTACTCCTGCTTGGAG	β-Actin

<sup>a</sup> The qRT-PCR product length is controlled at about 120–130 bp to minimize the possibility of dimer interference.

(see also Fig. 3). To induce oxidative stress, 1 mM *t*-BuOOH or 80  $\mu$ M cumene hydroperoxide was incorporated into the medium.

### 2.5. tsa1∆ bioassay

To confirm oxidant (i.e.,  $H_2O_2$ )-specific response/activity of Tsa1p [thioredoxin peroxidase (cTPx I)] of yeast, *S. cerevisiae tsa1*  $\Delta$  mutant (serially diluted as described for "antioxidant bioassays") was grown on YPD agar plate containing different types of oxidative stressors, i.e., 0.35 mM H<sub>2</sub>O<sub>2</sub>, 0.35 mM *t*-BuOOH or 40  $\mu$ M cumene hydroperoxide, in the presence of 100  $\mu$ g/ml of antimycin A [inhibitor of mitochondrial respiration; See also reference (Demasi et al., 2001) for the method]. Each chemical, when applied independently at these concentrations, had little effect on normal cell growth. Cell growth was monitored at 30 °C for three days.

# 3. Results

### 3.1. Anti-aflatoxigenic activity of caffeic acid

The effect of caffeic acid on production of aflatoxin by *A*. *flavus* was evaluated on days 4, 5 and 6, after inoculation of fungal spores onto the medium. Aflatoxin production in caffeic acid (12 mM)-treated plates was reduced >95% compared to non-treated control plates (i.e., day 4: 4.9  $\mu$ g aflatoxin vs. 0.03  $\mu$ g/control vs. caffeic; Day 5: 15.7  $\mu$ g vs. 0.3  $\mu$ g; day 6: 46  $\mu$ g vs. 1.7  $\mu$ g) (Fig. 1A). However, mass of the fungal mat was similar between the treatments (Fig. 1B), indicating that caffeic acid did not inhibit fungal growth at the dose applied. From these results, caffeic acid appears to be a potent anti-aflatoxigenic agent. Also, because this compound had no significant effect on fungal growth, we concluded caffeic acid could serve as a useful chemogenomic tool to narrow global gene expression to genes associated with repression of aflatoxin



Fig. 1. Comparison of (A) aflatoxin production by *A. flavus*, and (B) fungal growth (dry weight) with (b) or without (a) caffeic acid treatment. Values are the average of triplicate treatments from each time point (i.e., 4, 5 and 6 days after spore inoculation on plates). Bar, standard deviation (SD).

Table 2

biosynthesis. Analysis of such expression, using microarray hybridizations, would potentially enable us to identify target genes for repressing aflatoxin biosynthesis.

# 3.2. Effects of caffeic acid on global expression of A. flavus genes

Effects of caffeic acid on global expression was examined using microarray-based gene expression profiling. RNAs were isolated from control and caffeic acid-treated (4-day-old) *A*. *flavus* cultures as probes for microarray hybridizations. Level of change (i.e., either increase or decrease) in gene expression was categorized based on the following range in log<sub>2</sub> transformed ratios: "low"  $\geq -1.0$  to  $\leq 1.0$ ); "medium"  $\geq -2.0$  to <-1.0, or >1.0 to  $\leq 2.0$ ; "high" <-2.0, or >2.0 (See Tables). The most noteworthy results from microarray analysis of global responses of *A. flavus* cells to caffeic acid treatment are outlined below.

# 3.2.1. Down-regulation of genes in the aflatoxin biosynthetic pathway

Practically all the genes within the aflatoxin biosynthetic gene cluster were significantly down-regulated (viz., showed declines in relative mRNAs) from the caffeic acid treatment (Table 2). The  $\log_2$  levels in the decline of mRNAs in pathway genes ranged from low (-0.04; cypA; P450 monooxygenase) to high (-3.13; sterigmatocystin 7-O-methyltransferase precursor). Relative levels of mRNA of the transcription enhancer of the pathway, *aflJ*, also exhibited a low-level reduction (-0.58)(we were unable to detect aflR, a pathway regulator, in our microarray analysis). The only cluster gene that exhibited a departure from reduced mRNA levels was norB (dehydrogenase), showing a low increase (0.19). A pathway regulatory gene of secondary metabolism, laeA (Bok and Keller, 2004) which is located on a different chromosome from the aflatoxin pathway gene cluster, also showed a low increase in relative mRNAs (0.23). It should be noted that microarray data can be inherently vagarious, with low-level changes in expression. The low-level changes (absolute values less than 1) of the log<sub>2</sub> values listed in Table 2 without validation by qRT-PCR may not reflect their true expression level or direction (up- or down-regulation).

### 3.2.2. Down-regulated transcripts

A total of 36 transcripts, other than those in the aflatoxin biosynthetic pathway, were down-regulated ( $\log_2 < -1.0$ ) by caffeic acid treatment (Table 3). These genes were grouped into 5 categories, based on the following cellular functions: lipid metabolism (4 genes); cell wall structure/integrity (7 genes); transporter/pump (3 genes); and oxidoreductase/oxygenase (4 genes). Also included were hypothetical proteins (8 genes). The remaining 10 down-regulated genes participate in various functions, such as mitochondrial metabolism (aconitate hydratase), developmental stage expression (NAGCV29TV), DNA mismatch repair, etc.

## 3.2.3. Up-regulated transcripts

Twenty-eight genes were identified as exhibiting upregulation (viz., increased mRNA ratios) ( $\log_2$  ratio>1.0)

Transcripts in the aflatox	n biosynthesis	pathway (or	r gene clust	er) regulated	by
caffeic acid <sup>a</sup>					

Gene/ORF <sup>b</sup>	Description	Log <sub>2</sub> ratio <sup>c</sup>
name		
		Low-level
		changes
laeA	Regulator	0.23
norB	Dehydrogenase; NOR $\rightarrow$ AVN	0.19
cypA	P450 monooxygenase	-0.04
verA	Monooxygenase; VERA $\rightarrow$ DMST	-0.19
ordB	Monooxygenase/oxidase	-0.33
aflT	Transmembrane protein	-0.34
aflJ	Transcription enhancer, pathway regulator	-0.58
ordA	Oxidoreductase/P450 monooxygenase;	-0.93
	$OMST \rightarrow AFB_1$ and $AFG_1$ ,	
	DHOMST $\rightarrow$ AFB <sub>2</sub> and AFG <sub>2</sub>	
		Medium-leve
		changes
estA	Esterase; $VHA \rightarrow VAL$	-1.12
NAFAC21TV	O-methylsterigmatocystin oxidoreductase	-1.21
avfA	Oxidase; $AVF \rightarrow VHA$	-1.22
avnA	P450 monooxygenase; AVN→HAVN	-1.28
moxY	Monooxygenase	-1.33
hypA	Hypothetical protein	-1.63
vbs	VERB synthase; VAL $\rightarrow$ VERB	-1.82
	•	High-level
		changes
adhA	Alcohol dehydrogenase	-2.05
verB	Desaturase: VERB $\rightarrow$ VERA	-2.06
hexB	FAS beta subunit: acetate $\rightarrow$ polyketide	-2.34
norA	Dehvdrogenase	-2.61
pksA	Polyketide synthase A: acetate $\rightarrow$ polyketide	-2.68
NAFAD40TV	Aflatoxin biosynthesis ketoreductase <i>nor-1</i> :	-2.72
	reductase: NOR $\rightarrow$ AVN	
NAGCU50TV	VER1: dehvdrogenase/ketoreductase:	-2.85
1010000011	$VERA \rightarrow DMST$	2.00
cvnX	P450 monooxygenase	-2.94
omtR	<i>O</i> -Methyltransferase I or <i>O</i> -methyltransferase	-2.98
0	$B^{-}DMST \rightarrow ST^{-}DHDMST \rightarrow DHST^{-}$	2.70
NAFER 13TV	Norsolorinic acid reductase	-3.11
NAFAF89TV	omt 4: sterigmatocystin 7-0-methyltransferase	-3.13
1111111071V	precursor	5.15

<sup>a</sup> AFB<sub>1</sub> (aflatoxin B<sub>1</sub>), AFB<sub>2</sub> (aflatoxin B<sub>2</sub>), AFG<sub>1</sub> (aflatoxin G<sub>1</sub>), AFG<sub>2</sub> (aflatoxin G<sub>2</sub>), AVF (averufin), AVN (averantin), DHDMST (dihydrodemethylsterigmatocystin), DHST (dihydrosterigmatocystin), DMST (demethylsterigmatocystin), FAS (fatty acid synthase), HAVN (5'-hydroxyaverantin), NOR (norsolorinic acid), OMST (*O*-methylsterigmatocystin), ST (sterigmatocystin), VAL (versicolal), VERA (versicolorin A), VERB (versicolorin B), VHA (versiconal hemiacetal acetate).

<sup>b</sup> Open reading frame.

<sup>c</sup> The level of changes (i.e., either increase or decrease) in gene expression was determined as: (a) "low"  $(-1.0 \le \log_2 \text{ ratio} \le 1.0)$ , (b) "medium"  $(-2.0 \le \log_2 \text{ ratio} < -1.0 \text{ or } 1.0 < \log_2 \text{ ratio} \le 2.0)$  or (c) "high" (log<sub>2</sub> ratio < -2.0 or 2.0 < log<sub>2</sub> ratio) (See text).

from the caffeic acid treatment (Table 4). These genes were grouped into 3 categories, as follows: enzymes (10 genes) including dihydrodipicolinate synthase and imidazoleglycerol phosphate synthase (amino acid metabolism), hydroxyquinol-1,2-dioxygenase (aromatic metabolism), glutathione-*S*-transferase family, etc.; hypothetical proteins (11 genes); and other proteins (7 genes), including FAD-binding protein, 50S ribosomal protein, integral membrane protein, etc. Log

Table 3

Gene/ORF<sup>a</sup>

Transcripts down-regulated ( $\log_2$  ratio <-1.0) by caffeic acid [See Table 2 for the transcripts in the aflatoxin biosynthesis pathway]

Description

name	•	ratio <sup>b</sup>
Lipid metabolis	n	
NAFBZ36TV	Diacylglycerol lipase ( <i>mdlA</i> ) { <i>Aspergillus oryzae</i> }	-1.20
NAFFI12TV	Alkaline ceramidase	-1.22
NAFCJ13TV	Acyl-CoA dehydrogenase	-1.45
NAFCT20TV	Triacylglycerol lipase ( <i>tglA</i> )	-2.55
Cell wall/integr	ity	
NAFDU66TV	Oxalate decarboxylase	-1.03
NAFBZ28TV	Mixed-linked glucanase precursor { <i>Cochliobolus carbonum</i> }	-1.15
NAFFA65TV	Related to alpha-1, 2-galactosyltransferase	-1.16
NAFAA75TH	Trehalose phosphate synthase subunit	-1.21
NAGCP37TV	Antigen, p83/100, putative (outer surface protein)	-1.23
NAFDF61TV	Cell wall surface anchor family protein, putative	-1.52
NAFDH31TV	Antigenic cell wall protein MP1	-1.64
	-	
Transporter/pun	<i>ıp</i>	
NAFCM30TV	Drug transport protein, putative	-1.04
NAFAT07TV	Plasma-membrane proton-efflux P-type ATPase	-1.32
NAFEV60TV	Major facilitator family transporter, putative	-1.43
Oxidoreductase/	loxygenase	
NAGAB40TV	Cytochrome P450 monooxygenase	-1.68
NAFDF68TV	Oxidoreductase, aldo/keto reductase family, putative	-2.15
NAFCI74TV	Oxidoreductase, GMC family	-2.63
NAFBA56TV	Monooxygenase, flavin-binding family	-2.85
II	- 4 - Î	
Hypothetical pro	Concernation and humathetical protain	1.01
NAFAQOITV	Lise at a straight and the straight and	-1.01
NAGDJ65TV	Concerned how other inclusion	-1.05
NAFCK821V	Conserved hypothetical protein	-1.00
NAFEKOOIV	L'une and a mateir and dust autotive	-1.07
NAFE1/21V	Unnamed protein product, putative	-1.94
NAGDUOITV	Lunamed protein and duct	-2.18
NAGEE201V	Conserved hypothetical protein	-2.30
NAGC W411 V	Conserved hypothetical protein	-2.34
Other proteins		
NAFDH80TV	Neutral protease ii precursor	-1.01
NAGAG67TV	Clock-controlled gene-9 protein	-1.05
NAGCV29TV	Related to blastomyces yeast phase-specific protein 1	-1.14
NAGAE09TV	Aconitate hydratase mitochondrial	-1.23
NAFAD89TV	Methyl-accepting chemotaxis protein putative	-1.29
NAFER26TV	Isotrichodermin c-15 hydroxylase	-1.45
NAGDW16TV	Dihydropteroate synthase putative	-1.52
NAGAP67TV	IgE-binding protein	-1.85
NAGAB77TV	Related to mismatched base pair and cruciform dna	-1.92
	recognition protein	1.72
NAFAB75TV	Reticulocyte binding protein	-3 55
	Freedom Protein	5.55

<sup>a</sup> Open reading frame.

<sup>b</sup> The level of changes (i.e., either increase or decrease) in gene expression was determined as: (a) "low"  $(-1.0 \le \log_2 \text{ ratio} \le 1.0)$ , (b) "medium"  $(-2.0 \le \log_2 \text{ ratio} < -1.0 \text{ or } 1.0 < \log_2 \text{ ratio} \le 2.0)$  or (c) "high" ( $\log_2 \text{ ratio} < -2.0 \text{ or } 2.0 < \log_2 \text{ ratio}$ ) (See text).

### 3.2.4. Sugar utilization gene cluster

Four genes involved in sugar utilization are also clustered in *A. flavus*, adjacent to the aflatoxin pathway gene cluster (Yu et al., 2000). Transcription levels of three of these genes showed only low-level changes, as follows: *hxtA*, hexose transporter

protein for hexose uptake (0.08), *glcA*, glucosidase (-0.16) and *nadA*, NADH oxidase (-0.56). An expression profile for *sugR*, the sugar utilization regulatory gene in this cluster, was not detected (data not shown). Due to their low level of change in expression, as determined by microarray analysis, the changes in expression levels of these genes are classified as inconclusive or not responding to antioxidant treatment.

## 3.2.5. Antioxidative enzymes

A number of genes encoding antioxidative enzymes were identified by microarray analysis, but had low levels of change in expression profiles between treated and untreated cohorts (Table 5). Due to the inconclusive nature of low expression levels of these genes according to microarray analysis, qRT-PCR was performed on each one for validation. The log<sub>2</sub> transformed qRT-PCR results are listed in Table 5, together with the log<sub>2</sub> values detected by microarray analysis. Of these antioxidative genes, four peroxiredoxin gene families were found in *A. flavus* showing 38% to 50% sequence identity and

Table 4

Transcripts	up-regulated	$(\log_2)$	ratio>	1.0)	by	caffeic	acid
	1 0	< O2			~		

Gene/ORF <sup>a</sup> name	Description	Log <sub>2</sub> ratio <sup>b</sup>
Enzymes		
NAGAD56TV	Dihydrodipicolinate synthase, putative	1.74
NAFFB03TV	Imidazoleglycerol phosphate synthase, cyclase	1.61
NAGAG06TV	Hydroxyquinol-1, 2-dioxygenase	1.55
NAFFA29TV	Monooxygenase, flavin-binding family	1.37
NAGAD24TV	Uracil phosphoribosyltransferase	1.25
NAGDI75TV	Galactose oxidase precursor	1.16
NAFDK60TV	Methylenetetrahydrofolate dehydrogenase	1.16
NAFCG24TV	Glutathione S-transferase family protein, putative	1.08
NAGAP18TV	COG3485: Protocatechuate 3,4-dioxygenase beta subunit	1.05
NAFFN34TV	DNA damage response protein kinase dun1	1.05
Hypothetical pro	otein	
NAGDF05TV	Protein of unknown function family	1.89
NAFFK58TV	Protein of unknown function family	1.88
NAFCF89TV	Hypothetical protein	1.85
NAGDE40TV	Hypothetical protein	1.67
NAFFM56TV	Hypothetical protein	1.21
NAFFK51TV	Hypothetical protein	1.19
NAGAM50TV	Hypothetical protein	1.14
NAFEX18TV	Conserved hypothetical protein	1.08
NAGAM87TV	Hypothetical protein	1.04
NAGAD66TV	Hypothetical protein	1.03
NAFAQ58TV	Hypothetical protein	1.01
Other proteins		
NAGDX80TV	Bll6462 a_oryzae chr_0 Con0277 20114	1.43
NAGAG15TV	FAD-binding protein, putative	1.31
NAGAR04TV	50S ribosomal protein L24	1.31
NAGAP69TV	P4 protein {Ustilago maydis virus P4}	1.11
NAGAH65TV	Integral membrane protein, putative	1.04
NAFBF33TV	XynG1 {Aspergillus oryzae}	1.02
NAFCT07TV	GRAAL protein	1.01

<sup>a</sup> Open reading frame.

<sup>b</sup> The level of changes (i.e., either increase or decrease) in gene expression was determined as: (a) "low"  $(-1.0 \le \log_2 \text{ ratio} \le 1.0)$ , (b) "medium"  $(-2.0 \le \log_2 \text{ ratio} < -1.0 \text{ or } 1.0 < \log_2 \text{ ratio} \le 2.0)$  or (c) "high" ( $\log_2 \text{ ratio} < -2.0 \text{ or } 2.0 < \log_2 \text{ ratio}$ ) (See text).

Table 5

Gene/ORF <sup>a</sup> name	Description	Log2 ratio <sup>b</sup> (qRT-PCR)	Log2 ratio <sup>b</sup> (microarray)
Antioxidative enzymes			
NAFCN83TV 50% (26/52) <sup>c</sup> 69% (36/52) <sup>d</sup>	Antioxidant, AhpC/Tsa family	2.65: high-level increase	0.23
sNAGAD20TV 42% (69/161) <sup>c</sup> 60% (97/161) <sup>d</sup>	Antioxidant, AhpC/Tsa family	1.44: medium-level increase	0.02
NAFDD05TV 44% (44/99) <sup>c</sup> 60% (60/99) <sup>d</sup>	Antioxidant, AhpC/Tsa family	1.31: medium-level increase	-0.32
NAFAC53TV 38% (54/139) <sup>c</sup> 58% (81/139) <sup>d</sup>	Antioxidant, AhpC/Tsa family	1.08: medium-level increase	0.67
NAFDK26TV	Copper/zinc superoxide dismutase	-0.77: low-level decrease	0.58
Other genes			
NAGAG06TV	Hydroxyquinol-1,2-dioxygenase/aromatic metabolism	2.65: high-level increase	1.56
NAFFB03TV	Imidazoleglycerol phosphate synthase/L-histidine biosynthesis	1.88: medium-level increase	1.62
NAGAD56TV	Dihydrodipicolinate synthase/L-lysine biosynthesis	0.95: low-level increase	1.74
laeA	Secondary metabolite pathway regulator	0.90: low-level increase	0.23
verB	Desaturase	-0.06: low-level decrease	-2.06
omtB	O-methyltransferase I or O-methyltransferase B	-1.08: medium-level decrease	-2.99

Transcripts of antioxidative enzymes and other genes of interest identified by microarray analysis of caffeic acid-treated Aspergillus flavus comparing microarray to aRT-PCR analyses

<sup>a</sup> Open reading frame.

<sup>b</sup> The level of changes (i.e., either increase or decrease) in gene expression was determined as: (a) "low"  $(-1.0 \le \log_2 \text{ ratio} \le 1.0)$ , (b) "medium"  $(-2.0 \le \log_2 \text{ ratio} < -1.0 \text{ or } 1.0 < \log_2 \text{ ratio} \le 2.0)$  or (c) "high" ( $\log_2 \text{ ratio} < -2.0 \text{ or } 2.0 < \log_2 \text{ ratio}$ ) (See text).

<sup>c</sup> Amino acid sequence identity to S. cerevisiae Ahp1p (Numbers in parentheses: number of amino acids matched/number of amino acids aligned).

<sup>d</sup> Amino acid sequence similarity to S. cerevisiae Ahp1p (Numbers in parentheses: number of amino acids matched/number of amino acids aligned).

58% to 69% sequence homology, at the amino acid level, to Ahp1p (alkyl hydroperoxide reductase) of *S. cerevisiae* (Table 5). No genes in our analysis were found that showed sequence homology to Tsa1p, the other type of peroxiredoxin in *S. cerevisiae*. Alkyl hydroperoxide reductases play an important role in detoxifying organic and lipid peroxides.

The changes in expression levels of the A. flavus orthologs to these peroxiredoxin genes were confirmed to be at a medium to high level according to qRT-PCR (Table 5), yet, the microarray analysis showed only low levels of change with undetermined ranges for these genes. Noticeably, the antioxidant gene homolog (NAFDD05TV) to AhpC/Tsa family was shown as slightly down-regulated by microarray detection. However, the sensitive detection by qRT-PCR demonstrated that this gene was actually up-regulated at the medium level. The changes in expression of some other antioxidant genes were also detected by microarray analysis to be at low levels. These are gene (NAFDK26TV; log<sub>2</sub> 0.58) for copper/zinc superoxide dismutase, gene (NAFAX17TV;  $\log_2 -0.43$ ) for mitochondrial superoxide dismutase, and gene (NAFBL81TV;  $log_2 - 0.65$ ) for a catalase. Validation using qRT-PCR was performed for one of the three genes (NAFDK26TV). The qRT-PCR result demonstrated that this gene was actually slightly down-regulated instead of up-regulated as detected by microarray (Table 5).

The levels of change in expression for additional genes identified from microarray assays were further confirmed by qRT-PCR. The results of these additional qRT-PCR analyses are summarized, as follows (See also Table 5 and Fig. 2): NAGAG06TV, hydroxyquinol-1,2-dioxygenase/aromatic metabolism, 2.65; NAFFB03TV, imidazoleglycerol phosphate synthase/L-histidine biosynthesis, 1.88; NAGAD56TV, dihydrodipicolinate synthase/L-lysine biosynthesis, 0.95; *laeA*, secondary metabolite pathway regulator, 0.90; and *omtB*, *O*-methyltransferase I or *O*-methyltransferase B, -1.08. The  $\beta$ -actin gene (TC11908) was used as an internal control. The changes in expression levels of these genes according to the qRT-PCR results were generally concordant with the microarray results, in terms of their trends and level.



Fig. 2. Summary of qRT-PCR analysis compared to microarray results. (a) NAGAD56TV (1.74 microarray  $log_2$  ratio/0.95 qRT-PCR  $log_2$  ratio; dihydrodipicolinate synthase/L-lysine biosynthesis), (b) NAFFB03TV (1.62/1.88; imidazoleglycerol phosphate synthase/L-histidine biosynthesis), (c) NAGAG06TV (1.56/2.65; hydroxyquinol-1,2-dioxygenase/aromatic metabolism), (d) NAFAC53TV (0.67/1.08; AhpC/Tsa family/antioxidation), (e) NAFCN83TV (0.23/2.65; AhpC/Tsa family/antioxidation), (f) *laeA* (0.23/ 0.90; pathway regulator), (g) *verB* (-2.06/-0.06; desaturase), (h) *omtB* (-2.99/ -1.08; *O*-methyltransferase I/O-methyltransferase B).



Fig. 3. Responses of *S. cerevisiae* wild type and gene deletion mutants showing "gene–stress" and/or "compound–stress" relationship under the treatments of different organic peroxides (*t*-BuOOH, cumene) and antioxidative compounds (phenolics, glutathione, ascorbic acid). See Table 6 for summary of results.

# 3.3. Protection of fungi from hydroperoxide-induced oxidative stress by caffeic acid

Caffeic acid was tested for its antioxidant capacity as a protectant from different types of organic peroxides (*t*-BuOOH and cumene hydroperoxide). *S. cerevisiae* served as a model fungus for this study. We used the following strains: wild type and gene deletion mutants,  $yap1\Delta$ ,  $gsh1\Delta$ ,  $gsh2\Delta$ ,  $glr1\Delta$ ,  $ahp1\Delta$  and  $tsa1\Delta$  (see Materials and methods). We specifically compared responses of two different peroxiredoxin mutants,  $ahp1\Delta$  and  $tsa1\Delta$ , to the hydroperoxide treatments. Four additional antioxidant compounds, chlorogenic, gallic, ascorbic acids and glutathione, were also examined for their effects on attenuating organic peroxide stresses. The results of these assays are as follows:

# 3.3.1. Gene-stress relationship

All mutants were sensitive to *t*-BuOOH, while only  $ahp1\Delta$ and  $yap1\Delta$  mutants were sensitive to cumene hydroperoxide (Fig. 3; Table 6 for summary). Also, the level of sensitivity of  $ahp1\Delta$  and  $yap1\Delta$  mutants to peroxides was much higher (growth scores 1 and 0, respectively) than that of other mutants, including  $tsa1\Delta$  (growth scores 2 to 4). These results suggest that Ahp1p and Yap1p play a more significant role than the other peroxiredoxin, cTPxI (thioredoxin peroxidase encoded by *TSA1*), in detoxifying or responding to organic peroxides in *S. cerevisiae*. It was previously shown that expression of *AHP1* in yeast was induced by either *t*-BuOOH or H<sub>2</sub>O<sub>2</sub>, and the *ahp1*\Delta mutant was hypersensitive to *t*-BuOOH (Lee et al., 1999). However,  $tsa1\Delta$  showed higher sensitivity to H<sub>2</sub>O<sub>2</sub> (Lee et al., 1999). In composite, the results of these yeast studies dem-

Table 6

Responses of S. cerevisiae deletion mutants to different oxidants and effectiveness of various antioxidants for recovery a

Oxidants (conc.)	Mutants $^{b} \rightarrow$	$ahp1\Delta$	tsal∆	$glr1\Delta$	yap1∆
t-BuOOH (1 mM)	Response	Sensitive	Sensitive	Sensitive	Sensitive
	Effective	Caffeic	Caffeic	Caffeic	Caffeic
		Chlorogenic	Chlorogenic	Chlorogenic	Chlorogenic
		Gallic	Gallic	Ascorbic	Ascorbic
		Ascorbic	Ascorbic		
	Not effective	GSH	GSH	GSH, Gallic	GSH, Gallic
Cumene (80 µM)	Response	Sensitive	Not sensitive	Not sensitive	Sensitive
	Effective	Caffeic	N/A <sup>c</sup>	N/A <sup>c</sup>	Caffeic
		Chlorogenic			Chlorogenic
		Gallic			Gallic
		Ascorbic			Ascorbic
		GSH			GSH

<sup>a</sup> GSH (glutathione) 1 mM; *t*-BuOOH (*tert*-butyl hydroperoxide) 1 mM; caffeic acid, chlorogenic acid, gallic acid and ascorbic acid, 5 mM; CHP (cumene hydroperoxide) 80 μM.

<sup>b</sup>  $gsh1\Delta$  and  $gsh2\Delta$  data are not shown since their sensitivities are mainly due to GSH auxotrophy.

<sup>c</sup> Not applicable due to lack of sensitivity to the cumene hydroperoxide treatment.

onstrate a potentially unique role each peroxiredoxin gene plays in response to a different organic peroxide or hydrogen peroxide (See below for *tsa1* $\Delta$  bioassay). Also, Yap1p regulates at least four genes in the downstream oxidative stress response pathway (*TRX1*, thioredoxin; *TRR1*, thioredoxin reductase; *GLR1*, glutathione oxidoreductase; *GSH1*,  $\gamma$ -glutamyl cystein synthetase) in *S. cerevisiae* (Dumond et al., 2000). The hypersensitive response of *yap1* $\Delta$  to both organic peroxides in our study verifies the critical role *YAP1* plays in attenuating organic peroxide stress.

#### 3.3.2. Compound-stress relationship

Treatment of the yeast mutants with the phenolics, caffeic, chlorogenic and gallic acids, or ascorbic acid (used as a positive control) resulted in full recovery of the cells to both *t*-BuOOH and cumene hydroperoxide stress. The exception, however, was only the partial or no recovery of  $glr1\Delta$  or  $yap1\Delta$  mutants, respectively, with gallic acid to *t*-BuOOH treatment (Fig. 3 and Table 6). Alternatively, GSH recovered cell growth of  $ahp1\Delta$  and  $yap1\Delta$  under cumene hydroperoxide stress, but not under *t*-BuOOH. The differential recovery of mutants to the different organic peroxides suggests there is a compound–stress/gene relationship. Moreover, caffeic acid serves as a strong antioxidant under both organic peroxide stresses.

Growth of  $gsh1\Delta$  and  $gsh2\Delta$  mutants was poor, even on control plates (i.e., GSH auxotrophy). However growth of these mutants was recovered when GSH was provided exogenously, even under *t*-BuOOH or cumene hydroperoxide stress. None of other compounds tested could complement the GSH auxotrophy of both mutants. These results indicate the gsh mutants are less responsive to organic peroxide stress when GSH auxotrophy can be complemented.

# 3.4. Hypersensitive response of $tsal \Delta$ mutant to hydrogen peroxide

Thioredoxin peroxidase (cTPxI), encoded by *TSA1*, is essential for antioxidative defense of yeast having dysfunctional mitochondria (Demasi et al., 2001). The cTPx I gene deletion  $(tsa1\Delta)$  renders cells hypersensitive to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress compared to the wild type strain (Demasi et al., 2001). We performed bioassays to verify that hypersensitivity of the  $tsa1\Delta$  mutant is greater to H<sub>2</sub>O<sub>2</sub> than to organic peroxides. Growth of *S. cerevisiae*  $tsa1\Delta$  mutant was inhibited by H<sub>2</sub>O<sub>2</sub> in the presence of antimycin A, a drug interrupting respiratory complex III in mitochondria (Fig. 4). However, no growth inhibition was detected when this mutant was exposed to *t*-BuOOH or cumene hydroperoxide. These results further suggest the differing roles of individual antioxidative genes under different types of oxidative stressors, such as *AHP1* or *TSA1* to organic peroxide or hydrogen peroxide, respectively.

### 4. Discussion

In this study, we show that caffeic acid is a potent antiaflatoxigenic agent in *A. flavus*. Based on results from microarray expression profiling and validation using qRT-

Fig. 4. Response of *S. cerevisiae*  $tsa1\Delta$  mutant to different sources of oxidative stress from peroxides (H<sub>2</sub>O<sub>2</sub>, *t*-BuOOH, cumene) and in combination with the antibiotic, antimycin A, which disrupts mitochondrial respiration and, thus, affects oxidative stress response.

PCR, we also attempt to elucidate the genes/systems involved in the regulatory activity of this antioxidative compound. Expression of almost all of the genes in the aflatoxin biosynthetic gene cluster was repressed by caffeic acid. The mode of action of this anti-aflatoxigenic activity appears to be associated with attenuation of the oxidative stress response of the fungus to organic peroxides. A link between increased aflatoxin production and oxidative stress based on chemical induction has been documented (Javashree and Subramanyam, 2000; Reverberi et al., 2005). Interestingly, it was previously shown that activities of several antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidase, etc.) and the content of GSH increased as cells of aflatoxigenic aspergilli proceeded from early to late logarithmic growth phases, indicating cellular responses to oxidative stress (Jayashree and Subramanyam, 2000).

We have also observed in prior research that aflatoxin production by A. flavus was greatly enhanced when incubated under oxidative stress induced by t-BuOOH (Molyneux et al., 2006). The addition of tannic acid, a commercial hydrolysable tannin containing only gallic acid moieties, to the stressed cells reduced aflatoxin levels well below those observed without induced oxidative stress (Molyneux et al., 2006). In a separate study, culture filtrates from the mushroom, Lentinula edodes also inhibited aflatoxin production by A. parasiticus, wherein the activation of *aflR*, the pathway regulator gene, and *norA*, an aflatoxin biosynthesis cluster gene, was delayed by the compounds in the filtrate (Reverberi et al., 2005). Interestingly, the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, were stimulated by this filtrate, which was accompanied by activation of a yeast hsf2-like antioxidative transcription factor (Reverberi et al., 2005). These results suggest natural compounds can act as inhibitors of aflatoxin production, where the anti-aflatoxigenic mechanism involves activation of cellular antioxidant enzymes in Aspergillus.

Caffeic acid has been shown to have potential hepatoprotective effects against oxidative damage induced by *t*-BuOOH (i.e., lipid peroxidation, DNA damage, lower levels of GSH



(Lima et al., 2006). Caffeic acid treatment (and also other phenolic compounds) significantly decreased lipid peroxidation and prevented GSH depletion, resulting in reduced cell death (Lima et al., 2006). Several reports also showed caffeic, chlorogenic and gallic acids possess excellent antioxidant activity by eliminating ROS that is triggered by environmental stressors such as t-BuOOH, H<sub>2</sub>O<sub>2</sub> and metals (Pavlica and Gebhardt, 2005; Soobrattee et al., 2005). With our model yeast studies, we showed caffeic acid exerted strong antioxidant activity against organic peroxides, wherein the hypersensitive response of *ahp1* $\Delta$  and *vap1* $\Delta$  mutants were relieved by caffeic acid (and chlorogenic or gallic acids). As summarized in Table 6, the specific relationship between "gene-stress", "compound-stress" and/or "gene-compound interaction" reflects a complex intracellular network responding differentially at the molecular level to different environmental cues. Such differential responses of cells to different stresses/ compounds could be advantageous to fungi, since minimum expenditure of energy will be required if cells can operate only specific responsive or detoxification system to the triggers.

Data resulting from microarray analyses can provide reliable insights into "trends" of changes in gene expression, such as upregulation, no change, or down-regulation. The reliability of microarray-based trends is contingent upon the quantitative change in mRNA levels of the respective genes, and the accuracy with which the gene is detected after hybridization. Microarray analysis is a useful tool for initial, large-scale screening of gene expression profiles. However, there are cautionary deficiencies in conclusions solely drawn from such large-scale analysis without further validation. Quantitative PCR provides a more sensitive, accurate and quantitative method of detecting levels of mRNA and thus changes in gene expression. Thus, qRT-PCR is absolutely a requisite approach for validating microarray-based data, especially when the microarray signal is at a low range. Our data presented in this report (see Table 5) clearly demonstrated the necessity of such qRT-PCR validation.

The qRT-PCR results clearly show that four orthologs of the yeast AHP1 gene, encoding alkyl hydroperoxide reductase identified in A. flavus, were induced by caffeic acid. Induced expression of other antioxidant enzymes [i.e., superoxide dismutases (manganese and copper/zinc) and catalase] by the caffeic acid treatment was either unlikely or inconclusive (Table 5). Alternatively, the anti-aflatoxigenic culture filtrate of L. edodes activated superoxide dismutase and catalase in A. parasiticus (Reverberi et al., 2005). These differences in gene expression profiles by different anti-aflatoxigenic compounds further suggest that there is specificity between gene induction (or response) and treatment compounds. AHP1 orthologs in A. flavus appear to play important roles for both anti-aflatoxigenesis as well as tolerance to organic peroxides generated by fatty acid/lipid-rich substrates. We suggest that the AHP1 orthologs in A. flavus are associated with suppression of upstream signals (i.e., oxidative stress) that induce aflatoxin biosynthetic pathway genes.

We also examined the activity of other known antioxidants, in addition to the phenolic compounds tested. For example, in the yeast bioassay, ascorbic acid was shown to be an effective antioxidant against organic peroxide stress. GSH treatment resulted in only slight recovery of wild type cells under *t*-BuOOH stress and, in general was not a potent antioxidant for organic peroxide-induced oxidative stress. However, it appeared to have relatively better antioxidant activity, as reflected by cell recovery, when  $H_2O_2$  was used to induce oxidative stress (Kim et al., 2005).

There were a number of genes down-regulated by caffeic acid treatment involved in lipid metabolism, cell wall/integrity, transporter, oxidoreductase/oxygenase activity and more (Table 3). The functions of eight proteins whose genes were down-regulated are unknown, at this time. Two genes involved with lipid metabolism, diacyl and triacylglycerol lipases, were repressed at medium (-1.20) to high (-2.55) levels, respectively. Lipases (lipolytic enzymes) hydrolyse triglycerides, an important process for energy homeostasis and for providing precursors for membrane biosynthesis (Kurat et al., 2006 and references therein). Considering metabolism of lipids/fatty acids (e.g., saturated free fatty acids) or its metabolites such as lipophilic epoxy fatty acids also supports/stimulates aflatoxin production (Fanelli et al., 1983; Fanelli and Fabbri, 1989), the repression of these lipases (hence inhibition in lipid metabolism) may also be linked to the anti-aflatoxigenesis by caffeic acid. Caffeic acid may, as an antioxidant, directly inhibit enzymes involved in oxidation of cellular molecules (e.g., lipids, proteins, DNA, etc.); which in turn may limit oxidative stress response signaling. For example, methyl gallate, a compound that also inhibits aflatoxin biosynthesis (Mahoney and Molyneux, 2004) and relieves peroxide-induced oxidative stress in yeast (Kim et al., 2005), was found to directly inhibit cyclooxygenase-2 (COX-2), an enzyme involved in lipid hydroperoxide formation (Kim et al., 2006). In view that certain lipid peroxides from the host-plant are capable of modulating aflatoxin production (Burow et al., 1997), disruption of normal lipoxygenase activity, either in the substrate or in the fungus, directly, could alter aflatoxin biosynthesis. Thus, modulation in lipoxide production by the antioxidative activity of caffeic acid probably plays a major part in its ability to inhibit aflatoxin biosynthesis.

The microarray results also showed that the acyl-CoA dehydrogenase gene in lipid metabolism that catalyzes the  $\alpha$ , $\beta$ -dehydrogenation of acyl-CoA thioesters to the corresponding *trans* 2,3-enoyl CoA-products, was also moderately down-regulated by caffeic acid. This enzyme participates in  $\beta$ -oxidation, a crucial process for polyketide synthesis of mycotoxins, including aflatoxin (Maggio-Hall et al., 2005).

At the dosages we applied to the fungus, although aflatoxin biosynthesis was almost completely repressed, fungal growth was not affected. Our analyses of the microarray profiles also concur with this observation. For example, there were a number of genes involved in amino acid metabolic pathways (i.e., Llysine and L-histidine biosynthesis), aromatic metabolism, etc., that were actually up-regulated to some extent by the caffeic acid treatment. This would indicate that the caffeic acid treatment at the levels provided actually conferred a positive effect on fungal cell growth. Eleven genes of unknown function were also up-regulated. In conclusion, a number of researchers have already shown that oxidative stress to the fungus triggers aflatoxin production. Our study shows that caffeic acid (and also other types of phenolic antioxidants) can act as a potent anti-aflatoxigenic agent. From our study, it appears that the mechanism for this anti-mycotoxigenic activity may be associated with the attenuation of upstream oxidative stress responses. Further functional genomic studies incorporating induction of oxidative stress responses should reveal signaling pathways that are involved in the up-regulation of aflatoxin production.

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