Gene targets for fungal and mycotoxin control

J. H. Kim[1], B. C. Campbell[1], R. Molyneux[1], N. Mahoney[1], K. L. Chan[1], J. Yu[2], J. Wilkinson[2], J. Cary[2], D. Bhatnagar[2], T. E. Cleveland[2]

[1]Plant Mycotoxin Research, USDA-ARS Western Regional Research Center, 800 Buchanan St., Albany, CA 94710, USA
[2]Food and Feed Safety Research, USDA-ARS Southern Regional Research Center, 100 Robert E. Lee Blvd., New Orleans, LA 70124, USA

Abstract

It was initially shown that gallic acid, from hydrolysable tannins in the pellicle of walnut kernels, dramatically inhibits biosynthesis of aflatoxin by *Aspergillus flavus*. The mechanism of this inhibition was found to take place upstream from the gene cluster, including the regulatory gene, *aflR*, involved in aflatoxin biosynthesis. Additional research using other antioxidant phenolics showed similar antiaflatoxigenic activity to gallic acid. Treatment of *A. flavus* with *tert*-butyl hydroperoxide resulted in an almost doubling of aflatoxin biosynthesis compared to untreated samples. Thus, antioxidative response systems are potentially useful molecular targets for control of *A. flavus*. A high throughput screening system was developed using yeast, *Saccharomyces cerevisiae*, as a model fungus. This screening provided an avenue to quickly identify fungal genes that were vulnerable to treatment by phenolic compounds. The assay also provided a means to quickly assess effects of combinations of phenolics and certain fungicides affecting mitochondrial respiration. For example, the *S. cerevisiae sod2*Δ mutant was highly sensitive to treatment by certain phenolics and strobilurins/antimycin A, fungicides which inhibit complex III of the mitochondrial respiratory chain. Verification of stress to this system in the target fungus, *A. flavus*, was shown through complementation analysis, wherein the mitochondrial superoxide dismutase (Mn-SOD) gene (*sodA*) of *A. flavus* in the ortholog mutant, *sod2*Δ, of *S. cerevisiae*, relieved phenolic-induced stress. Mitochondrial antioxidative stress systems play an important role in fungal response to antifungals. Combined treatment of fungi with phenolics and inhibitors of mitochondrial respiration can effectively suppress growth of *A. flavus* in a synergistic fashion.

Keywords: aflatoxin, *Saccharomyces cerevisiae*, oxidative stress, antioxidants, mitochondrial respiration

Introduction

The filamentous fungi *Aspergillus flavus* and *A. parasiticus* are noted producers of hepatocarcinogenic aflatoxins that can contaminate a number of agricultural commodities. Even at very low quantities (parts per billion) this contamination can cause a significant negative effect on food safety/human health and economic value of affected crops (1).
Previously, Jayashree and Subramanyam (2) reported oxidative stress induces aflatoxin biosynthesis in *A. parasiticus*. Hydrolysable tannins significantly inhibit aflatoxin biosynthesis with one of the antiaflatoxigenic constituents identified as gallic acid (3). Gallic acid hinders expression of genes within the aflatoxin biosynthetic cluster except for transcription of the positive aflatoxin pathway gene regulator, *aflR* (4). This observation suggests gallic acid disrupts signal transduction pathway(s) for aflatoxigenesis. Experiments to demonstrate this possibility are currently underway. Hydrolysable tannins and gallic acid are known antioxidants in living cells (5) and gallotannins prevent cell death under oxidative stress (6, 7). Recently, it was shown that inhibition of aflatoxin biosynthesis by *A. parasiticus* was achieved through activation of an *hsf*2-like transcription factor that triggered antioxidative enzyme production (8).

Presented at the EU-USA Bilateral Workshop on Toxigenic Fungi & Mycotoxins, New Orleans, USA, July 5-7, 2005

Correspondence: Bruce C. Campbell, 800 Buchanan St., Albany, CA 94710, USA (bcc@pw.usda.gov)

Financial support: Intramural CRIS projects 5325-410-032-00D and 6435-41420-004-00D; Washington Tree Fruit Research Commission project AH-04-420

The antiaflatoxigenic activity of gallic acid appears to attenuate the oxidative stress responses in aspergilli. Thus, disruption of upstream stress-responses in aflatoxigenic aspergilli could be a useful means of preventing aflatoxin contamination of food commodities. To better understand the mode of action of gallic acid and other antioxidant natural compounds, we examined how oxidative stress affected stress responses of fungi using *Saccharomyces cerevisiae* Meyen ex. E.C. Hansen. The genome of this model fungus has been fully sequenced and well annotated, as has its oxidative stress response pathways (9). We developed a new high throughput bioassay system based upon singular gene deletion mutants of signaling and transduction of stress responses of *S. cerevisiae*. The availability of an *A. flavus* Expressed Sequence Tag (EST) database (10) allowed us to perform complementation analysis of antioxidative stress response genes of *A. flavus* in orthologous deletion mutants of *S. cerevisiae*. Yeast deletion mutants have been useful for examining the target genes of antifungal compounds. Parsons *et al.* (11) were successful in using such mutants to identify targeted molecular pathways and genes of a number of fairly diverse inhibitory compounds. We have already shown that application of vanillyl acetone and strobilurins disrupted mitochondrial respiration and effectively controlled *A. flavus* (12).

Material and Methods

Effects of peroxide and phenolics on aflatoxigenesis

Protocols mainly followed those outlined in Mahoney *et al.* (3) and are summarized as follows: Test phenolics were obtained from Sigma-Aldrich (St. Louis, MO) and purified by passage through a column of LH-20 in ethanol. Semi-solid media prepared from finely ground pistachio kernels (5% pistachio in 1.5% agar, 10 ml per 60 mm petri dish) was inoculated with *A. flavus* NRRL 25347, ~200 spores in a single point, and incubated at 28 °C. Aflatoxin was quantified from fungal mats, including spores and media, by extraction with MeOH (50 mL). A 1 mL aliquot was removed and evaporated with N_2 at 40 °C, the residue was derivatized with hexane and trifluoroacetic acid, evaporated to dryness with N_2 and redissolved in H_2O/CH_3-CN (9:1; 1 mL). Aliquots (20 µL) were analyzed for aflatoxin by reversed-phase HPLC and fluorescence detection, with excitation at 365 nm and detection of emission at 455 nm. Oxidative stress was introduced into this same medium with *tert*-butyl hydroperoxide (100 µM final concentration). Aflatoxin production for cultures with and without oxidative stress were measured in triplicate by HPLC, as outlined above, over a time course of 1 to 9 days. Antioxidants (12 mM final concentration) were added to pistachio agar with and without oxidative stress. Aflatoxin values were measured in triplicate after 5 days of incubation.

High throughput yeast and A. flavus *bioassays*

Protocols for the yeast high throughput bioassay are outlined in Kim *et al.* (12) and are summarized as follows: Wild-type, and deletion mutants of the yeast, *S. cerevisiae,* were purchased from Invitrogen (Carlsbad, CA). Yeast cells were grown in rich medium (YPD: Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) at 30 °C overnight. Approximately 1×10^6 cells were serially diluted from 10-fold to 10^5-fold. Cells from each serial dilution were spotted adjacently on SG (Yeast nitrogen base w/o amino acids 0.67%, glucose 2% with appropriate supplements: uracil 0.02 mg/ml, amino acids 0.03 mg/ml) agar plates incorporated with each natural compound (5, 15, 25 mM) to be examined. Yeast cell growth was determined after incubation at 30 °C for 7 days. For examination of effects of phenolics and fungicides, *A. flavus* NRRL 3357 was cultured on either YES (yeast extract 2%, sucrose 6%, pH 5.8) or Potato Dextrose Agar (PDA; Sigma, St. Louis, MO) media at 28 °C. Test phenolic compounds were obtained from Sigma, and dissolved in dimethyl sulfoxide (DMSO; absolute amount <200µl/10mL media) or water.

Complementation analysis

Protocols for the complementation analysis are outlined in Kim *et al.* (12) and are summarized as follows: A representative bioassay for examining orthology of genes for Mn-SOD between *S. cerevisiae* and *A. flavus* was performed by complementing yeast deletion mutant *sod2*Δ with an *A. flavus sodA*. *A. flavus* was cultured in YES liquid medium,

28 °C, overnight, total RNA was isolated, *sodA* cDNA was synthesized by PCR and cloned into pYES2, a yeast expression vector (Invitrogen, Carlsbad, CA). Functional expression of *sodA* in the *sod2*Δ strain was verified by comparison of phenotype to oxidative stress using *sod2*Δ-pYES2 empty vector (negative control), wild-type-pYES2 empty vector (positive control) and *sod2*Δ-*sodA*. Yeast cells were cultured, overnight, as described above and ~1×10^6 cells were serially diluted from 10-fold to 10^5-fold in SGAL (yeast nitrogen base w/o amino acids 0.67%, galactose 2%, amino acids 0.03 mg/ml) liquid medium. Cells from each serial dilution were spotted adjacently on SGAL agar plates. Functionality of *sodA* was determined based on cell growth in presence of vanillyl acetone (5, 10, 15, 20 mM) and/or strobilurin, antimycin A fungicides to demonstrate *sodA* is responsible for vanillyl acetone/strobilurin tolerance.

Results and Discussion

Treatment of *A. flavus* with *tert*-butyl hydroperoxide resulted in a significant increase in aflatoxin production (Figure 1). Aflatoxin production under the standard test regimen that we use progressively increases over a four-day period, peaks at day five, then begins to decline. The 155 µg of aflatoxin at day five and declining to 79 µg at day nine for the untreated *A. flavus* are basically the standard levels we see under the culture conditions we use (3). However, the *A. flavus* cohorts subjected to oxidative stress clearly showed an increase in aflatoxin production. By day five there was a >30% greater level and by day nine a >100% greater level of aflatoxin in the oxidatively stressed compared to unstressed cohorts of *A. flavus* (Figure 1).

Interestingly, when certain phenolics or other antioxidants, such as ascorbic acid, were added to oxidatively stressed *A. flavus*, aflatoxin production significantly declined, without any effect on the level of fungal growth (Figure 2). For example, when no antioxidants were added (solvent controls), there was no decline in aflatoxin production. Alternatively, when ascorbic acid was added to the media, aflatoxin production was completely inhibited under the oxidatively stressed conditions. Chlorogenic and gallic acids inhibited aflatoxin production by 92% and 80%, respectively, compared to cohorts that were oxidatively stressed but not provided with these antioxidants (Figure 2).

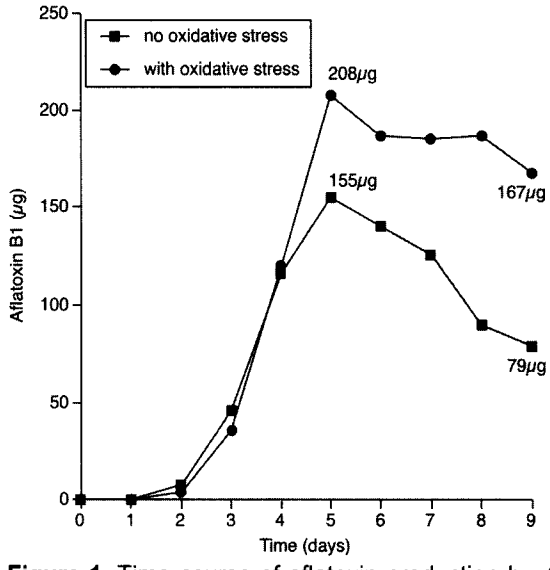

Figure 1. Time course of aflatoxin production by *A. flavus* on pistachio kernel agar with and without oxidative stress (100 µM *tert*-butyl hydroperoxide). Oxidative stress not only increased peak aflatoxin production, but high aflatoxin levels were sustained

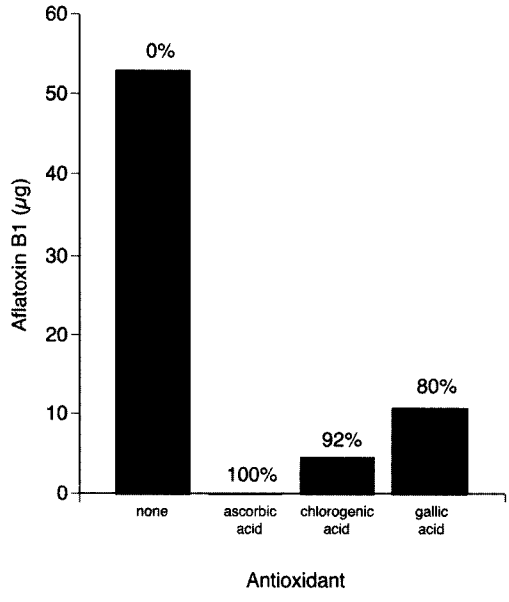

Figure 2. Antioxidants (12mM) counteract the aflatoxin stimulatory effect of oxidative stress (100 µM *tert*-butyl hydroperoxide). Histogram columns represent the difference in aflatoxin production on pistachio kernel agar with and without oxidative stress. Ascorbic acid was the most effective antioxidant, reducing 100% of the aflatoxin, while the phenolic natural products chlorogenic acid and gallic acid reduced 92% and 80%, respectively, of the aflatoxin produced as a result of oxidative stress

Results using the yeast high throughput bioassay to identify potential fungal genes involved in oxidative stress responses or genes that could serve as promising targets for fungal control are more completely presented in Kim *et al.* (13). The use of this system and representative results are depicted in Figure 3 showing how deletion mutants of *S. cerevisiae* can be used to ascertain candidate fungal genes that may be vulnerable targets to oxidative stress resulting from treatment with phenolics. In this example, treatment by 2-hydroxycinnamic acid clearly affects survivability of certain deletion mutants, depending upon the deleted gene.

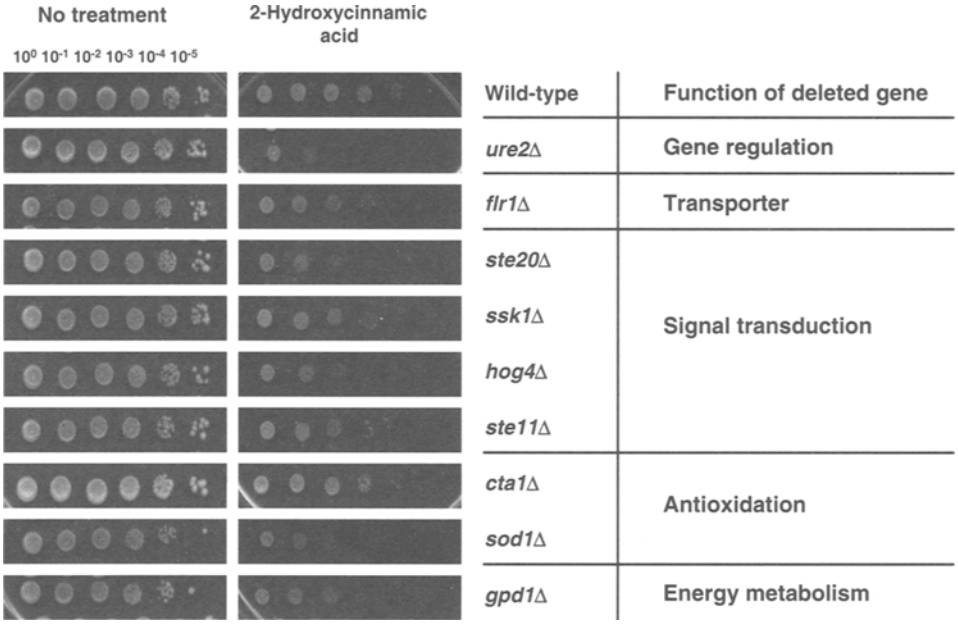

Figure 3. Representative results of the yeast high throughput bioassay. This figure illustrates how the effect of a natural compound, represented in this case by 2-hydroxycinnamic acid, can be categorized according to gene function and how a particular gene target can be identified. The assay utilizes deletion mutants of *S. cerevisiae*. The deletion mutants used in this example are listed (*e.g.*, *ureΔ*, *flr1Δ*, *ste20Δ*, *etc.*) according to function (*e.g.*, gene regulation, *etc.*). The response of the various strains of yeast used are scored according to the level of cell dilution at which a colony becomes visible. The greater the dilution (shown at the top left under "No treatment") at which a colony is visible, the less sensitivity that strain has towards the test compound (*e.g.*, *flr1Δ* is approximately 100 times less sensitive to 2-hydroxycinnamic acid than is *sod1Δ*). Also, note, that all strains grew normally (similar to that of the wild-type) under control conditions

Using this type of bioassay and testing a number of different phenolic compounds, we have essentially found that the most fruitful gene targets for fungal control generally include those genes involved in oxidative stress responses. In particular, we have found that genes regulating mitochondrial respiration are particularly vulnerable (14). Also, we have found that targeting the oxidative stress system of fungi, both "upstream" and "downstream", can result in highly lethal effects. These lethal effects can be accomplished by using a combination of commercial fungicides and phenolics, simultaneously, as depicted in Figure 4. This figure also shows how complementation analysis can be used to demonstrate the validity of using *S. cerevisiae* as a model fungal system. A combination of strobilurin and cinnamic acid is 100 to 1000 times more toxic, depending on the strain (Figure 4). For example in the *sod2Δ*-pYES2 (empty vector), the combined treatment was almost 1000-fold more toxic (denoted by "*") than either of the fungicides, alone. A similar trend in toxicity as seen in the yeast strains is also reflected in the bioassays using *A. flavus,* where combined treatments were completely lethal (denoted by*). Successful complementation of the *sod2Δ* by *A. flavus* Mn-SOD gene (*sodA*) is confirmed by almost 100-fold less sensitivity in the complemented yeast than in the deletion mutant without *sodA*.

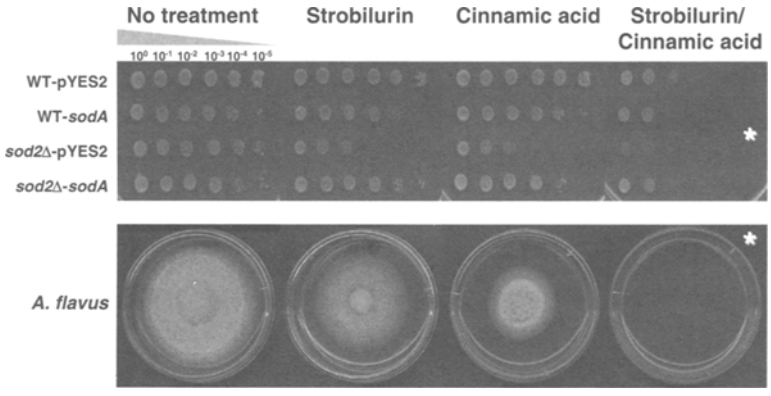

Figure 4. Bioassays of yeast and *A. flavus* showing increased fungicidal activity of combined treatments of a phenolic (cinnamic acid) and a fungicide (strobilurin) that target the oxidative stress response system of fungi, particularly Mn-SOD. The top section of the figure shows results of yeast dilution bioassays and includes results of complementation of the Mn-SOD gene of *A. flavus* (*sodA*) in the orthologous deletion mutant of *S. cerevisiae* (*sod2Δ*). WT-pYES2= wild-type yeast with empty vector; WT-*sodA*= wild-type yeast with Mn-SOD gene (*sodA*) from *A. flavus*; *sod2Δ*-pYES2= yeast deletion mutant lacking the Mn-SOD gene plus empty vector; *sod2Δ-sodA*= yeast deletion mutant complemented with orthologous Mn-SOD gene from *A. flavus*

Once we determined the potential to target the oxidative stress response system of fungi as a means for control, we began to investigate more gene targets in the fungal oxidative stress response system and additional natural phenolics. As shown in Figure 5 we found that vanillyl acetone affects the normal function of mitochondria based on its effects on the *tsa1Δ* mutant of *S. cerevisiae* lacking the gene for Tsa1p (Thioredoxin peroxidase; cTPxI). Thioredoxin peroxidase is essential for an antioxidative response in yeast having dysfunctional mitochondria (15). Thus, deletion of this gene results in heightened sensitivity of cells to oxidative stress. Vanillyl acetone inhibited growth of the *tsa1Δ* mutant when exposed to oxidative stress (*e.g.*, hydrogen peroxide) compared to the wild-type strain. This growth reduction shows that vanillyl acetone disrupts mitochondrial function. For purposes of comparison, use of antimycin A and strobilurin, known inhibitors of mitochondrial function, by inhibiting electron transfer in the mitochondrial respiratory chain, produced similar effects to those of vanillyl acetone (Figure 5). However, vanillyl acetone presumably targeted a different system in the mitochondrial oxidative stress system than that of strobilurin and antimycin A. As shown previously, vanillyl acetone disrupts mitochondrial Mn-SOD activity (12).

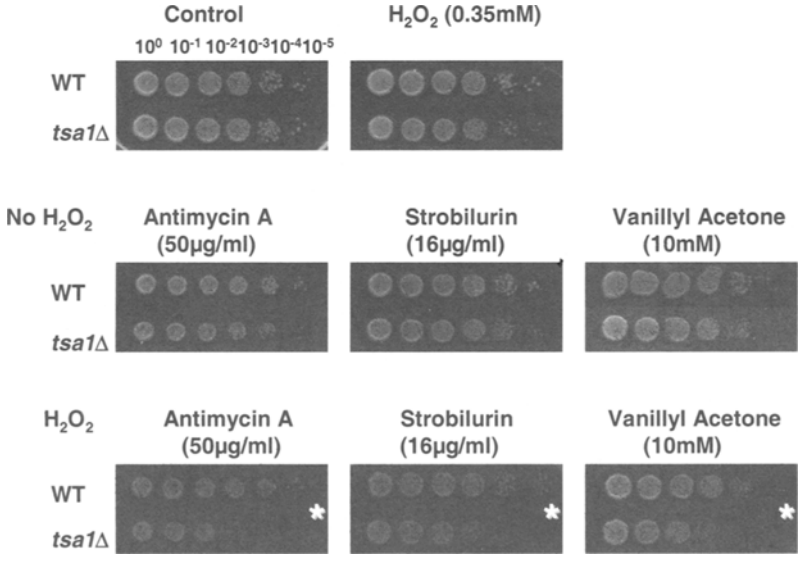

Figure 5. Yeast dilution bioassays showing deleterious effects of commercial fungicides (*e.g.*, antimycin A and strobilurin) and a test natural phenolic, vanillyl acetone, on the *tsa1Δ* mutant under oxidative stress

Conclusions

The ability of antioxidants to reduce aflatoxin production has been observed before (2, 3). Moreover, the relationship between inducing an antioxidative stress response with concurrent reduction of aflatoxin biosynthesis in *A. parasiticus* was presented by another participant of this conference (16). The advantage conferred on aspergilli by biosynthesis of aflatoxins has not been established with any degree of certainty. That the biosynthetic precursors of aflatoxins are phenolics, which have predictable antioxidant activity, one could speculate that they could contribute to alleviation of oxidative stress. The fact that oxidative stress induces aflatoxin biosynthesis and that the compounds synthesized in the course of making aflatoxin are antioxidants argues that the aflatoxin biosynthetic pathway might provide some evolutionary advantage to combating oxidative stress. Our research results to date suggest that the use of chemicals that weaken the oxidative stress response system of fungi is a promising fungicidal strategy. With combined use of strobilurins and phenolics, one disrupts the cytochrome complex in the mitochondrial respiratory chain and other antioxidative responses (*e.g.,* Mn-SOD), respectively, resulting in a synergistic fungicidal effect.

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