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Identification of Phenolics for Control of *Aspergillus flavus* Using *Saccharomyces cerevisiae* in a Model Target-Gene Bioassay

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The yeast *Saccharomyces cerevisiae* was used in a high-throughput bioassay to identify phenolic agents for control of the aflatoxigenic fungus *Aspergillus flavus*. Veratraldehyde, **1**, cinnamic acid, **5**, and the respective benzoic acid derivatives vanillin, **2**, vanillic acid, **3**, and vanillylacetone, **4**, and cinnamic acid derivatives *o*-coumaric acid, **6**, *m*-coumaric acid, **7**, and *p*-coumaric acid, **8**, showed significant antifungal activities (from highest to lowest, **2**, **5** > **1** > **6**, **7** > **4** > **3**, **8**) in the yeast system, with caffeic acid, **9**, having little to no effect. Antifungal activity levels against *A. flavus* were similar. This similarity in antifungal activity demonstrated the usefulness of the *S. cerevisiae* bioassay for screening antifungal compounds. Assays using deletion mutants of yeast identified signal transduction and antioxidative stress response genes important to fungal tolerance. Targeting the antioxidative stress response system with certain compounds (e.g., **4**) in combination with strobilurin fungicides had a synergistic effect against both fungi.

KEYWORDS: ***Aspergillus flavus*; *Saccharomyces cerevisiae*; oxidative stress response; fungicide; aflatoxin; phenolic; antifungal; strobilurin; superoxide dismutase**

INTRODUCTION

Aflatoxins are hepatocarcinogenic difuranocoumarins produced by certain ascomycetous fungi in the genus *Aspergillus*. *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare are the most common species associated with aflatoxin biosynthesis. Contamination of food by aflatoxins is a significant food safety issue. Low levels of contamination by aflatoxin B_1 (1−2 ppb) can result in rejection of agricultural products intended for human consumption. Such constraints and potential rejections generate economic losses, especially to exporters of peanut, cottonseed, corn, fig, and tree nut products (*1*). Because of increasingly stringent regulations among importing nations, there is an urgent need to develop ways of reducing aflatoxin contamination in both pre- and postharvest production of agricultural commodities.

Natural compounds could potentially serve as effective alternatives to conventional antifungal or antimycotoxigenic agents. Conventional fungicides are frequently perceived to present a hazard to human health or the environment (*2*−*5*). Natural compounds that do not have any significant medical or environmental impact could serve as useful alternatives to fungicides. For example, a phenolic compound from walnut seed coats, gallic acid, was recently shown to prevent aflatoxin biosynthesis by *A. flavus* (*6*). Treatment of *A. flavus* with gallic acid results in complete shutdown in expression of "early" and "late" genes (e.g., *nor-1* and *ver-1*) in the aflatoxin biosynthetic pathway (J. Cary, personal communications). On the basis of bioassays using yeast deletion mutants, it appears gallic acid acts as a potent antioxidant, suggesting there is a link between oxidative stress and aflatoxin biosynthesis in *A. flavus* (*7*). Prior studies also showed that derivatives of benzoic or cinnamic acid inhibit growth of various filamentous fungi, including *Rhizoctonia*, *Aspergillus*, *Penicillium*, and *Pythium*, growth of food spoilage yeasts, and biosynthesis of mycotoxins (*8*−*11*).

Cellular targets of several conventional fungicides are already known. Examples include macromolecular synthesis (e.g., nucleic acids, amino acids, cell wall, etc.), cell division, signal transduction, and respiration (*12*). In particular, there are at least seven target sites in the fungal respiration system (e.g., complexes I, II, and III, oxidative phosphorylation, ATP synthesis, etc.). Defects in any of these systems can lead to oxidative stress, with a resultant decrease in cell viability. Therefore, genes responsible for normal mitochondrial functioning (respiration/antioxidation) can be useful targets for fungal pathogen control. Recently, we isolated and functionally characterized the *sodA* gene of *A. flavus*, an orthologue of the *sod2* gene of *Saccharomyces cerevisiae* encoding mitochondrial manganese superoxide dismutase (MnSOD; EC 1.15.1.1) (*13*). This enzyme is crucial for detoxifying strong oxidants such as reactive oxygen species (ROS; e.g., superoxide anion, hydrogen peroxide, etc.). Such strong oxidants can react with lipid side

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chains of the mitochondrial membrane, causing malfunctioning of the mitochondrial respiratory chain and oxidative stress (*14*).

The yeast *S. cerevisiae* Meyen ex E. C. Hansen is a useful tool for examining antifungal compounds and identifying gene targets in the view that the entire genome of *S. cerevisiae* has been sequenced and well annotated. Many genes in yeast are orthologues of genes of fungal plant pathogens (*15*). Moreover, structural homology of several signal transduction and antioxidative stress genes between *S. cerevisiae* and the aflatoxigenic, filamentous fungus *A. flavus* Link has already been confirmed (*7*). Also, yeast deletion mutants have proven to be very useful for determination of the mode of action of antifungal compounds and identification of target genes for antimicrobial agents. Parsons et al. (*16*) used viable deletion mutants of *S. cerevisiae* to identify molecular pathways and genes that were targets of 12 diverse inhibitory compounds. There are approximately 6000 strains of yeast deletion mutants based upon genes identified in the yeast genome sequencing project [Stanford Genome Technology Center (*17*)].

In this study, we describe a new bioassay that uses the yeast *S. cerevisiae* as a model for screening phenolic compounds for antifungal activity. Compounds identified as being effective against yeast were then examined for their antigrowth and aflatoxigenic activities against *A. flavus*. The yeast-based bioassay thus provided a framework to examine structure−activity relationships of test compounds and use fungal functional genomics to identify promising molecular targets for control of other fungi.

MATERIALS AND METHODS

Strains and Media. Strain BY4741 (*mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), the wild-type, and deletion mutants of the yeast *S. cerevisiae* were purchased from Invitrogen (Carlsbad, CA), and are described in **Table 2**. Yeast cells were grown in rich medium (YPD: Bacto yeast extract, 1%; Bacto peptone, 2%; glucose, 2%), minimal medium (SG: yeast nitrogen base without amino acids, 0.67%; glucose, 2%; with appropriate supplements: uracil, 0.02 mg/mL; amino acids, 0.03 mg/mL), and complementation medium (SGAL: yeast nitrogen base without amino acids, 0.67%; galactose, 2%; amino acids, 0.03 mg/mL). Yeast cells were grown at 30 °C. *A. flavus* NRRL 3357 was grown in either yeast extract sucrose agar (YES; yeast extract, 2%; sucrose, 6%; pH 5.8) or potato dextrose agar (PDA) (Sigma, St. Louis, MO) at 28 °C. Phenolic compounds were obtained from Sigma, and dissolved in dimethyl sulfoxide (DMSO; absolute amount <200 μL/10 mL of medium) or water [i.e., vanillylacetone for testing effects on aflatoxin biosynthesis] before use.

In Vitro Susceptibility Test. Yeast cells were cultured in YPD liquid medium overnight, and ~1×10^6 cells were serially diluted from 10-fold to 10^5-fold in SG liquid medium. The cells from each serial dilution were spotted adjacently on SG agar plates incorporated with each natural compound (5, 10, 15, and 25 mM) to be examined and with nutritional supplements (e.g., amino acids, uracil, etc.) depending on the experiment. Veratraldehyde, **1**, and cinnamic acid, **5**, their phenolic compound derivatives, i.e., benzoic acid derivatives vanillin, **2**, vanillic acid, **3**, and vanillylacetone, **4**, and cinnamic acid derivatives *o*-coumaric acid, **6**, *m*-coumaric acid, **7**, *p*-coumaric acid, **8**, and caffeic acid, **9** (**Figure 1**), were tested. Yeast cell growth was measured on the basis of the highest cell dilution (lowest number of cells) from which a colony became visible after incubation at 30 °C for 7 days.

A. flavus NRRL 3357 (~200 spores) was spotted on the center of PDA plates containing natural compounds (5, 10, 15, and 25 mM), and growth was observed after 7 days at 28 °C. To test the synergistic effects of vanillic acid and azoxystrobin or kresoxim-methyl on the growth of *A. flavus*, fungal strains (~200 spores) were spotted on PDA plates containing compounds (vanillylacetone, 5, 10, 15, 20 mM; azoxystrobin or kresoxim-methyl, 64 μg/mL). Colony growth was measured on the basis of percent radial growth compared to that of control colonies grown on PDA plates receiving only DMSO.

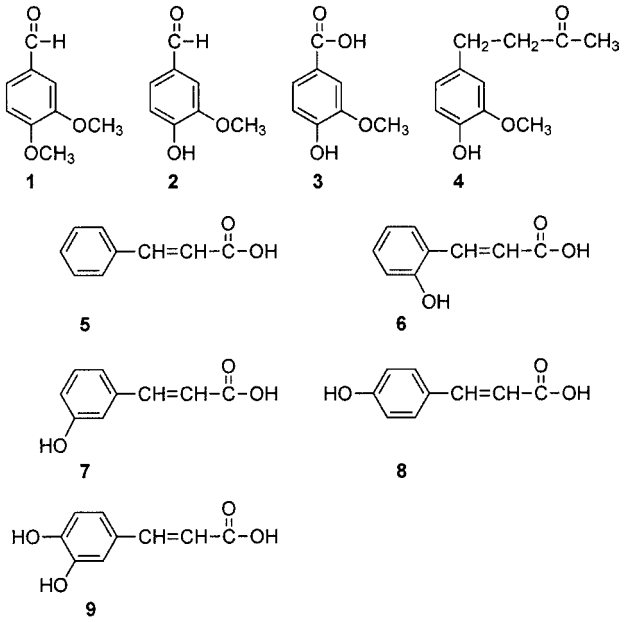

Figure 1. Chemical structures of phenolic compounds examined in this study: veratraldehyde, **1**; vanillin, **2**; vanillic acid, **3**; vanillylacetone, **4**; cinnamic acid, **5**; *o*-coumaric acid, **6**; *m*-coumaric acid, **7**; *p*-coumaric acid, **8**; caffeic acid, **9**.

Functional Expression of *sodA* in the *S. cerevisiae sod2*Δ Strain Sensitive to Vanillylacetone. To demonstrate the method of identifying genes in *A. flavus* targeted by antifungal compounds in the yeast bioassays, a functional complementation assay was performed. Complementation was tested in a *sod2*Δ yeast deletion mutant using the *sodA* orthologue from *A. flavus*. The *sodA* gene was isolated by first obtaining a reverse-transcribed cDNA template using PCR. *A. flavus* was cultured in YES liquid medium, 28 °C, overnight, and total RNA was isolated from this culture using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA). The first-strand cDNA of *sodA* was synthesized using protocols outlined in the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Beverly, MA) using the PCR primer "*sodA*-C", tctagatctagaCCGAGGCGTATCCAATTGCATTAGGGCTAC (lowercase letters indicate the *Xba*I site). PCR cycles used were 95 °C (2 min)−1 cycle, 95 °C (30 s), 55 °C (30 s), 68 °C (1.5 min)−25 cycles, and 68 °C (15 min)−1 cycle (primers "*sodA*-N", ggatcc**g**gat**cc**-ATGAACTCAATTCCATTGATACATAAAAATC [ATG = initiation codon, bold letters indicate the Kozak sequence (*18*), and lowercase letters indicate the *Bam*HI site], and *sodA*-C). The PCR-amplified *sodA* cDNA clone was treated with *Bam*HI and *Xba*I restriction enzymes, and purified DNA (QIAEX II Gel Extraction Kit, QIAGEN) was ligated into pYES2, a yeast expression vector (Invitrogen).

The complementation bioassay to determine functional expression of *sodA* in the *sod2*Δ strain was performed as follows: The *sod2*Δ strain having a pYES2 empty vector (negative control), wild-type strain having a pYES2 empty vector (positive control), and *sod2*Δ strain containing PCR-amplified *sodA* in the pYES2 vector were cultured in raffinose medium (0.67% yeast nitrogen base without amino acids; 2% raffinose; amino acids, 0.03 mg/mL), 30 °C, overnight, and ~1×10^6 cells were serially diluted with raffinose liquid medium and spotted adjacently on SGAL agar plates, as described above. Functional expression of *sodA* was achieved under the GAL1 promoter (30 °C, 10 days). Functionality of *sodA* was assessed on the basis of yeast cell growth in the presence of vanillylacetone (5, 10, 15, and 20 mM) and/or strobilurin fungicides (64 μg/mL). If cell growth was similar to that of the positive control or better than that of the negative control, *sodA* was considered to have functionally complemented the *S. cerevisiae sod2*Δ strain by relieving sensitivity to the tested compounds.

Analysis of Aflatoxin. The effect of vanillylacetone on aflatoxin production was examined as follows: Vanillylacetone was dissolved in H_2O and incorporated into PDA (5, 10, 15, 20, and 25 mM). The media were then inoculated with ~200 spores of *A. flavus* NRRL 3357,

Table 1. Responses of Wild-Type *S. cerevisiae* and *A. flavus* to Natural Compounds[a]

	concn (mM)	*S. cerevisiae* log scale	*A. flavus* growth (%)
control (DMSO only)		6	100
	5	6	49
veratraldehyde	10	5	0
	15	2	0
	25	0	0
vanillin	5	5	11 ± 8.5
	10	1	0
	15	0	0
	25	0	0
vanillic acid	5	6	91
	10	6	79
	15	5[b]	70
	25	3	49
vanillylacetone	5	6	77
	10	6	53
	15	6[b]	26
	25	2	0
cinnamic acid	5	1	47
	10	0	0
	15	0	0
	25	0	0
o-coumaric acid	5	6[b]	79
	10	1	57
	15	na[c]	40 ± 8.5
	25	na[c]	na[c]
m-coumaric acid	5	6	83
	10	2	70
	15	1	57
	25	1	19
p-coumaric acid	5	6[b]	85
	10	3	68
	15	na[c]	62
	25	na[c]	na[c]
caffeic acid	5	6	94
	10	6	87
	15	6	85
	25	6	79

[a] Responses for yeast growth are represented as a logarithmic number (e.g., 1 indicates a 100000-fold decline in cell growth relative to 6, etc.) and for growth of *A. flavus* as a percentage of radial growth of the fungal mat of treated compared to control (DMSO only) samples. Values are means of three replicates. Standard deviations of all measurements were <0.1 except where noted. Examples of bioassays used to construct this table are depicted in **Figure 2**. [b] Smaller colonies. [c] Not applicable due to precipitation of natural compounds.

and the culture was incubated at 28 °C for 7 days. Aflatoxin B_1 was quantitated as described previously (*19*), with each treatment replicated three times.

RESULTS AND DISCUSSION

Testing Antifungal Activities of Natural Compounds Using Model Yeast Bioassays and *A. flavus*. All phenolic acids tested, except caffeic acid, inhibited the growth of wild-type yeast cells proportional to the concentration of the compounds (5−25 mM) examined. Cinnamic acid and vanillin showed the highest levels of growth inhibition, followed by other members of each phenolic group. For example, cinnamic acid and vanillin almost completely inhibited the growth of yeast cells at 10−15 mM, while other structural derivatives (e.g., veratraldehyde and all coumaric acids) showed moderate levels of toxicity at this concentration (**Table 1**). Veratraldehyde, however, completely inhibited the growth of yeast cells at 25 mM.

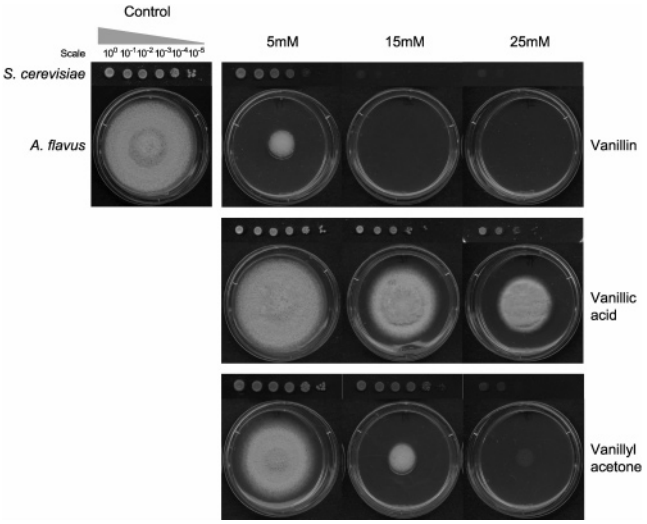

Figure 2. Examples of bioassays showing growth inhibition of wild-type *S. cerevisiae* and *A. flavus* by benzoic acid derivatives vanillin, vanillic acid, and vanillylacetone. The scale indicates the level of dilution of yeast cells from approximately 1×10^6 cells (10^0 dilution) to 10 cells (10^{-5} dilution). The extent of growth of *A. flavus* was based on the diameter of the fungal mat in Petri dish cultures (see Materials and Methods).

The growth of cells treated with vanillylacetone or vanillic acid was similar to that of the control at 5 mM. Although vanillic acid was more toxic than vanillylacetone at 15 mM, vanillylacetone exerted higher toxicity than vanillic acid at 25 mM. Likewise, all three coumaric acids were less inhibitory than cinnamic acid, and *o*-coumaric acid was more inhibitory than *m*- and *p*-coumaric acids at 10 mM. Both *o*- and *p*-coumaric acids, however, precipitated at concentrations >15 mM, whereas *m*-coumaric acid did not precipitate at any of the concentrations tested. These differences in solubility should be taken into consideration for future, practical use of these compounds. Since the coumaric acids differ only in the position of the −OH group on the aromatic ring, this must be the structural feature that affects their structure−activity relationships and physicochemical properties. Caffeic acid showed almost no antifungal activity at any concentration tested in the yeast system. The overall order of highest to lowest antifungal activity in the yeast bioassays was cinnamic acid, vanillin > veratraldehyde > *o*-, *m*-coumaric acids > vanillylacetone > vanillic acid, *p*-coumaric acid > caffeic acid (**Table 1**; **Figure 2**).

The compounds screened using the yeast bioassay were, then, examined for their effects on *A. flavus*. As shown in **Figure 2** and **Table 1**, cinnamic acid, vanillin, and veratraldehyde severely inhibited hyphal growth of *A. flavus* at 5 mM, and completely inhibited spore germination at 10 mM. Vanillylacetone, vanillic acid, and all three coumaric acids inhibited hyphal growth at 5−25 mM, while caffeic acid showed only limited inhibition of fungal growth even at the highest concentration tested. Vanillylacetone, which was only moderately toxic in yeast bioassays, was highly toxic to *A. flavus* relative to the vanillic or coumaric acid derivatives at 15−25 mM. All three coumaric acids showed similar levels of inhibiting fungal growth at 5−15 mM. The relative toxicities of the phenolic compounds in the *A. flavus* bioassays (cinnamic acid, vanillin, veratraldehyde > vanillylacetone > vanillic acid, all three coumaric acids > caffeic acid) as well as their structure−activity relationships were quite similar to those observed in the *S. cerevisiae* bioassays. This similarity in results shows that *S. cerevisiae* can serve as a useful tool for the high-throughput screening of

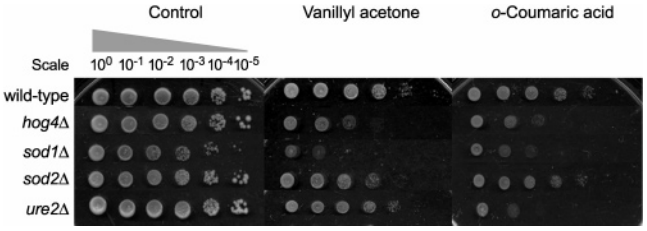

Figure 3. Examples of bioassays showing responses of the wild-type and deletion mutants *hog4*Δ, s*od1*Δ, *sod2*Δ, and *ure2*Δ of *S. cerevisiae* to vanillylacetone (15 mM) and *o*-coumaric acid (5 mM). The scale indicates the level of dilution of yeast cells from approximately 1×10^6 cells (10^0 dilution) to 10 cells (10^{-5} dilution). The response to treatment is measured on the basis of the highest dilution from which a yeast colony becomes visible.

antifungal natural compounds with the added benefit of determining gene targets using deletion mutants.

A total of 44 deletion mutants of *S. cerevisiae* were selected for bioassays to identify target genes for fungal pathogen control. Yeast strains having defects in genes responsible for cellular detoxification of the test compounds will fail to grow or be more sensitive than wild-type yeast. The selected mutants were classified into five groups depending on which particular gene was lacking in function. These groups were (1) signal transduction, (2) gene regulation, (3) antioxidation, (4) DNA damage control, and (5) energy metabolism. The rationale for selecting these groups of deletion mutants was that phenolic compounds strongly influence cellular redox potentials. The selected deletion mutants had defective genes that are normally involved in oxidative stress-response pathways, considered to play key roles in relieving the toxic effects of phenolic acids. These pathways play a very important role in fungal cell survival, especially when a fungal pathogen infects a host plant that possesses a variety of natural phenolic compounds (*20*).

Vanillylacetone (15 mM) and *o*-coumaric acid (5 mM) were selected for a representative study of their effects on the growth of yeast deletion mutants. At these concentrations both compounds showed threshold effects on wild-type yeast cells (minimum effective concentration) (**Table 1**). Vanillin and cinnamic acid were not included in the deletion mutant assays because of their high level of toxicity, even in wild-type yeast cells. This high degree of toxicity would make it difficult to determine structure−activity relationships when higher concentrations of compounds were applied.

Heightened sensitivity exhibited by certain deletion mutants to natural products, as shown in **Figure 3** and **Table 2**, made it possible to identify functional defects that would be reflected in genetic and molecular targets for pathogen control. For both compounds (vanillylacetone and *o*-coumaric acid), gene deletions in efflux pumps (*PDR5* for vanillylacetone, *FLR1* and *YOR1* for *o*-coumaric acid), signal transduction, and antioxidative stress pathways affected cellular sensitivity. The genes *URE2* (gene regulation) and *GPD1* and *HOR2* (energy metabolism) were shown to be especially associated with tolerance to *o*-coumaric acid. The fact that growth of a higher number of different deletion mutants was sensitive to *o*-coumaric acid than vanillylacetone suggests *o*-coumaric acid (and possibly *m*- and *p*-coumaric acids) affects more target genes than vanillylacetone, in yeast.

Synergistic Effects of Vanillylacetone and Strobilurin-Related Fungicides on Fungal Growth. In eukaryotic cells, MnSOD (commonly named Sod2) is located in the mitochondrial matrix, and detoxifies superoxide radicals generated by the mitochondrial respiratory chain. Along with CuZnSOD (Sod1), which resides mainly in the cytosol, Sod2 plays a key role in antioxidative stress responses in cells (*21*) [a new Sod3 was recently identified in the cytosol of *Candida albicans* (Robin) Berkhout (*22*)].

Results using the 44 deletion mutants of *S. cerevisiae* (**Table 2**; **Figure 3**) indicated that the antioxidative stress response plays an important role in detoxification after fungi are exposed to vanillylacetone and *o*-coumaric acid. Because of these findings, the *sod2* deletion mutant (*sod2*Δ) of *S. cerevisiae* was selected for complementation analysis using the *sodA* orthologue of *A. flavus*. Complementation would be demonstrated if *sodA* relieved sensitivity of the *S. cerevisiae sod2*Δ strain to vanillylacetone, lowering the mortality from oxidative stress. If complementation does occur, then *sodA* should serve as a promising gene target for controlling *A. flavus* using natural compounds to cause oxidative stress. As mentioned above, there are at least seven target sites in respiratory machinery for fungal control, and Sod2 can be a good target for this purpose (see below). We have demonstrated functional complementation of the *A. flavus sodA* gene in the *S. cerevisiae sod2*Δ strain in relieving H_2O_2-induced oxidative stress (*13*).

Strobilurin fungicides are derived from strobilurin A, a secondary metabolite produced by the basidiomycete *Strobilurus tenacellus* (Pers. ex Fr.) Singer (*23*). The molecular target for strobilurin-related fungicides, such as azoxystrobin or kresoxim-methyl, is the respiratory *bc1* complex (complex III; ubiquinol−cytochrome *c* oxidoreductase, EC 1.10.2.2). These fungicides specifically bind to the Q_P (Q_O) center of cytochrome *b* (*23*−*26*) and inhibit the functioning of the respiratory chain. This inhibition eventually leads to cellular oxidative stress caused by abnormal release of electrons from the respiratory chain.

As shown in **Figure 4**, the *S. cerevisiae sod2*Δ strain with an empty pYES vector is more sensitive to the combined treatment of vanillylacetone/kresoxim-methyl and also to vanillylacetone/azoxystrobin (data not shown) than the wild-type control. However, the *S. cerevisiae sod2*Δ strain with *sodA* is 1000 times less sensitive to the combined vanillylacetone/fungicide exposure, with cell growth in the complemented *sod2*Δ strain almost equivalent to that of the wild-type having an empty pYES2 vector. Neither vanillylacetone nor the fungicides affected yeast cell growth when applied individually at concentrations chosen for the complementation analysis. The fact that the combination of vanillylacetone and fungicide inhibited cell growth, and *sodA* alleviated toxicity in the *sod2*Δ strain, indicates oxidative stress is the major cause of toxicity triggered by vanillylacetone (and *o*-coumaric acid). Also, the successful complementation clearly shows genes involved in antioxidative stress play important roles for fungal responses to phenolic compounds.

As in yeast bioassays, treatment of *A. flavus* with combined vanillylacetone and azoxystrobin or kresoxim-methyl showed greater toxicity than separate treatments by these compounds. Combined treatments should affect both the mitochondrial respiratory chain and the activity of SodA. By using combined treatment of vanillylacetone and fungicide, SodA must contend with oxidative stress caused by electrons released from the respiratory chain (due to the treatment of azoxystrobin or kresoxim-methyl) and detoxify vanillylacetone. Thus, the natural activity level of this enzyme is overwhelmed under this synergistic treatment.

Effects of Vanillylacetone on Aflatoxin Production. Since vanillylacetone inhibited growth of *A. flavus*, its effect on aflatoxin biosynthesis was also examined. Cellular sensitivity to this phenolic compound was dramatically elevated as the

Table 2. Responses of Wild-Type and Various Deletion Mutants of *S. cerevisiae* to Vanillylacetone and *o*-Coumaric Acid[a]

gene	function	GenBank accession GI (yeast)	vanillylacetone (15 mM)	*o*-coumaric acid (5 mM)
wild-type			5	5
	A. Gene Regulation			
569 (*yap1*Δ)	*jun*-like transcription factor	854483	5	5
7117 (*msn2*Δ)	transcription factor (zinc finger)	695717	5	5
4911 (*msn4*Δ)	transcription factor	486083	5	5
6957 (*hot1*Δ)	transcription factor, RNA polymerase II	854442	5	5
2045 (*sko1*Δ)	transcription factor, RNA polymerase II	1302140	5	5
1983 (*ure2*Δ)	glutathione transferase transporter regulator	1302253	5	2
	B. Transport			
3143 (*flr1*Δ)	multidrug transporter	536200	5	4
5933 (*yor1*Δ)	ABC transporter	1323513	5	4.5
2409 (*pdr5*Δ)	multidrug transporter		4	5
4969 (*vph2*Δ)	assembly of vacuolar H(+)ATPase	486199	3	1.5
	C. Signal Transduction			
6116 (*sho1*Δ)	transmembrane osmosensor	603357	5	5
22306 (*sln1*Δ)	histidine kinase osmosensor	6322044	5	5
3439 (*ste50*Δ)	protein kinase regulator	5332	5	4.5
956 (*ste20*Δ)	protein Ser/Thr kinase	508679	5	3
23932 (*ypd1*Δ)	transferase activity	1431398	5	5
1561 (*ssk1*Δ)	two-component response regulator activity	1360296	4	4
2464 (*ptp2*Δ)	protein tyrosine phosphatase	1420487	5	5
215 (*ptp3*Δ)	protein tyrosine phosphatase	603312	4.5	4.5
2724 (*hog1*Δ)	mitogen-activated protein kinase (MAPK)	1360508	4	4
7101 (*hog4*Δ)	MAPK kinase (MAPKK)	1008328	3	3
7195 (*ssk22*Δ)	MAPKK kinase (MAPKKK)	1907212	5	4
5406 (*ssk2*Δ)	MAPKKK	1302527	5	5
5271 (*ste11*Δ)	MAPKKK	4554	5	4
	D. Antioxidation			
4718 (*ctt1*Δ)	catalase T	1323129	5	5
3615 (*cta1*Δ)	catalase A	1136211	5	4.5
2737 (*glr1*Δ)	glutathione oxidoreductase	1151235	5	5
829 (*osr1*Δ)	glutathione metabolism	736309	5	5
24190 (*trr1*Δ)	thioredoxin reductase	849175	5	5
1934 (*trr2*Δ)	thioredoxin reductase	529123	5	5
545 (*tsa1*Δ)	thioredoxin peroxidase	575691	5	5
6681 (*grx1*Δ)	glutaredoxin	5328	5	4
4347 (*grx2*Δ)	glutaredoxin	927781	5	4
2654 (*trx1*Δ)	thioredoxin	1360373	5	5
4839 (*trx2*Δ)	thioredoxin	1323375	5	5
7097 (*gsh1*Δ)	γ-glutamyl cysteine synthetase	1008282	5	5
1740 (*gsh2*Δ)	glutathione synthetase	1419854	5	5
6913 (*sod1*Δ)	copper zinc superoxide dismutase	1015812	1	2.5
6605 (*sod2*Δ)	manganese superoxide dismutase	500704	4	5
2720 (*ahp1*Δ)	alkyl hydroperoxide reductase	1360500	5	5
	E. DNA Damage Control			
4530 (*rad54*Δ)	DNA-dependent ATPase	1322760	5	5
775 (*sgs1*Δ)	ATP-dependent DNA helicase	349194	5	5
	F. Energy Metabolism			
25391 (*acc1*Δ)	acetyl CoA carboxylase	1302498	5	5
3718 (*gpd1*Δ)	glycerol-3-phosphate dehydrogenase	1430995	5	3.5
199 (*hor2*Δ)	glycerol-1-phosphatase	603298	5	4

[a] Responses are represented as a logarithmic number (e.g., 1 indicates a 10000-fold decline in cell growth relative to 5, etc.; 6 when the strains were untreated). Yeast strains are the wild-type and deletion mutants lacking various genes associated with signal transduction and stress responses. Examples of bioassays used to construct this table are depicted in **Figure 3**. Note that the *sod2*Δ strain was further used for vanillylacetone/strobilurin antifungal and functional complementation assays (**Figure 4**).

concentration increased. As shown in **Table 3**, vanillylacetone (5, 10, 15, 20, and 25 mM) inhibited aflatoxin biosynthesis in addition to fungal growth (**Table 1**). However, while there was only a negligible decline in the diameter of the fungal mat between the controls and the 5, 10, and 15 mM treatments of vanillylacetone, there was more than a 10-fold decline in aflatoxin production. Even at 5 mM, aflatoxin production was reduced by ~82%, and almost complete inhibition of aflatoxin production was reached at 20 mM. Therefore, we concluded that vanillylacetone is effective for preventing both fungal cell growth and aflatoxin biosynthesis.

Identifying fungal-specific antimicrobials is a much needed but laborious task. Devising a high-throughput bioassay could greatly facilitate identification of antifungal compounds. Yeast has served as a useful tool for screening therapeutic compounds and genes associated with a number of human diseases. Modes of action for therapeutic compounds have been discovered using genome-wide screening of yeast heterozygous deletion strains (*16*, *27*, *28*). Systematic screening for human disease genes in yeast (*29*) was also described, where deletions of 466 genes impaired mitochondrial respiration. By using hypersensitive phenotypes of a yeast α-tubulin mutant to antimitotic agents,

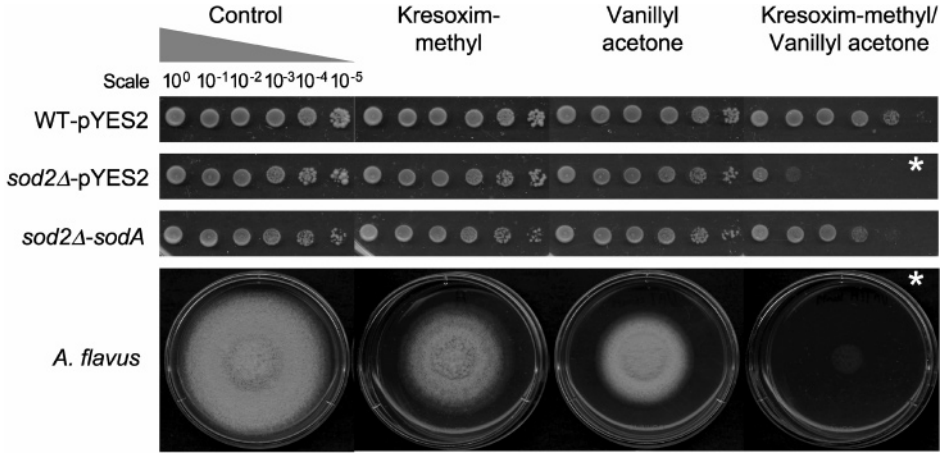

Figure 4. Synergistic effects of vanillylacetone and kresoxim-methyl on the growth of *A. flavus* and strains of *S. cerevisiae*. The scale shows serial 10-fold dilutions of approximately 10^6 cells of wild-type *S. cerevisiae* with an empty vector (WT-pYES2), *S. cerevisiae sod2* deletion mutant with an empty vector (*sod2*Δ-pYES2), and *S. cerevisiae sod2* deletion mutant with a vector containing *A. flavus sodA* (*sod2*Δ-*sodA*) spotted on plates with test compounds prior to a 10-day incubation at 30 °C.

Table 3. Effect of Vanillylacetone on Aflatoxin Production and Fungal Mat Diameter in *A. flavus* NRRL 3357 (Mean ± Standard Deviation)

vanillylacetone concn (mM)	aflatoxin production (μg/plate)	fungal mat diameter (cm)
0	210 ± 24	5.0 ± 0
5	37 ± 3.3	4.8 ± 0
10	24 ± 2.2	4.6 ± 0
15	16 ± 6.2	4.3 ± 0.23
20	0.03 ± 0.05	3.0 ± 0.16
25	0	1.6 ± 0.66

compounds specifically inhibiting fungal cell mitosis or polymerization of fungal tubulin were identified (*30*).

In this study, we applied a new approach for identifying antifungal natural compounds against aflatoxigenic *Aspergillus*. We demonstrated that *S. cerevisiae* is a useful model organism for use in a target-gene-based bioassay for fungal pathogen control. Our results also clearly show a synergistic inhibition of targeted molecular pathways using natural compounds (e.g., vanillylacetone) and strobilurin fungicides. This synergism appears to be an effective strategy for fungal pathogen control. Strobilurin fungicides are known to be vulnerable to development of fungal resistance. Potential mechanisms of resistance to these fungicides are (1) amino acid changes within interhelical regions of cytochrome *b*, (2) induction of alternative respiratory pathways following inhibition of mitochondrial respiratory electron transport, and (3) increased efflux of fungicides from fungal cells through the pleiotropic drug resistance (PDR) network (*24*−*26*). According to our observations, vanillylacetone and *o*-coumaric acid are also regulated through the *PDR5* network or other efflux pumps (e.g., *YOR1*, *FLR1*) (**Table 2**). Therefore, increased cellular toxicity to these antifungal compounds could be achieved by inhibiting efflux pumps as a supplemental strategy. Such cellular transport systems can be inhibited by application of vanadate, cyclosporin A, verapamil, etc. Use of azoxystrobin, however, could suppress activities of natural microorganisms that are antagonistic to a particular fungal pathogen and thereby reduce natural biological control (*31*). However, the compounds we used in our study are of very low toxicity to humans or other nontarget animals, and could serve as promising lead compounds.

Results showed that, like vanillin, veratraldehyde almost completely inhibited the growth of *A. flavus* at 10 mM. We speculate that the aldehyde group in these molecules is responsible for the higher antifungal activities. Another recent study (*32*) showed vanillin acts as a novel family of DNA-dependent protein kinase (DNA-PK) inhibitors in human cells, and nonaldehyde analogues did not inhibit DNA-PK. This difference in inhibition suggests that the aldehyde group plays an important role in inhibiting this enzyme or possibly other biological functions.

Results from using cinnamic acid derivatives showed that cinnamic acid (without an −OH group) has the highest antifungal activity, while caffeic acid (with two −OH groups) did not affect the growth of either *S. cerevisiae* or *A. flavus* significantly at any of the concentrations tested. Alternatively, all three coumaric acids (with one −OH group) showed moderate levels of antifungal activities. The results with the coumaric acids further suggested that the number of hydroxyl groups in phenolic molecules might affect the level of antifungal activity.

Recent studies on a number of fungal pathogens have demonstrated the effectiveness of natural phenolic compounds as antimicrobials or antimycotoxigenic agents. Beekrum et al. (*33*) reported naturally occurring phenols, such as vanillic and caffeic acids, inhibited the growth of fumonisin-producing *Fusarium verticillioides* (Saccardo) Nirenberg. Mycotoxin production by this organism was also reduced by vanillic, caffeic, and ferulic acids. Curir et al. (*34*) found a number of phenolics (e.g., benzoic acid derivatives) from cultivars of carnation were effective as antimicrobials against *Fusarium oxysporum*. Aziz et al. (*35*) reported that phenolic compounds, including vanillic and caffeic acids, effectively inhibited the growth of several fungi and bacteria. These studies demonstrate the potential for natural phenolic compounds to serve as antimicrobials. However, none of these studies clearly determined any cellular targets for their activities. Hence, our approach will be applicable for the future identification of antimicrobial natural compounds as well as their cellular targets.

Our high-throughput bioassay system not only identifies antimicrobial activity but also incorporates functional fungal genomics (i.e., functional identification of target genes in the pathogen). This approach can be used to exploit comparative genomics among related fungi such as *Aspergillus fumigatus* etc. Use of the model yeast *S. cerevisiae* enhances efforts to develop methods for target-gene-based fungal pathogen control.

SAFETY

Aflatoxins are classified as hepatotoxins and carcinogens and should be handled with appropriate precautions.

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