

ORIGINAL ARTICLE

Targeting antioxidative signal transduction and stress response system: control of pathogenic *Aspergillus* with phenolics that inhibit mitochondrial function

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antifungal, antioxidative stress, *Aspergillus*, mitochondrial superoxide dismutase, phenolics, *Saccharomyces cerevisiae*.

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Abstract

Aims: The aim of this study was to show whether antioxidative response systems are potentially useful molecular targets for control of *Aspergillus fumigatus* and *Aspergillus flavus*. Selected phenolic agents are used in target-gene-based bioassays to determine their impact on mitochondrial respiration.

Methods and Results: Vanillyl acetone, vanillic acid, vanillin, cinnamic acid, veratraldehyde, *m*-coumaric acid (phenolic agents to which *Saccharomyces cerevisiae sod2Δ* mutant showed sensitivity), carboxin (inhibits complex II of the mitochondrial respiratory chain), strobilurins/antimycin A (inhibits complex III of the mitochondrial respiratory chain) and fludioxonil/fenpiclonil [antifungals potentiated by mitogen-activated protein kinase (MAPK)] were examined in *A. fumigatus*, *A. flavus* and *S. cerevisiae*. Individual or combined application of phenolics with inhibitors of mitochondrial respiration showed some of the phenolics effectively inhibited fungal growth. Target-gene bioassays were performed using a *sakAΔ* (MAPK deletion) strain of *A. fumigatus* and a complementation analysis using the mitochondrial superoxide dismutase (Mn-SOD) gene (*sodA*) of *A. flavus* in the ortholog mutant, *sod2Δ*, of *S. cerevisiae*. The results demonstrated that mitochondrial antioxidative stress system plays important roles in fungal response to antifungal agents tested.

Conclusions: Antioxidative response systems of fungi can be an efficient molecular target of phenolics for pathogen control. Combined application of phenolics with inhibitors of mitochondrial respiration can effectively suppress the growth of fungi.

Significance and Impact of the Study: Natural compounds that do not pose any significant medical or environmental risks could serve as useful alternatives or additives to conventional antifungals. Identifying the antioxidative response systems in other pathogens could improve methods for fungal control.

Introduction

The filamentous fungi in genus *Aspergillus* are most notable as sources of highly debilitating human diseases, such as aspergillosis, and production of mycotoxins (Denning 1998; Campbell *et al.* 2003). *Aspergillus fumigatus* and *Aspergillus flavus* are ubiquitous opportunistic pathogens. They are capable of forming highly invasive infections, especially in immunocompromised individuals

or in those suffering chronic granulomatosis (Roilides *et al.* 1993; Marr *et al.* 2002; Anderson *et al.* 2003). *Aspergillus flavus* (and *Aspergillus parasiticus*) also produces 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 the economic value of affected crops (Campbell *et al.* 2003).

A number of antioxidative signal transduction and stress response systems of fungal pathogens have been implicated as virulence factors (Holdom *et al.* 1996; Hamilton and Holdom 1999). These include mitogen-activated protein kinase (MAPK), two-component histidine kinase (e.g. *fos-1*), superoxide dismutase (SOD), catalase and melanin biosynthesis, melanin being a potent free radical scavenging pigment. In *A. fumigatus*, Cu and Zn-SOD can trigger allergenic responses in infected individuals, but the main role of this protein is a mean of combating reactive oxygen radicals produced by the host defence system (Washburn *et al.* 1987). For example, patients with chronic granulomatous disease (CGD) are highly susceptible to invasive infections by *Aspergillus*. CGD patients are defective in the ability for phagocytic immune cells to cause an oxidative burst by lacking NADPH oxidase needed to generate superoxide, the precursor to the antimicrobial reactive oxygen species hydrogen peroxide (H₂O₂) (Dinauer 1993). From the perspective of the pathogen, antioxidative response system such as mitochondrial SOD (Mn-SOD) plays an important role in protecting the pathogen from reactive oxygen species.

Precise control of antioxidative enzyme activity and regulation of its expression are necessary for normal pathogen growth. Harris *et al.* (2003) showed that overproduction of Mn-SOD leads to dramatic Sir2p-independent shortening of the replicative lifespan in the model yeast cells. The failure for segregation of the mitochondrion from the mother to daughter cells during replication is the proposed mechanism of this defect. Therefore, proper regulation of antioxidative systems in fungi plays a critical role not only for protecting the pathogen from the host's defensive responses during infection but also for normal cell growth. Thus, targeting the destabilization of antioxidative stress systems should be an effective way for controlling fungal pathogens.

Recent studies on a number of fungal pathogens have demonstrated the effectiveness of natural compounds, such as derivatives of benzoic or cinnamic acid, as antifungals or antimycotoxigenic agents (Tawata *et al.* 1996; Florianowicz 1998; Beekrum *et al.* 2003). In a prior study, we found that combined application of vanillyl acetone and strobilurins disrupted mitochondrial respiration and was highly effective in controlling *A. flavus* (Kim *et al.* 2004b). The results indicated that Mn-SOD plays an important role for fungal tolerance to phenolic compounds and that targeting the mitochondrial antioxidative stress system is a promising approach to control pathogenic fungi. Moreover, the fundamental differences between human and pathogen mitochondrial respiratory chains are exploitable for development of pharmaceuticals for fungal control (Tudella *et al.* 2003).

In this study, we provide further evidence that Mn-SOD (SodA) of *A. flavus*, using a yeast model system, plays a protective role against phenolic agents and inhibitors of the mitochondrial respiratory chain. Targeting this gene, we show that certain phenolic agents can be useful sources of antifungals for the control of *A. fumigatus* and *A. flavus*. The role of the MAPK signal transduction for regulating antioxidative stress response system in *Aspergillus* with the treatment of the redox-active antifungal agents tested is also discussed.

Materials and methods

Micro-organisms and culture conditions

Aspergillus fumigatus AF293, wild type, and *A. fumigatus sakAA*, a MAPK deletion mutant (Xue *et al.* 2004), were grown at 37°C on potato dextrose agar (PDA) medium. *Aspergillus flavus* NRRL3357, wild type, was cultured at 28°C on PDA medium. *Saccharomyces cerevisiae* wild-type BY4741 (*mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants were obtained from Invitrogen (Carlsbad, CA, USA). Yeast strains (without plasmids) were maintained on rich medium (YPD: 1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) at 30°C without light.

Chemicals and reagents

The fungicides fludioxonil, fenpiclonil, carboxin, strobilurin (azoxystrobin, kresoxim-methyl) and antimycinA, and phenolic agents vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), vanillyl acetone (3-methoxy-4-hydroxybenzyl acetone), cinnamic acid (*trans*-3-phenylacrylic acid), *m*-coumaric acid (3-hydroxycinnamic acid) and veratraldehyde (3,4-dimethoxybenzaldehyde) were purchased from Sigma Co. (St Louis, MO, USA). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute amount <20 μl ml⁻¹ media) before use.

Aspergillus bioassays

Aspergillus flavus NRRL3357, *A. fumigatus* AF293 and *A. fumigatus sakAA* (*c.* 200 spores) were diluted in phosphate-buffered saline solution and spotted on the centre of PDA plates containing test phenolic agents and/or antifungal compounds. Growth was observed for 7 days. For testing the effects of the combined treatment of test compounds, phenolic agents were added to the growth medium together with carboxin or strobilurin. Colony growth was measured based on per cent radial growth compared with control colonies grown on PDA plates receiving only DMSO.

Functional expression of *A. flavus sodA* in *S. cerevisiae*: yeast dilution bioassay

Yeast dilution bioassays were carried out as described previously (Kim *et al.* 2005). The *S. cerevisiae sod2Δ* strain with pYES2 empty vector (*sod2Δ* + pYES2; negative control), wild-type strain with pYES2 empty vector (WT + pYES2; positive control), wild-type strain with pYES2 vector containing PCR-amplified *sodA* (WT + *sodA*; SodA overexpression) and *sod2Δ* strain with pYES2 vector containing PCR-amplified *sodA* (*sod2Δ* + *sodA*) were cultured in raffinose medium (0.67% yeast nitrogen base without amino acids, 35 $\mu\text{mol l}^{-1}$ raffinose, amino acids 200 $\mu\text{mol l}^{-1}$), 30°C, overnight. Around 1×10^6 cells were serially diluted with raffinose liquid medium and spotted adjacently on SGAL (0.67% yeast nitrogen base without amino acids, 110 $\mu\text{mol l}^{-1}$ galactose with appropriate supplements: 180 $\mu\text{mol l}^{-1}$ uracil, 200 $\mu\text{mol l}^{-1}$ amino acids) or SG (0.67% yeast nitrogen base without amino acids, 110 $\mu\text{mol l}^{-1}$ glucose with appropriate supplements: 180 $\mu\text{mol l}^{-1}$ uracil, 200 $\mu\text{mol l}^{-1}$ amino acids) agar plates. This provided six samples of yeast cells from approximately 10^6 to 10 cells. Functional expression of *sodA* was achieved under the yeast *GAL1* promoter (30°C, 10 days). Functionality of *sodA* was assessed on the basis of yeast cell growth in the presence of phenolic agents and/or strobilurin/antimycin A. If cell growth was similar to the positive control or better than the negative control, *sodA* was considered to functionally complement the *S. cerevisiae sod2Δ* strain, and, by definition, relieved sensitivity to the tested compounds.

Results

Overexpression of *A. flavus* Mn-SOD gene (*sodA*) inhibited the growth of *S. cerevisiae*

Our previous study showed that *A. flavus sodA* functionally complemented the yeast *sod2Δ* mutant, enabling this strain to recover from H_2O_2 -mediated oxidative stress (Kim *et al.* 2005). However, in this study, we found that when the *A. flavus sodA* gene was overexpressed in the *S. cerevisiae* wild type (WT + *sodA*) in the absence of oxidative stress, yeast cell growth on SGAL medium was inhibited relative to wild type with empty vector (WT + pYES2) on the same medium (Fig. 1). The '*sodA*' transfectant (WT + *sodA*) grown on SGAL also showed significantly lower growth at lower dilutions, especially during the first 3–4 days, than the same transfectant grown on SG medium. Colony size was also negatively affected, indicating that overexpressed SodA may result in loss of redox homeostasis in these cells. Overexpression of Mn-SOD is known to shorten the replicative lifespan of yeast cells (MacLean *et al.* 2001, 2003). Overexpression of human Mn-SOD in mouse NIH/3T3 cells alters the mitochondrial redox state through rapid increase in levels of reactive oxygen species (Kim *et al.* 2004a). Although we do not rule out the possibility that producing more Mn-SOD protein uses up more nutrients that would otherwise be utilized for cellular growth, strict regulation of the levels of this protein in the mitochondria is necessary for normal cellular function. These results further suggest that destabilizing SOD activity could be a viable molecular target/strategy for control of fungal pathogens.

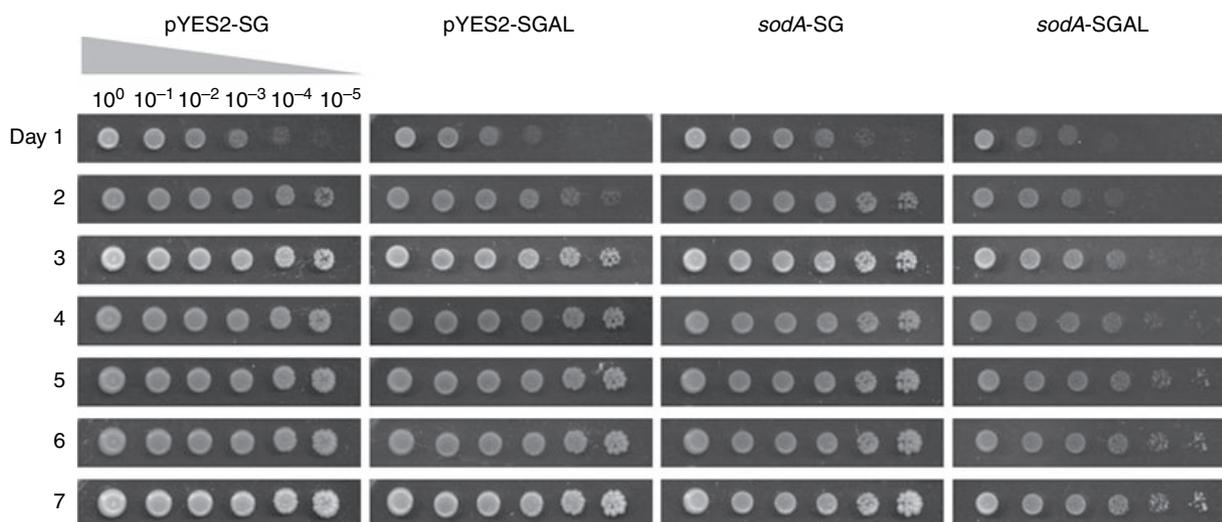


Figure 1 Growth of *Saccharomyces cerevisiae* wild-type (WT) cells with empty vector (pYES2) or transformed with *Aspergillus flavus sodA* on SG and SGAL media over a 7-day period using a yeast cell dilution bioassay. Legend signifies the position of yeast colonies developing from tenfold serial dilutions of yeast cells from approximately 1 million (10^0) to 10 (10^{-5}) cells.

Targeting mitochondrial SOD using phenolic agents and inhibitors of the respiratory chain

Targeting the mitochondrial antioxidative stress system with phenolic compounds (e.g. vanillyl acetone) in combination with strobilurins had a synergistic antifungal effect against *S. cerevisiae* and the filamentous fungus *A. flavus* (Kim *et al.* 2004b). The fact that *sodA* alleviated sensitivity of the *sod2Δ* strain indicated oxidative stress is the major cause of toxicity triggered by vanillyl acetone. Our recent study using *S. cerevisiae tsa1Δ* (deletion mutant for the thioredoxin reductase gene) showed vanillyl acetone inhibited normal mitochondrial respiration (Kim *et al.* 2006).

In this study, we found cinnamic acid, *m*-coumaric acid, veratraldehyde, vanillin and vanillic acid also inhibited

the growth of the *sod2Δ* mutant (10^2 – 10^5 times of growth inhibition compared with nontreated control). All phenolic agents exerted positive interaction with the inhibitors of complex III in the mitochondrial respiratory chain (i.e. strobilurins and antimycin A) for inhibiting the growth of *S. cerevisiae* (Table 1, Fig. 2). The exemplary bioassay described in Fig. 2 shows that absence of Mn-SOD in *sod2Δ*-pYES2 results in significant reduction of cell growth with treatment of cinnamic acid compared with other yeast strains. As in the test with vanillyl acetone (Kim *et al.* 2004b), the *A. flavus sodA* gene effectively recovered *sod2Δ* yeast cells from the toxic effect of these phenolic agents. Similar to that observed in yeast cells, growth inhibition of *A. flavus* was enhanced by the use of these compounds in combination with fungicides

Phenolic	Strains	w/o Antimycin A	w/ Antimycin A (10 µg ml ⁻¹)	w/o Strobilurin	w/ Strobilurin (200 µmol l ⁻¹)
DMSO only	A	6	6	6	5.5
	B	6	5	6	5
	C	6	2.5	6	4
	D	6	5	6	4
Vanillin 1 mmol l ⁻¹ (3 mmol l ⁻¹)	A	6	4.5	6	3.5
	B	6	4	5.5	3
	C	5	1	5	2
	D	6	4	6	3
Vanillyl acetone 10 mmol l ⁻¹	A	6	0	6	1
	B	5.5	0	6	1
	C	3	0	4	0
	D	6	0	6	0
Vanillic acid† 3 mmol l ⁻¹	A	6	0	6	0
	B	6	0	5.5	0
	C	6	0	5.5	0
	D	6	0	5.5	0
Veratraldehyde 5 mmol l ⁻¹ (10 mmol l ⁻¹)	A	6	2	6	1.5
	B	5	2	5.5	1.5
	C	4	0	4	0
	D	5	2	5	0
Cinnamic acid 0.1 mmol l ⁻¹ (0.3 mmol l ⁻¹)	A	6	3	6	2.5
	B	6	3	5.5	2.5
	C	3	0	3	0
	D	6	3	5	2.5
<i>m</i> -Coumaric acid 5 mmol l ⁻¹	A	6	0	4	0
	B	5	0	3	0
	C	4	0	1.5	0
	D	5.5	0	3	0

Table 1 Responses of yeast strains to antifungal compounds (antimycin A, strobilurin and/or phenolic agents) using the high-throughput yeast dilution bioassay (Fig. 1)*

A, wild type (WT) + pYES2; B, WT + *sodA*; C, *sod2Δ* + pYES2; D, *sod2Δ* + *sodA*. Concentrations in parenthesis: the concentration at which no yeast growth was observed with combined application of test compounds. Antimycin A (Sigma catalog#A8674) used was a mixture of antimycins; hence, the concentration was noted as µg ml⁻¹.

*Numerical scoring for the sensitivity using the yeast dilution bioassay is as follows: 6 – colonies are visible in all dilutions, 0 – no colonies are visible in any dilution, 1 – only the undiluted colony is visible, 2 – the undiluted and tenfold diluted colonies are visible, etc. Values represent the mean of two replicates.

†The growth of *Saccharomyces cerevisiae sod2Δ* + pYES2 was slightly better than *sod2Δ* without plasmid.

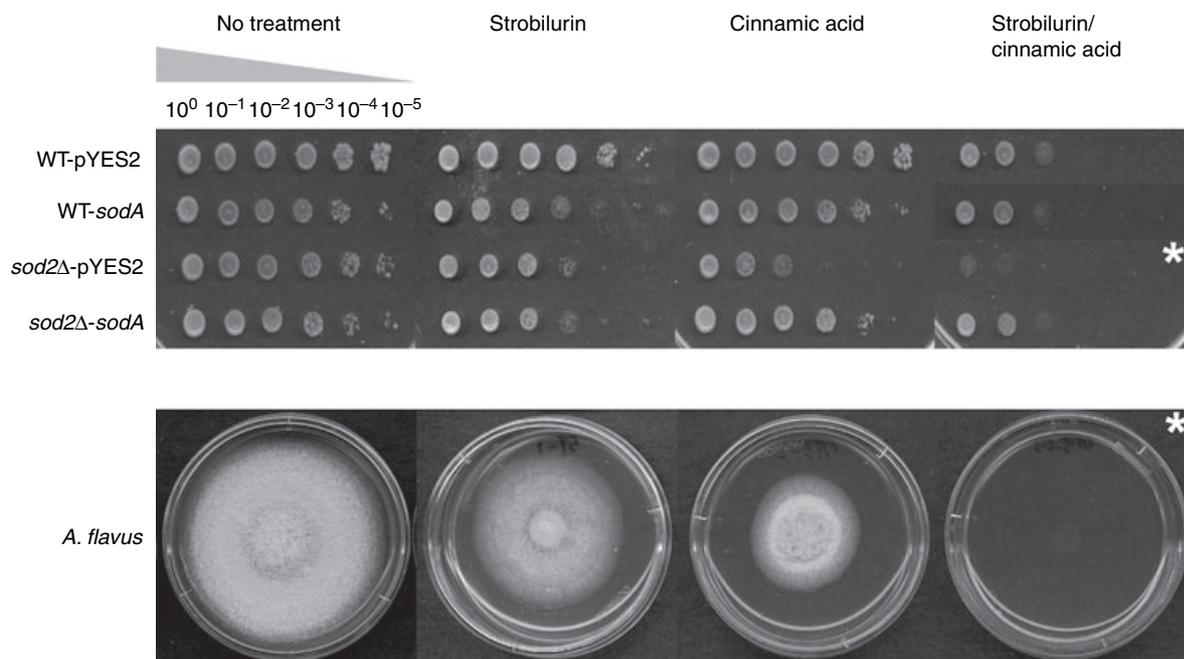


Figure 2 Bioassays showing effects of cinnamic acid and/or strobilurin on the growth of *Aspergillus flavus* and *Saccharomyces cerevisiae*. Yeast cell dilution bioassay (see Fig. 1) includes wild type with empty vector (WT-pYES2), wild type with *sodA* from *A. flavus* (WT-*sodA*), yeast *sod2Δ* mutant with empty vector (*sod2Δ*-pYES2) and *sod2Δ* mutant transformed with *sodA* (*sod2Δ*-*sodA*).

(Table 2; see also Fig. 2 for the exemplary bioassay using cinnamic acid and strobilurin). These parallel results between yeast and *A. flavus* supported the usefulness of the high-throughput yeast bioassay for screening antifungal compounds to find potential gene targets viable for control of fungal pathogens.

Inhibitory effect of phenolic agents against *A. fumigatus*: role of *A. fumigatus* MAPK system for tolerance to vanillyl acetone and fludioxonil

We identified a number of orthologous genes in the antioxidative signal transduction and stress response systems between *A. flavus* and *A. fumigatus* based on BLAST searches of available EST and genomic sequence databases. The orthologs shared a high level of identity/similarity at the amino acid level (Kim *et al.* 2004c). For example, Mn-SOD of both organisms showed 51% of identity and 64% of similarity, while MAPK showed 84% of identity and 91% of similarity (*A. fumigatus saka*; *A. flavus* TC4614). On the basis of the identified presence for homologous gene activity, we reasoned the responses of *A. fumigatus* and *A. flavus* to phenolic agents would likely be similar.

Six phenolic agents used in the *A. flavus* bioassays (Table 2) were tested against *A. fumigatus* AF293 and *sakAΔ* strains. We initially applied the same concentra-

Table 2 Enhanced inhibition of the growth of *Aspergillus flavus* by combined application of strobilurin or antimycin A with six different phenolic agents*

	Conc. (mmol l ⁻¹)	None	Strobilurin (200 μmol l ⁻¹)	Antimycin A (1.6 μg ml ⁻¹)
Control (DMSO only)	–	100	75	82
Cinnamic acid	0.5	98	78	82
	1	98	62 ± 7	82
	5	58 ± 9	0	51
<i>m</i> -Coumaric acid	5	96	44	69
	8	91	40	64
	10	82	36	62
Veratraldehyde	1	100	64	84
	5	62	0	29 ± 11
Vanillin	1	100	64	80
	3	82	4	56
	5	31 ± 7	0	7 ± 11
Vanillic acid	5	100	51	64
	10	96	38	56
	15	87	27	38 ± 13
Vanillyl acetone	10	56	16	36 ± 7
	15	40	13 ± 22	13 ± 11
	20	24	0	0

Antimycin A (Sigma catalog#A8674) used was a mixture of antimycins; hence, the concentration was noted as μg ml⁻¹.

*Responses for growth of fungi as a percentage of radial growth of the fungal mat of treated compared with control (DMSO only). Values represent the mean of three replicates, and standard deviations of all measurements were <3% except where noted.

tions of the phenolics as were used in *A. flavus* assays (i.e. vanillyl acetone 15 mmol l⁻¹, vanillic acid 15 mmol l⁻¹, vanillin 3 mmol l⁻¹, cinnamic acid 5 mmol l⁻¹, *m*-coumaric acid 10 mmol l⁻¹, veratraldehyde 5 mmol l⁻¹). At these concentration levels, the growth inhibition of both *A. fumigatus* strains was similar to that in *A. flavus* with the exception of cinnamic acid (data not shown). While 3 mmol l⁻¹ cinnamic acid inhibited *A. flavus* growth by

Table 3 Antifungal activity of vanillyl acetone against the wild type, AF293 and *sakAΔ* strains of *Aspergillus fumigatus**

	Vanillyl acetone (mmol l ⁻¹)							
	0	0.5	1	5	10	15	20	25
AF293	100	100	100	89	45	21	16	0
<i>sakAΔ</i>	100	100	100	86	44	~0	0	0

*Responses for growth of fungi as a percentage of radial growth of the fungal mat of treated compared with control (DMSO only). Values represent the mean of two replicates, and standard deviations of all measurements were <3%.

Bold type indicates that antifungal activity of vanillyl acetone is higher in *sakAΔ* than in AF293.

approximately 40%, germination of *A. fumigatus* AF293 and *sakAΔ* was completely inhibited at 2 mmol l⁻¹ (data not shown). This higher tolerance by *A. flavus* indicates that it probably possesses a more efficient system(s) for detoxifying cinnamic acid than *A. fumigatus*. Although the sensitivity of the *A. fumigatus sakAΔ* strain to the tested compounds was similar to that of the wild-type strain (AF293), there were differences in cell growth between the strains when treated with vanillyl acetone. While colony growth was almost identical between the two strains at lower concentrations, the AF293 strain showed higher tolerance to vanillyl acetone than the *sakAΔ* strain at 15–20 mmol l⁻¹ levels (Table 3, Fig. 3). Complete inhibition of germination for both the strains was observed at 25 mmol l⁻¹. This difference in sensitivities between the strains to vanillyl acetone indicates a possible role for *sakA* in regulation of the antioxidative stress response system in *A. fumigatus*, perhaps involving mitochondrial function/respiration. This stems from our observation that normal mitochondrial function/respiration of the *sod2Δ* mutant of *S. cerevisiae* was recovered from treatment with vanillyl acetone when complemented by *A. flavus* Mn-SOD (SodA).

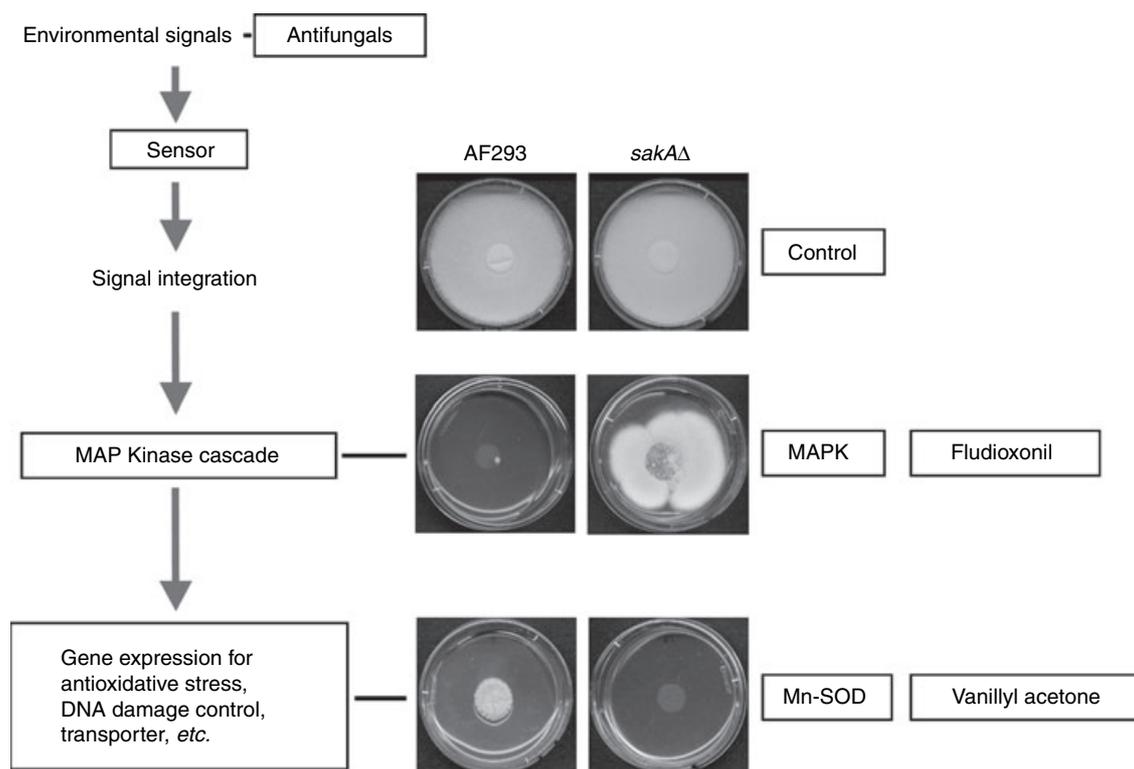


Figure 3 Role of fungal antioxidative signalling pathway in response to antifungal compounds. On the right are bioassays of strains of *Aspergillus fumigatus* wild type (AF293) and *sakAΔ* showing differences in phenotypic response to treatment with the fungicide fludioxonil and the phenolic vanillyl acetone.

To further understand the role of *A. fumigatus* MAPK (SakA) in response to antifungal compounds, we tested the effects of fludioxonil on the wild-type and *sakAΔ* strains. In certain fungal pathogens, the fungicidal effect of fludioxonil (or fenpiclonil) is activated by the MAPK signalling pathway (Kojima *et al.* 2004). These compounds are known to induce osmotic stress response in fungal cells. Interestingly, the *sakAΔ* mutant was also tolerant to treatment with fludioxonil at 50 $\mu\text{mol l}^{-1}$ (i.e. escaping the toxicity), while the wild-type strain (AF293) was hypersensitive (Fig. 3). The fact that toxicity of fludioxonil was not triggered in the *sakAΔ* mutant but that it was more sensitive to vanillyl acetone than the wild type indicates *sakA* of *A. fumigatus* plays an important role in the response mechanism to sensing/detoxifying antifungal compounds such as fludioxonil. This result also indicates that the *A. fumigatus* Mn-SOD might be regulated by SakA activity. The signalling pathway initiated by *sakA* likely leads to the activation of numerous downstream genes involved in antioxidation, for example, DNA damage control responses.

Positive interaction of test compounds for inhibiting the growth of *A. fumigatus* AF293 and *sakAΔ* strains

We observed the enhanced antifungal effect of six phenolic agents on the growth of AF293 and *sakAΔ* strains of *A. fumigatus* when combined with either carboxin or strobilurin. Using *Aspergillus niger*, *A. flavus* and *Penicillium expansum*, we recently showed that inhibition of complex III of the mitochondrial respiration system with strobilurin was more effective for the control of fungal pathogens than targeting complex II (unpublished data). To confirm this effect on *A. fumigatus*, we treated the *A. fumigatus* strains (AF293 and *sakAΔ*) with equimolar concentrations of carboxin or strobilurin (100 $\mu\text{mol l}^{-1}$) in the presence of vanillyl acetone (5 or 10 mmol l^{-1}). Vanillyl acetone at 5 mmol l^{-1} effectively inhibited (>75%) the growth of both the strains of *A. fumigatus* when combined with strobilurin, with complete inhibition of germination achieved when vanillyl acetone was applied at 10 mmol l^{-1} with strobilurin (Table 4). As with *A. flavus*, carboxin was far less effective than strobilurin for the control of *A. fumigatus*, further demonstrating complex III of the mitochondrial respiratory chain is a better target than complex II for fungal control. Other phenolic agents tested (i.e. vanillic acid, vanillin, cinnamic acid, *m*-coumaric acid and veratraldehyde) also showed synergistic inhibition of cell growth when applied with either strobilurin or carboxin, again greater inhibition was observed when applied with strobilurin (data not shown).

Table 4 Enhanced inhibition of the growth of *Aspergillus fumigatus* using vanillyl acetone in combination with the fungicides carboxin or strobilurin*

Inhibitors (100 $\mu\text{mol l}^{-1}$)	Vanillyl acetone (mmol l^{-1})					
	0		5		10	
	AF293	<i>sakAΔ</i>	AF293	<i>sakAΔ</i>	AF293	<i>sakAΔ</i>
No inhibitors	100	100	95	90	52	46
Carboxin	88	81 ± 9	81	79	44	42
Strobilurin	47	47	24	16	0	0

*Responses for growth of fungi is a percentage of radial growth of the fungal mat of treated compared with control (DMSO only). Values represent the mean of two replicates, and standard deviations of all measurements were <3% except where noted.

Discussion

In the present study, we show the results of bioassays wherein the antioxidative signal transduction and stress response pathways of *A. fumigatus* and *A. flavus* are targeted to achieve fungal control. Much of the predictive knowledge for demonstrating this was initially achieved by studies using *S. cerevisiae*. Strains of *S. cerevisiae* are available whose individual open reading frames are functionally deleted (c. 6000 open reading frames; Winzeler *et al.* 1999). Such strains can be used for determining mode of action and/or identification of target genes for antifungal agents (Parsons *et al.* 2004; Tucker and Fields 2004). Hence, to initially verify the utility of targeting the signal transduction and antioxidation response systems of the aspergilli in this study, we used *S. cerevisiae*, as a model system in order to exploit the availability of these mutant strains. Moreover, *A. flavus* and *S. cerevisiae* responded similarly to antifungal effects of a number of structurally related phenolic compounds in our previously designed high-throughput bioassays (Kim *et al.* 2004b), further demonstrating the usefulness of *S. cerevisiae* as a screening tool for antifungal compounds. Finally, we also showed that many genes in antioxidative signal transduction and stress response system of yeast are orthologs of genes in fungal pathogens. Structural homology of several genes in this system in *S. cerevisiae*, the filamentous fungi *A. flavus* and *A. fumigatus* has been confirmed (Kim *et al.* 2004c, 2005).

We identified six phenolic agents that can inhibit the growth of the *S. cerevisiae* *sod2Δ* mutant and pathogenic species of *Aspergillus*. Combined treatments of these phenolic agents and commercially available fungicides that are inhibitors of the mitochondrial respiratory chain should affect both mitochondrial respiration and activity of Mn-SOD. In such combined applications, Mn-SOD must contend with oxidative stress caused by both the release of electrons from the respiratory chain in the form

of reactive oxygen species (because of the inhibition of respiratory chain by the fungicides) and by the presence of phenolic agents. The natural activity level of this enzyme may not be sufficient for detoxifying the oxidative stress caused by such a combined treatment. Functional expression of *sodA* from *A. flavus* in the *S. cerevisiae* *sod2Δ* mutant successfully recovered the cells from the fungitoxic effect of these phenolics, proving this mitochondrial antioxidative enzyme plays an important role in mitigating oxidative stress to achieve normal fungal growth.

Many defensive phenolic compounds are produced or released by plants during fungal infection (Kobayashi *et al.* 1994; Yao *et al.* 1995; von Ropenack *et al.* 1998). These compounds must be detoxified by infecting fungi in order to achieve pathogenesis, thus, illustrating how phenolic compounds can be useful sources of antifungal agents. We surmise that disruption of cellular redox homeostasis using phenolics may inhibit fungal growth. Because of their structural characteristics, phenolics can act as antioxidants under oxidative stress conditions. However, if phenolics are applied to cells under normal growth condition (i.e. without oxidative stress), the redox-active phenolic compounds can interfere with the cellular redox homeostasis, resulting in growth inhibition from oxidative stress. This effect of phenolic compounds on inducing cellular oxidative stress has been recently documented (Shvedova *et al.* 2000). The antifungal activities of polyphenols (e.g. catechin and epigallocatechin gallate) with regard to inducing cellular oxidative stress responses has also been shown to occur in *S. cerevisiae* (Izawa and Inoue 2004; Takatsume *et al.* 2005). Yap1 is a redox-responsive transcription factor of yeast that normally resides in the cytosol but is predominantly translocated into the nucleus under conditions of oxidative stress. When *S. cerevisiae* was treated with these polyphenols, green-fluorescent-protein-tagged Yap1 was concentrated into the nucleus of yeast cells, thus indicating polyphenols cause oxidative stress in *S. cerevisiae*.

The *sakA* gene of *A. fumigatus* was shown to be responsive to osmotic stress and to be involved in nutritional sensing (Xue *et al.* 2004). SakA regulates conidial germination in response to nitrogen levels and is activated upon depletion of either carbon or nitrogen during vegetative growth. In our study, responses of *A. fumigatus* *sakAΔ* to either fludioxonil or vanillyl acetone demonstrated that this MAPK might also play an important role for the response to certain antifungals. Thus, the *A. fumigatus* *sakA* gene should be a good molecular target for the control of this pathogen. This targeting strategy should also hold true for the ortholog of this gene in *A. flavus* (*A. flavus* MAPK showed high similarity to SakA at the amino acid level). Our high-throughput bioassays also identified

several other yeast mutants, such as those defective in signal transduction [*hog1Δ* (MAPK), *hog4Δ* (MAPK kinase, MAPKK), *ssk2Δ* (MAPKK kinase, MAPKKK)] and certain other cellular processes [*ure2Δ* (glutathione transferase-transporter regulator), *pdr5Δ* (multidrug transporter), *vph2Δ* (assembly of vacuolar H(+)ATPase)], which were hypersensitive (>100 times) to fenpiclonil/fludioxonil compared with the wild type (data not shown). Targeting these other gene systems with natural products may result in synergism of the activity of the fungicides for the control of pathogenic fungi.

In summary, we showed antioxidative signal transduction (e.g. MAPK) or stress response systems (e.g. MnSOD) of fungi could be efficient molecular targets of phenolic agents for pathogen control. Combined application of redox-active phenolic agents with inhibitors of the mitochondrial respiratory chain, many of which are already available as commercial fungicides, can effectively suppress the growth of *A. fumigatus* and *A. flavus*.

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