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Response of *Saccharomyces cerevisiae* to a monoterpene: evaluation of antifungal potential by DNA microarray analysis

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Plant-derived essential oils with monoterpenoids have been used as antifungal drugs since ancient times, but the mode of action of these natural hydrocarbons at the molecular level is not understood. In order to understand the mechanisms of toxicity of α-terpinene (a cyclic monoterpene), a culture of *Saccharomyces cerevisiae* was exposed to 0.02% α-terpinene for 2 h and transcript profiles were obtained using yeast DNA arrays. These profiles, when compared with transcript profiles of untreated cultures, revealed that the expression of 793 genes was affected. For 435 genes, mRNA levels in treated cells compared with control cells differed by more than two-fold, whereas for 358 genes, it was <0.5-fold. Northern blots were performed for selected genes to verify the microarray results. Functional analysis of the up-regulated genes indicates that, similar to commonly used antifungal drugs, α-terpinene exposure affected genes involved in ergosterol biosynthesis and sterol uptake. In addition, transcriptional induction of genes related to lipid metabolism, cell wall structure and function, detoxification and cellular transport was observed in response to terpinene toxicity. Notably, the functions of 192 up-regulated genes are still unknown, but their characterization will probably shed light on the mechanisms of drug resistance and sensitivity. Taken together, this study showed that α-terpinene has strong antifungal activities and its modes of action resemble those of presently used antifungal drugs.

Keywords: essential oils, ergosterol, stress response, toxicity, genomic expression

Introduction

α-Terpinene (1-isopropyl-4-methyl-1,3-cyclohexadiene) is a monocyclic monoterpene with the pleasant odour of lemons. It is universally present in the essential oils of a large variety of useful aromatic plants, such as cardamom (*Elletaria cardamomum*), marjoram (*Origanum majorana*) and coriander (*Coriandrum sativum*), as well as tea tree oil (from *Melaleuca alternifolia*) and palmarosa oil (from *Cymbopogon martini*).[1–3] Plants and essential oils have been recognized as antimicrobial agents in folk medicine for centuries.[4,5] The antimicrobial properties of these compounds are attributable largely to the presence of monoterpenes, sesquiterpenes and related alcohols or other hydrocarbons.[6]

These plant-derived essential oils have been reported to show antimicrobial activity against a wide range of bacteria including antibiotic-resistant species.[5,7] In addition to bactericidal or bacteriostatic activities, monoterpenes are also used to treat fungal infections, in particular genital and oral candidiasis, dermatophytoses, etc.[2,8] All of these studies demonstrated that the activity of essential oils containing monoterpenes is both inhibitory and fungicidal. The antimicrobial activities and toxicity of monoterpenes (natural hydrocarbons) and other synthetic hydrocarbons have been well noted, but their modes of interaction with cells and the mechanism(s) of toxicity are largely unknown. Several studies concluded that, as lipophilic agents, they execute their action at the level of the membrane and membrane-embedded enzymes.[9,10] Most recently, it has been reported that complete inhibition of *Saccharomyces cerevisiae* after exposure to palmarosa oil occurred due to a change in the fatty acid composition of the yeast cell membrane.[3] Disruption of membrane integrity and the permeability barrier caused by tea tree oil was implicated as a mode of antimicrobial action against *Candida albicans* and different

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bacterial species.[11,12] However, the response of a living cell to these compounds at the molecular level is not yet known.

To maintain the internal milieu at optimal conditions, a cell has to employ a specific genomic expression programme, in which a set of specific genes remains active whereas others remain switched off. When a cell faces a change in its surroundings caused by either a harmful chemical or drug, it reprogrammes its genomic expression to an adaptive response. Thus, measurement of changes in gene expression as an adaptive response upon exposure to a drug or chemical can help us to understand the mechanism of how drugs and drug candidates work in cells and organisms. DNA microarray analysis is powerful enough to provide a fast and systematic high-throughput analysis of gene expression at the level of the whole genome.[13] *S. cerevisiae* is a good model organism for this type of analysis, because it adapts easily to changes in its environment and mimics many of the properties of higher organisms. Most notably, its genome sequence has already been completed and the functions of almost 70% of the genes are known, at least in part.[14] Thus, the post-genomic era of *S. cerevisiae* has facilitated the identification of mechanisms of adaptive response of a whole organism to certain external or internal stimuli. Taking this advantage, yeast-based DNA microarrays have been used by our group and others to monitor global responses to environmental stresses and a variety of chemical agents with environmental health risk potential.[15–18] In addition, genomic profiling studies have been carried out to ascertain global responses to the toxicity of amphotericin B, 5-fluorocytosine and various azole compounds in both *S. cerevisiae* and *C. albicans*[19–22] at the molecular level. These studies have resulted in the development of a framework for predicting the mode of action of novel agents with antifungal activities.[20,22] In this study, we report the global response of *S. cerevisiae* to α-terpinene by monitoring the altered gene expression profiles using genome scale DNA microarrays.

Materials and methods

Strain, chemicals and growth conditions

S. cerevisiae strain S288C (α *SUC2 mal gal2 CUP1*) was used as an indicator strain for cDNA microarray analysis. It was grown in YPD medium (2% polypeptone, 1% yeast extract, and 2% glucose) at 25°C. α-Terpinene was purchased from Sigma Chemical Co. (USA). To optimize the culture conditions, exponentially growing cultures of yeast cells were exposed to various concentrations (0.0%, 0.01%, 0.02%, 0.03% and 0.04%) of α-terpinene for 24 h, and growth of the cells was measured every 2 h by counting colony forming units on YPD agar medium and also by measuring optical density at 660 nm (OD_{660}). For transcriptional analysis, yeast cells diluted in YPD medium were incubated overnight at an OD of 1.0, and then 40 μL of terpinene was added to a 200 mL culture. After incubation for 2 h, cells were harvested by centrifugation and pellets were washed three times with DEPC-treated H_2O before processing for RNA extraction.

Preparation of mRNA and cDNA probes

Total RNA was extracted by a hot-phenol method as described elsewhere.[15] Poly(A)$^+$ RNA was purified from total RNA with an Oligotex – dT30 mRNA purification kit (Takara, Kyoto, Japan). Fluorescently labelled cDNA was synthesized by oligo(dT)primed polymerization using PowerScript reverse transcriptase (Clontech, CA, USA) in the presence of Cy3 (green) or Cy5 (red)-labelled deoxyuridine triphosphate (dUTP). The cDNA made from the poly(A)$^+$ RNA of the control was fluorescently labelled with Cy3 and that of the terpinene-treated sample was labelled with Cy5. For each labelling, 2–4 μg of poly(A)$^+$ RNA was used, and the same amount of each poly(A)$^+$ RNA was used in one slide. For more details see Kitagawa *et al*.[16]

Hybridization and washing

Microarray hybridization was conducted using yeast DNA chips obtained from DNA Chip Research, Inc, Yokohama, Japan. The two labelled cDNA pools were mixed and hybridized with a yeast DNA chip for 24–48 h at 65°C. When hybridization was complete, the labelled array was washed with 2 × SSC, 0.1% SDS, and with 0.2 × SSC, 0.1% SDS (twice for 20 min), and rinsed with 0.2 × SSC and 0.05 × SSC for 10 min each, and then dried.

Microarray analysis

Afterwards, labelled arrays were scanned with a confocal laser ScanArray 4000 (GSI Lumonics, Billerica, MA, USA) system. Array images were analysed with GenePix 4000 (Inter Medical). Responses to terpinene toxicity were determined by calculating the expression ratios of normalized Cy5 and Cy3 intensities. Normalization was carried out using the intensity of the median as the positive control, after reducing the intensities of the background and the non-specific signal. The background was the intensity around each spot, and the non-specific signal was the intensity due to solvent (spots with 10 mM Tris–HCl 1mM EDTA, pH 8.0, buffer only). Gene Spring (Silicon Genetics, Redwood City, CA, USA) was used for further data analysis. For reliability of the data, changes in expression levels more than two-fold and <0.5-fold in at least two of the three independent experiments were considered to indicate induction and repression, respectively. The details of the microarray procedure have been described previously.[16] The relative fold changes in the ratio of fluorescence intensity represent the average change in gene expression caused by the terpinene treatment. More than a two-fold increase was accepted as a basal level of induction and <0.5-fold was considered to be repression in the three independent experiments. We used 'at least two of the three' in order to exclude genes that had high or low average values due to an irregularly high or low intensity in only one experiment.

Northern-blot analysis

Northern blotting was performed as described in Murata *et al*.[17] Total RNA (20 μg) isolated from control or terpinene-treated cells was subjected to electrophoresis through 1.0% formaldehyde denaturing agarose gels (4–5 h at 100V). RNA was transferred to a nylon membrane (Roche Diagnostics) and fixed by UV cross-linking (120 mJ). Blots were probed with the double-strand DNA probes of the significantly induced genes, for example, *INO1* and *OPI3*. Probes were made by PCR amplification using the chromosomal DNA as the template and the following primers: *INO1*, 5′-TCTGCAACAACGCTTGAAGGGG-3′ as the forward primer and 5′-AGCCATTCACCGGGTGAAATCC-3′ as the reverse primer; *OPI3*, 5′-ATGAAGGAGTCAGTCCAAGAGATCA-3′ as the forward primer and 5′-CATATTCTTTTTGGCCTTATCACGG-3′ as the reverse primer; *ACT1*, 5′-TAACGGTTCTGGTATGTGTAAAGCC-3′ as the forward primer and 5′-TGTAAGTAGTTTGGTCAATACCGGC-3′ as the reverse primer. Each denatured probe was hybridized to the membrane-bound RNA, and detected with anti-digoxigenin antibody according to the manufacturer's instructions (Roche Diagnostics).

Results

Experimental design

The purpose of this study was to assess the response of yeast cells to α-terpinene at the molecular level. To this end, at first we optimized the concentration of terpinene that inhibited growth by ~50% because strong or weak inhibition may cause undetectable responses. We found that 0.02% terpinene is enough to exert approximately

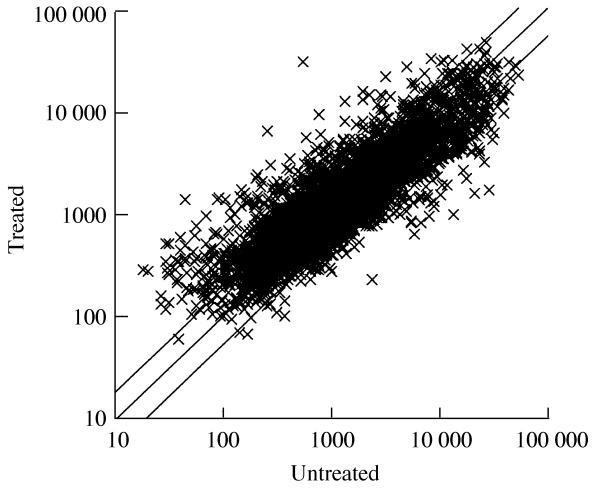

Figure 1. Scatter plot of signal intensities from yeast DNA microarray experiments. Yeast cells were treated with α-terpinene for 2 h at 25°C. Intensities of Cy3 and Cy5 show the amounts of mRNA in untreated and treated cells, respectively. Each spot corresponds to one gene. Each intensity represents data averaged from two of triplicate experiments.

half-maximal growth inhibition (IC_{50}) (data not shown). Based on previous reports in which one doubling time (90 min) was sufficient to detect specific gene expression changes in response to various antifungal drugs,[20–22] yeast cells were treated with 0.02% terpinene for 2 h and RNA was prepared for microarrays from both terpinene-treated and untreated cells. DNA arrays of almost all of the yeast open reading frames were hybridized with Cy5- and Cy3-labelled probes, as described above. In Figure 1 the normalized signal intensities of the terpinene-treated samples are plotted against the normalized signal intensities of the controls. Data points shown in the top left and bottom right regions represent those ORFs that were either induced or repressed by terpinene treatment, respectively. Thus, a total of 793 genes were identified as responsive genes, and of them, 435 genes responded with increases in transcript levels upon terpinene treatment (the most significantly induced genes are shown in Table 1), and 358 genes were shown to respond with decreased mRNA levels (not shown).

Functional categories of responsive genes

All of the responsive genes were annotated using the biological roles assigned by Munich International Centre For Protein Sequences (MIPS), and major representative classes of responsive genes were categorized according to their biological function as shown in Figure 2. We found that reprogramming of the genomic programme initiated the expression of proteins and enzymes related to lipid and fatty acid metabolism, transport facilitation, amino acid metabolism etc. in terpinene-treated cells. As was observed with commonly used antifungal drugs, we also clearly found that cell wall- and membrane-related genes were major targets of terpinene (Figure 2a). Notably, about 192 (44%) of the induced genes have not yet been functionally characterized, and their characterization will probably be helpful in understanding the mechanisms of antifungal drug resistance and sensitivity more clearly. Functional analysis of repressed genes showed that genes belonging to 'protein synthesis', 'carbohydrate metabolism' and 'transcription' categories were abundant among those genes that were repressed in terpinene-treated cells (Figure 2b).

Changes in expression level of genes in the ergosterol pathway

Microarray data revealed a global up-regulation of *ERG* genes along with other linked genes. This is in agreement with earlier studies showing that the ergosterol biosynthesis pathway is the target of azole derivatives in *S. cerevisiae* and its close relative *C. albicans*.[19,21,22] Similar to these studies, a set of genes, e.g. *ERG1*, *ERG3*, *ERG5*, *ERG6*, *ERG24*, *ERG28* (Figure 3), involved in the ergosterol biosynthesis pathway were transcriptionally activated in our experiment. In addition to the ergosterol biosynthesis genes, several other genes involved in this pathway also responded to terpene toxicity (Figure 3). For instance, overexpression of *CYB5*, which encodes cytochrome b5-reductase, was shown to cooperate with the ergosterol pathway downstream of *ERG11*. Thus, overexpression of *CYB5* may reduce the sensitivity of *S. cerevisiae* to terpinene, and this is consistent with previous observations reported with azole compounds.[21,23] Notably, and in agreement with previous studies,[20,21] *NCP1*, which encodes NADP-cytochrome reductase, was also induced in this study. This enzyme acts as the electron donor for the products of *ERG1*, *ERG11* and *ERG5*. Overexpression of *SAM2* (S-adenosyl methionine synthetase), which catalyses the formation of S-adenosylmethionine (*SAM*) from methionine, also indirectly helps the ergosterol pathway by providing the substrate for *ERG6* activity.[21] In addition, *FMS1*, a multicopy suppressor of fenpropimorph resistance (*Fen2* mutant) was up-regulated in response to terpinene action. In *S. cerevisiae*, fen2p was suggested to be a sensor of ergosterol levels in the membrane, thus allowing the cells to adjust to the growth conditions.[24]

Genes involved with phospholipid biosynthesis

Apart from the up-regulation of the ergosterol biosynthesis pathway, several other genes related to inositol and lipid biosynthesis have been shown to change expression upon terpinene treatment (Table 1). The gene with the largest increase in expression (58.2-fold) in response to terpinene toxicity was *INO1*. Inositol is the precursor of phosphatidylinositol (PI), which is an essential membrane component of *S. cerevisiae* that acts in the cellular signal transduction pathway.[25] The inositol that is required for PI synthesis is endogenously produced from glucose-6-phosphate, and the reaction is catalysed by Ino1p (inositol-1-phosphate-synthase).[26] In addition, cells can take up inositol from the medium by expressing two transporters *ITR1* and *ITR2*.[27] Gene expression profiles show that Itr1 expression is also highly responsive to terpinene treatment. Consistent with this finding, expression of *INO4*, a regulator of *ITR1* expression, was also significantly increased. Furthermore, *CHO2/PEM1* and *OPI3/PEM2,* which catalyse the three subsequent methylation steps in the phospholipid biosynthesis pathway,[17] were up-regulated in response to terpinene stress. In addition, the transcript level of *GIT1*, which encodes a transporter for glycerophosphoinositol (GroPIns), was also increased upon terpinene treatment. *GIT1* is used to transport GroPIns from the extracellular medium during inositol starvation[28] in terpinene-treated cells (Table 1).

Genes associated with cell wall organization

We have also found that a group of induced genes in terpinene-treated cells are implicated in cell wall biogenesis including *CRH1*, *CHS1*, *GSC2*, *AGA2*, *SCW10*, *PIR2 (HSP150)*, *KRE1*, *KRE26*, *ECM4*, *ECM13*, *ECM17*, *DAN1* etc. (Table 1). Crh1, whose expression is regulated differently during the life cycle,[29] is a novel member of a

Table 1. Specific changes (induced) in *S. cerevisiae* gene expression in response to a 2 h treatment with 0.02% α-terpinene

ORFs	Genes	Description	Fold induc.
Ergosterol biosynthesis and cell wall-linked genes			
YJL153C	*INO1*	L-myo-inositol-1-phosphate synthase	58.2
YJR150C	*DANI*	protein induced during anaerobic growth	26.9
YJR073C	*OPI3*	methylene-fatty-acyl-phospholipid synthase (unsaturated phospholipid N-methyltransferase	9.7
YOR237W	*HES1*	homology to human oxysterol binding protein	9.0
YDR502C	*SAM2*	*S*-adenosylmethionine synthetase	7.3
YDR213W	*UPC2*	zinc finger transcription factor of the Zn(2)-Cys(6) binuclear cluster domain type	6.4
YKR053C	*YSR3*	DHS-1-P phosphatase	6.3
YCR098C	*GIT1*	permease involved in the uptake of glycerophosphoinositol	5.7
YNL012W	*SPO1*	encodes a protein with high similarity to phospholipase B	5.0
YML008C	*ERG6*	S-adenosylmethionine: δ 24-methyltransferase	4.4
YGR032W	*GSC2*	catalytic component of 1,3-β-D-glucan synthase	4.1
YLR056W	*ERG3*	C-5 sterol desaturase	4.1
YGL032C	*AGA2*	adhesion subunit of a-agglutinin	3.9
YER044C	*ERG28*	involved in synthesis of ergosterol	3.7
YDR497C	*ITR1*	myo-inositol transporter	3.5
YMR020W	*FMS1*	multicopy suppressor of fenpropimorph resistance (fen2 mutant), shows similarity to *C. albicans* corticosteroid-binding protein CBP1	3.3
YNL169C	*PSD1*	phosphatidylserine decarboxylase 1	3.2
YGR189C	*CRH1*	cell wall protein	3.0
YGR157W	*CHO2*	phosphatidyl-ethanolamine N-methyltransferase	3.0
YDR058C	*TGL2*	triglyceride lipase	2.8
YIL160C	*POT1*	peroxisomal 3-oxoacyl CoA thiolase	2.7
YNL111C	*CYB5*	cytochrome b5	2.7
YKL188C	*PXA2*	peroxisomal ABC transporter 2	2.6
YGR175C	*ERG1*	squalene monooxygenase	2.6
YHR042W	*NCP1*	NADP-cytochrome P450 reductase	2.4
YPL148C	*PPT2*	phosphopantetheine:protein transferase (PPTase)	2.3
YMR015C	*ERG5*	cytochrome P450 involved in C-22 denaturation of the ergosterol side-chain	2.2
YGR060W	*ERG25*	C-4 sterol methyl oxidase	2.1
YGL055W	*OLE1*	δ-9-fatty acide desaturase	2.1
YER026C	*CHO1*	phosphatidylserine synthase	2.1
YNL280C	*ERG24*	sterol C-14 reductase	1.5
YDL041W	*KRE26*	killer toxin resistant	2.1
YMR305C	*SCW10*	soluble cell wall protein	3.0
Detoxification			
YGR213C	*RTA1*	involved in 7-aminocholesterol resistance	7.9
YIR038C	*GTT1*	glutathione transferase	3.9
YNL259C	*ATX1*	putative copper binding/homeostasis protein	3.5
YOR031W	*CRS5*	metallothionein-like protein	3.4
YNL241C	*ZWF1*	glucose-6-phosphate dehydrogenase	3.0
YDR453C	*TSA2*	strong similarity to thiol-specific antioxidant proteins	3.0
YHL047C	*TAF1*	triacetylfusarinine C transporter	3.0
YJL159W	*HSP150*	heat shock protein, secretory glycoprotein	2.9
YHR042W	*NCP1*	NADP-cytochrome P450 reductase	2.4
YHR176W	*FMO*	dimethylaniline monoxygenase	2.1
Other genes			
Cell wall maintenance and related genes			
YJR137C	*ECM17*	putative sulfite reductase	2.3
YKR076W	*ECM4*	(putative) involved in cell wall biogenesis	2.3
YBL043W	*ECM13*	(putative) involved in cell wall biogenesis	2.4
YAL062W	*GDH3*	NADP-linked glutamate dehydrogenase	7.0
YIL154C	*IMP2′*	transcription factor	2.8

Table 1. (*Continued*)

ORFs	Genes	Description	Fold induc.
Cellular import			
YBR294W	*SUL1*	probable sulfate transport protein	9.1
YDR497C	*ITR1*	myo-inositol transporter	3.5
YCL040W	*GLK1*	glucokinase	2.1
Phosphate transport			
YBR296C	*PHO89*	probable Na+/Pi symporter	4.2
YML123C	*PHO84*	inorganic phosphate transporter, transmembrane protein	2.2
Amino acid metabolism			
YGL184C	*STR3*	probable sulfate transport protein	8.1
YER091C	*MET6*	vitamin B12-(cobalamin)-independent isozyme of methionine synthase (also called N5-methyltetrahydrofolate homocysteine methyltransferase)	7.0
YLR303W	*MET17/25*	*O*-acetylhomoserine-*O*-acetylserine sulfhydralase	6.7
YBR213W	*MET8*	effector in the expression of PAPS reductase and sulfite	6.2
YGL125W	*MET13*	putative methylenetetrahydrofolate reductase (mthfr)	5.2
YLL061W	*MMP1*	high affinity s-methylmethionine permease	5.0
YKL001C	*MET14*	adenylylsulfate kinase	4.7
YKL015W	*PUT3*	zinc-finger transcription factor of the Zn(2)-Cys(6) binuclear cluster domain type	3.9
YNL277W	*MET2*	homoserine *O*-trans-acetylase	3.5
YFR030W	*MET10*	subunit of assimilatory sulfite reductase	3.5
YER042W	*MXR1*	peptide methionine sulfoxide reductase	3.4
YJR010W	*MET3*	ATP sulfurylase	3.1
YOR303W	*CPA1*	carbamoyl phosphate synthetase, arginine specific	2.8
YOL058W	*ARG1*	arginosuccinate synthetase	2.7
YLR438W	*CAR2*	ornithine aminotransferase	2.3
YAL012W	*CYS3*	cystathionine γ-lyase	2.1
YPL274W	*SAM3*	high affinity *S*-adenosyl methionine permease	2.1
C-compound and carbohydrate metabolism			
YIL162W	*SUC2*	invertase (sucrose hydrolysing enzyme)	8.5
YGL156W	*AMS1*	vacuolar α mannosidase	6.6
YKL217W	*JEN1*	carboxylic acid transporter protein homolog	5.2
YLR377C	*FBP1*	fructose-1,6-bisphosphatase	4.8
YNL192W	*CHS1*	chitin synthase 1	3.7
YGR177C	*ATF2*	alcohol acetyltransferase	3.5
YLR174W	*IDP2*	cytosolic form of NADP-dependent isocitrate	3.4
YOL110W	*SHR5*	involved in RAS localization and palmitoylation	3.4
YHL032C	*GUT1*	glycerol kinase (converts glycerol to glycerol-3-phosphate)	3.4
YCR010C	*ADY2*	accumulation of DYads; member of the TC 9.B.33 YaaH family of putative transporters	3.1
YKR097W	*PCK1*	phosphoenolpyruvate carboxylkinase	3.0
YDL037C	*BSC1*	strong similarity to glucan 1,4-α-glucosidase	2.8
YDL049C	*KNH1*	KRE9 homolog	2.7
YOR299W	*BUD7*	involved in bipolar bud site selection	2.7
YNL322C	*KRE1*	putatively involved in side-chain addition to β-1,6-glucan	2.4
YER024W	*YAT2*	similarity to carnitine *O*-acetyltransferase Yat1p	2.2
YGR288W	*MAL13*	MAL-activator protein	2.1
YDR043C	*NRG1*	transcriptional repressor which can bind to UAS-1 in the STA1 promoter and which can interact with Ssn6p	2.0
Energy			
YCR005C	*CIT2*	non-mitochondrial citrate synthase	5.3
YFL056C	*AAD6*	hypothetical aryl-alcohol dehydrogenase (AAD)	3.1
YDL021W	*GPM2*	phosphoglycerate mutase, involved in glycolysis	2.5
YBR145W	*ADH5*	alcohol dehydrogenase isoenzyme V	2.4
YGR192C	*TDH3*	glyceraldehyde-3-phosphate dehydrogenase 3	2.4
YCR105W	*ADH7*	strong similarity to alcohol dehydrogenases	2.2

Table 1. (*Continued*)

ORFs	Genes	Description	Fold induc.
Meiosis			
YOR198C	*BFR1*	involved in secretion	6.5
YKR046C	*PET10*	hypothetical protein	4.6
YNL250W	*RAD50*	contains a purine-binding domain, two heptad repeats and a hydrophobic tail	3.1
YHR124W	*NDT80*	DNA-binding transcription factor that activates middle sporulation genes	2.7
YIL072W	*HOP1*	DNA binding protein	2.3
YGL033W	*HOP2*	meiosis-specific gene required for the pairing of homologous chromosomes	2.1
YOR351C	*MEK1*	mRNA is induced in meiosis, encodes a meiosis-specific serine/threonine protein kinase which interacts with and is believed to phosphorylate Hop1p	2.0
Mitotic cell cycle and cell cycle control			
YOR198C	*BFR1*	involved in secretion	6.5
YNL012W	*SPO1*	encodes a protein with high similarity to phospholipase B	5.0
YMR052W	*FAR3*	involved in the cell cycle	3.2
YJL095W	*BCK1*	MEKK serine/threonine kinase	3.1
YGR049W	*SCM4*	suppressor of cdc4 mutations	2.5
YNR010W	*CSE2*	component of RNA polymerase II mediator subcomplex	2.5
Protein modification			
YAL039C	*CYC3*	cytochrome c heme lyase (CCHL)	12.4
YPL148C	*PPT2*	phosphopantetheine:protein transferase (PPTase)	3.7
YEL012W	*UBC8*	ubiquitin-conjugating enzyme; ubiquitin-protein ligase	2.7
Transcription			
YIR009W	*MSL1*	encodes YU2B, a component of yeast U2 snRNP	14.2
YBR182C	*SMP1*	probable DNA-binding transcription factor, homolog to R1m1p	12.8
YDL098C	*SNU23*	putative RNA binding zinc protein	5.1
YNL012W	*SPO1*	encodes a protein with high similarity to phospholipase B	5.0
YBR240C	*THI2*	probable Zn-finger protein	4.5
YLR116W5	*MSL5*	branchpoint bridging protein	4.4
YKL015W	*PUT3*	zinc-finger transcription factor of the Zn(2)-cys(6) binuclear cluster domain type	3.9

group of cell wall-related proteins, and encodes a putative glycosidase that plays an important role in cell wall organization and maintenance by remodelling the glucan.[30] It is known that *GSC1* (*FKS1*) and *GSC2* (*FKS2*) encode β 1–3 glucan synthase that plays important roles in glucan synthesis.[31,32] Mutations in the genes encoding these two proteins are lethal and, therefore, they are assumed to be essential for yeast survival.[33] Similarly, genes involved with synthesis of β 1–6 glucan, such as *KRE1*, were also up-regulated. Increased levels of transcripts of *GSC2* and *KRE1* in terpinene-treated cells could suggest that terpinene targets cell wall glucan synthesis and consequently may alter cell morphology and integrity. Our observation regarding the up-regulation of *CHS1* (which encodes chitin synthase 1) in response to terpinene stress is very interesting, because it has a function in cell wall repair.[34] In addition, we found that some of the cell wall mannoprotein-expressing genes significantly responded in terpinene-treated cells. Some of the mannoproteins (for example, Cwp1 and Cwp2) are expressed to adapt with environmental stress. One of the most highly expressed genes in terpinene-treated cells is *DAN1* (delayed anaerobic)—a gene whose expression is induced in response to stresses, such as anaerobiosis.[35] Consistent with this result, a regulatory factor controlling the expression of the *DAN/TIR* genes, *UPC2*, was also up-regulated [19,36] in our study. According to an earlier report,[35] the expression of Cwp1 (the major mannoprotein of the cell wall) was down-regulated due to anaerobic growth conditions, probably created by the terpinene treatment.

Our genomic data also revealed that the transcript level of *YSR3*, which encodes dihydrosphingosine 1-phosphate (DHS1-P) phosphatase, was significantly elevated. DHS1-P is a phosphorylated long chain (sphingoid) base (LCBP) and has been shown to be associated with the heat shock response.[37,38] A recent study has shown that the intracellular accumulation of these phosphorylated molecules results in growth inhibition in *S. cerevisiae*. To cope with this unfavourable condition, the cell induces the expression of genes encoding members of the phosphatase family, such as Ysr2p and Ysr3p, to dephosphorylate LCBPs to LCBs.[37]

Induction of genes associated with detoxification

Most of the cyclic hydrocarbons are non-polar lipophilic compounds. To be excreted from the cell or for their metabolism, these compounds have to be oxidized to more polar compounds.[39] These oxidation reactions are primarily mediated by the superfamily of cytochrome P450s and thus help in xenobiotic metabolism.[40] As mentioned above, *CYP60*, the second cytochrome P450 gene in yeast, was highly induced in terpinene-treated cells. Based on an

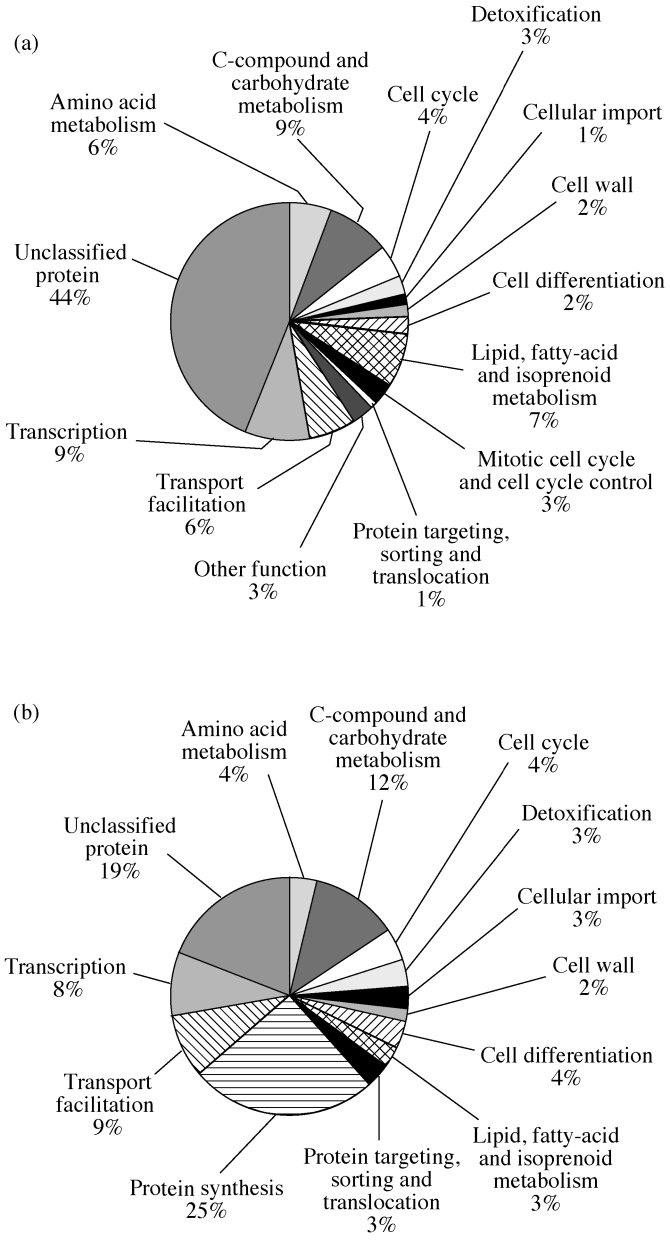

Figure 2. Distribution of α-terpinene responsive genes in the most representative classes, according to the MIPS classification system. (a) Induced. (b) Repressed.

earlier report, it is reasonable to argue that the observed induction of *CYP60* in response to terpinene could be a mechanism of xenobiotic resistance.[39] Consistent with this proposal, yeast cells induced the expression of the *FMO* genes, which encode dimethyline mono-oxygenase and catalyse the mono-oxygenation of phosphorus-, sulphur- and nitrogen-containing xenobiotics including drugs, pesticides and industrial pollutants.[41] Similarly, overexpression of *RTA1* might also help the cell to attain resistance against terpene-mediated toxicity. *RTA1* encodes a membrane-spanning protein that was reported to render resistance against the antifungal drug 7-aminocholesterol.[42] Interestingly, some plants such as tobacco express a type of protein, namely osmotin, belonging to the PR-5 family, that inhibits fungal growth as a defence mechanism.[43] However, further studies showed that the fungus becomes resistant to osmotin by inducing a gene family (PIR) encoding the membrane-embedded stress protein Hsp150. Therefore, increased expression of *HSP150* against terpene cytotoxicity could function as a mode of xenobiotic resistance in *S. cerevisiae*. Based on this observation, it could be hypothesized that plants produce terpinene to defend themselves from pathogenic fungi. These observations suggest that terpene compounds could be considered to be xenobiotics, and in response, the cell induces the expression of genes associated with drug metabolism and detoxification pathways.

Validation of microarray data by northern blotting

There have been arguments suggesting that microarray data may not always be reliable. To validate the differential expression of genes identified in the microarray experiments, the expression of selected genes can be tested either by northern-blot analysis or real-time PCR. In this study, the transcription of two abundantly induced ORFs identified in the microarray analysis, *INO1* and *OPI3*, along with *ACT1* (as a loading control), were verified by northern blotting. The results (Figure 4) are in complete agreement with the microarray experiments.

Discussion

Ergosterol is the predominant lipid molecule in yeast cells that functions to regulate membrane fluidity, permeability and the activity of many membrane-bound enzymes. It can thus be expected that the functional activities of ergosterol play an important role in cellular growth. Since monoterpenes and other plant-derived essential oils are lipophilic in nature, their presence in the culture medium must induce stress on the cellular lipid environment. This stress should induce an adaptive response that may result in the reprogramming of genomic expression in order to protect cell wall architecture from disintegration. In this study, we analysed the gene expression profiles by DNA microarray to obtain a comprehensive view of mode of actions of terpinene toxicity on yeast cells and the mechanisms of adaptive responses evoked by cells to avoid these stresses. Results of this analysis clearly showed that genes belonging to lipid and fatty acid metabolism, cell wall structure and organization, detoxification, cellular transport etc. were up-regulated in terpinene-treated cells. On the other hand, reprogramming of genomic expression led to down-regulation of numerous genes associated with protein synthesis, probably to save energy for other vital metabolic processes.

This study showed that global up-regulation of genes associated with the ergosterol biosynthesis pathway occurs in response to terpinene toxicity. This suggests that ergosterol synthesis was strongly inhibited in terpinene-treated cells. Interestingly, azole compounds such as fluconazole, miconazole and clotrimazole etc., commonly used as antifungals, target and inhibit the ergosterol biosynthesis pathway in *C. albicans* and *S. cerevisiae*. In addition to azoles, other classes of antifungal drugs, such as polyenes, allylamines and morpholines also target ergosterol biosynthesis as a mode of fungal growth inhibition. Most notably, similar microarray analysis conducted with these compounds[19,21,22] also showed a global up-regulation of *erg* genes, as observed with terpinene in our study. But, in contrast to these studies, expression of cytochrome P450 (*CYP51*) encoding *ERG11*, a major target of azole compounds, was not increased in terpinene-treated cells. Instead, another cytochrome P450-encoding gene, *ERG5* (*CYP61*), which encodes c-22 desaturase,[44] was responsive. It is believed that *ERG11* works under aerobic conditions when *ERG3* is not functional.[45] The observed induction of *ERG3* in our study suggests that *ERG11* function was silenced, prob-

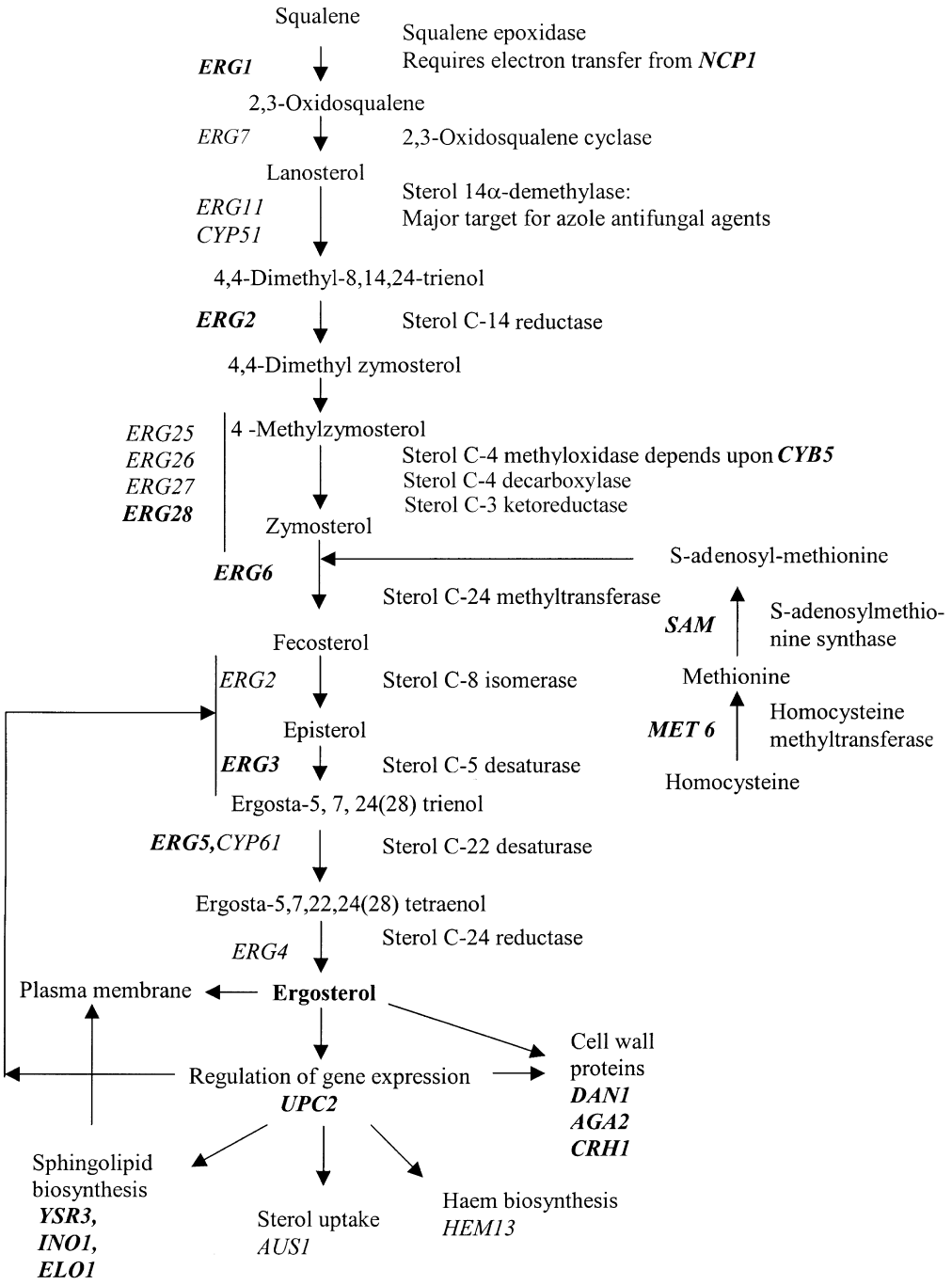

Figure 3. Effect of α-terpinene on the expression of *S. cerevisiae* genes involved in the ergosterol biosynthetic pathway; responsive genes are shown in bold. The relationship between ergosterol biosynthesis and *UPC2*-mediated gene regulation is summarized.

ably due to the anaerobic conditions created by terpinene treatment. But we do not have any direct evidence that culture conditions were anaerobic. However, abundant induction of *DNA1* and down-regulation of Cwp1 in terpinene-treated cells could be circumstantial evidence of anaerobic conditions.[35,36]

However, which mechanism is responsible for the up-regulation of the *ERG* genes in response to terpinene toxicity remains unknown. One study[46] has shown that ergosterol limitation in *S. cerevisiae* induces the expression of *ERG1*. A study reported by DeBacker *et al.*[21] suggested that sterol limitation may cause the up-regulation of *ERG1* in *C. albicans* in response to itraconazole treatment. Additionally, expression of the *ERG3* and *ERG9* genes was also found to be regulated by ergosterol availability.[20] Similarly, overexpression of *ERG1* and *ERG3* in our study indicates that sterol depletion in terpinene-treated cells may be the reason for up-regulation of ergosterol biosynthesis. This could be further supported by overexpression of

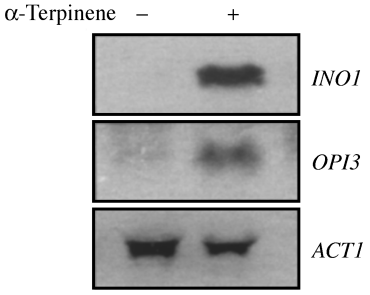

Figure 4. Transcriptional levels of *INO1* and *OPI3* genes induced by exposure to α-terpinene. Total RNA (20 µg) extracted from either untreated (–) or 0.02% α-terpinene treated (+) cells was run on a 1.0% agarose gel. Northern-blot analysis was performed as described in the Materials and methods section. *ACT1* was used as a loading control.

FMS1, which acts as a sensor of ergosterol levels in the cell membranes. The other hypothesis argues that accumulation of toxic sterol by-products, due to the inhibition of specific steps in the ergosterol biosynthesis pathway, induces *ERG* expression.

An interesting observation from our study is the overexpression of several genes associated with sulphur assimilation, methionine biosynthesis and AdoMet production. *MET6*, *MET17/25*, *MET8*, *MET13*, *MET14*, *MET10*, *SAM2*, *SAM3* etc. were found to be overexpressed in the presence of terpinene (Table 1). One explanation of this observation could be the continuous supply of a methyl group in the methylation reaction catalysed by *ERG6* in the ergosterol biosynthesis pathway, and also that three subsequent methylation reactions in the phospholipid biosynthesis pathway (not shown) may have led to the activation of AdoMet biosynthesis. A similar observation was also reported in DMSO-treated yeast cells by Murata *et al.*[17] Consistent with their study, we can also predict that overexpression of *INO1* and *OPI3,* two important genes of phospholipid biosynthesis, and simultaneous activation of the methionine biosynthesis pathway, may induce lipid proliferation in terpinene-treated cells. This proposal has yet to be proven.

Another important observation from our study is the significant up-regulation of a large number of genes associated with cell wall biogenesis. We could see that cell wall mannoproteins, β-glucan as well as chitin synthesis pathways were activated in terpinene-treated cells. It is well known that the complex structures of these three compounds define the yeast cell wall architecture, which is essential for the maintenance of cell shape, cellular integrity and protection against harmful environments.[47] Obviously, perturbation or damage of cell wall structures caused by environmental insults or any cell wall-targeting drug must be deleterious to fungal growth. Yeast has developed mechanisms to compensate for these attacks and one of these mechanisms is termed 'cell wall compensatory mechanism'.[48] Three main responses have been identified to explain how cell wall stresses induce a change in cell wall arrangement.[49] First, hyper-accumulation of chitin occurs to bring a change in cell wall polysaccharides. Second, a change in glucan and cell wall mannoprotein synthesis takes place to increase the mechanical strength of the cell wall, and third, cell wall synthesis and repairing machineries are redistributed all over the cell. Taking these into account, overexpression of a group of genes associated with cell wall biogenesis in terpinene-treated cells clearly indicates that it can affect cell wall structures, which in turn activates cell wall compensatory mechanism to overcome the stress.

Consistent with their lipophilic nature, many earlier studies have implicated the toxicity on membrane structures as a mode of antimicrobial action of essential oils and their monoterpenoid components.[9,10,50] Very recently, Prashar *et al.*[3] showed that the antimicrobial affect of palmarosa oil on *S. cerevisiae* led to a change in fatty acid composition of the yeast cell membrane, with more saturated and fewer unsaturated fatty acids in the membrane. Similarly, using a biochemical approach Cox *et al.*[12] found that tea tree oil targets cell membrane permeability and fluidity as a mode of antimicrobial action against Gram-negative bacteria and the yeast *C. albicans.* In our study, functional analysis of induced genes by yeast DNA microarray allowed us to monitor the antimicrobial activities of a monoterpene on *S. cerevisiae* at a molecular level. It is clear from our data, in addition to the ergosterol and lipid biosynthesis pathway, that cell wall structures were severely affected by terpinene toxicity. Taken together, we could say that the mechanisms of antifungal action depicted by microarray data are very consistent with other mechanisms, as described above.

In conclusion, a global view of changes in gene expression in response to the antifungal action of terpinene was obtained with DNA microarrays. Analysis of these data revealed that specific changes in gene expression were consistent with mechanisms of action of other commonly used antifungal drugs. In addition, some non-specific changes were also observed, along with changes in the expression of several genes of unknown function. Obviously, understanding the function of these unknown genes, in addition to known genes, will probably be useful to identify more targets for the design of antifungal drugs. In addition, our data will be useful for characterizing the mechanisms of antifungal activities of many widely used folk medicines. These data could also be helpful in obtaining a clear understanding of the mechanisms of toxicity of other monoterpenes and cyclic hydrocarbons.

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