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Disruption of fungal cell wall by antifungal *Echinacea* extracts

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In addition to widespread use in reducing the symptoms of colds and flu, *Echinacea* is traditionally employed to treat fungal and bacterial infections. However, to date the mechanism of antimicrobial activity of *Echinacea* extracts remains unclear. We utilized a set of ~4,600 viable gene deletion mutants of *Saccharomyces cerevisiae* to identify mutations that increase sensitivity to *Echinacea.* Thus, a set of chemical-genetic profiles for 16 different *Echinacea* treatments was generated, from which a consensus set of 23 *Echinacea*-sensitive mutants was identified. Of the 23 mutants, only 16 have a reported function. Ten of these 16 are involved in cell wall integrity/structure suggesting that a target for *Echinacea* is the fungal cell wall. Follow-up analyses revealed an increase in sonication-associated cell death in the yeasts *S. cerevisiae* and *Cryptococcus neoformans* after *Echinacea* extract treatments. Furthermore, fluorescence microscopy showed that *Echinacea*-treated *S. cerevisiae* was significantly more prone to cell wall damage than non-treated cells. This study further demonstrates the potential of gene deletion arrays to understand natural product antifungal mode of action and provides compelling evidence that the fungal cell wall is a target of *Echinacea* extracts and may thus explain the utility of this phytomedicine in treating mycoses.

Keywords *Echinacea,* antifungal mode of action, gene deletion array, chemical-genetic profile, yeast cell wall mutants, natural health product

Introduction

Echinacea has a rich tradition of use by First Nations Peoples of the North American plains [1]. *Echinacea* use was quickly adopted by European settlers in the 19th century [2] and is now one of the top-selling herbs in natural products' market surveys in North America [3,4]. The genus is native to the prairies of central and southeastern United States, extending north to southeastern Saskatchewan and southern Manitoba in Canada [2]. The taxonomy of *Echinacea* was recently reassessed using morphometric and AFLP analysis which provided evidence for four species and several varieties [5]. The two species commonly used as medicine are *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronquist (syn. *E. angustifolia*) [5,6]. Medicinal extracts of *Echinacea* are made from all parts of the plant, including the root, leaves, flowerheads, and seeds. These are commonly used to prevent, treat and reduce the symptoms and duration of colds, coughs, flu and other upper respiratory illnesses (review in: [7]). *Echinacea* is also used to treat infections and topical conditions such as candidiasis (including ethnobotanical reports of use for thrush [1]), inflammations caused by a variety of bacteria, viruses and other microbes associated with wound infections, and in the relief of swelling and pain [8–12].

A number of chemical entities are considered important contributors to the therapeutic effects of *Echinacea*. These include a volatile oil, alkamides, alkaloids, caffeic acid derivatives, flavonoids, essential oils, polyacetylenes and polysaccharides [13–17]. However, the alkamides, the major lipophilic compounds found in high concentrations in the roots of *Echinacea*, have a much higher bioavailability when compared with other *Echinacea* constituents such as cichoric acid and caffeic acid [18–20]. Therefore, they may be responsible for pharmacological effects reported for ethanolic extracts [21].

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In vivo and *in vitro* pharmacological investigations have shown immunomodulatory effects by *Echinacea* [12,22] which may account for the efficacy of using *Echinacea* to treat and prevent colds and flu [7]. However, the modes of action of *Echinacea* as an antifungal and for topical applications have not been examined in detail. Our objective was to study possible modes of antifungal activity of *Echinacea* extracts. We investigated eight different ethanolic extracts of *E. purpurea* and *E. angustifolia* for potential phototoxicity and light-independent (dark) toxicity to *Saccharomyces cerevisiae*, i.e., a total of 16 treatments. To study the possible mode of action of *Echinacea*, we used a set of ~4,600 viable gene deletion mutants of *S. cerevisiae*, each with a defined deletion of a characterized or putative gene. The mutants were grown with and without each treatment to provide a view of the chemical-genetic interactions and identify pathways affected by exposure to *Echinacea* extracts.

Our rationale for the above approach was three-fold. First, we investigated the mode of antifungal activity in extracts of all *E. purpurea* and *E. angustifolia* parts based on the traditional usage outlined above. Second, dark and near-UV-light-enhanced antifungal activity of ethanolic extracts from these plants is well-established, although the specific activities of the dozens of lipophilic compounds in these extracts is largely unknown [11,23]. Thus, we looked for consensus activities among diverse ethanol extracts of *Echinacea* with and without UV light activation. Third and finally, the chemical-genomic profiles of crude extracts can closely resemble those of the active, pure, constituent compounds [24]. Determination of mode of action by crude extracts may therefore help guide subsequent efforts to identify the active compound(s) in complex mixtures.

The notable trend in our *Echinacea* gene deletion array (GDA) data sets was an abundance of supersensitive yeast mutants with defects in cell wall-associated functions, suggesting that *Echinacea* extracts perturb fungal cell wall biogenesis/functions. Subsequently, we tested whether fungi grown in the presence of sub-inhibitory levels of *Echinacea* were more sensitive to sonication-based cell wall disruption.

Materials and methods

The series of steps employed in this study are illustrated in Fig. 1. The following sections describe the procedures in further detail.

Echinacea sources

Echinacea purpurea (U Ottawa voucher no. 010502-18) and *E. angustifolia* (U Ottawa # 010410-12 and 020607-01) plant materials were obtained from North American commercial growers and classified according to the most recent taxonomic revision [5]. Eight ethanol extracts (55% EtOH or 70% EtOH) were prepared using root, leaves+stems, leaves+stems+flowers (herb) and flowers exactly as described previously [10,25], using an accelerated solvent extraction system (DIONEX). All fractions were concentrated at 30°C in a rotary-evaporator and adjusted to 50 ml in the appropriate solvent and stored in the dark at −20°C or kept as dried powder until use.

Fungal strains and MIC assays

Wildtype (S288C, *MATa SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*) and mutant strains of *S. cerevisiae*, and *Cryptococcus neoformans* (Ontario Ministry of Health, OMH # FR2704) were grown in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SCM (synthetic complete medium, 2% glucose, 0.67% yeast nitrogen base) with or without 2% agar and supplements as required. For the MIC assays strains were grown to mid-log phase in YPD broth, adjusted to OD600 ~0.8 and diluted to obtain ~10^3 CFU/mL (colony forming units/ml) before aliquoting ~100 cells in 100 µl YPD into each well of a 96-well sterile plate. *Echinacea* extracts were serially diluted across the wells with the last column serving as a drug-free control. Each fraction was tested under two conditions, i.e., following UV light irradiation (10 W/m^2 for 2 h using three 20 W black-light blue tubes, 320–400 nm range) and without UV light irradiation (dark treatment), for which plates were wrapped in aluminum foil. The microtiter plates from both UV and dark treatments were then incubated in the dark at 30ºC and monitored at 48 h. Mean MIC values were based on optical density readings (OD600, Spectra Max 340PC, Molecular Devices, Sunnyvale CA) and calculated from multiple trials ($n > 3$) as the concentration at which there was ~80% growth reduction in comparison to wells with no inhibitor present.

Gene deletion array (GDA) analysis

Chemical-genomic profiling of *Echinacea* extracts was done using a yeast GDA as described by Parsons *et al*. [24]. The method is based on comparing the size of the colonies formed by yeast gene deletion strains with and without a semi-inhibitory concentration of the target drug incorporated in the medium. We used a set of haploid *S. cerevisiae* strains representing ~4600 gene mutants in the background strain BY4741, a derivative of S288C [26]. *Echinacea* extracts were added to 55°C molten YPD agar medium at a concentration that was ~80% of the MIC. YPD agar plates without (control) and with *Echinacea* extract (experimental) were inoculated by hand pinning sets of 384 (16 × 24) mutant strains per plate with a 384-floating pin replicator.

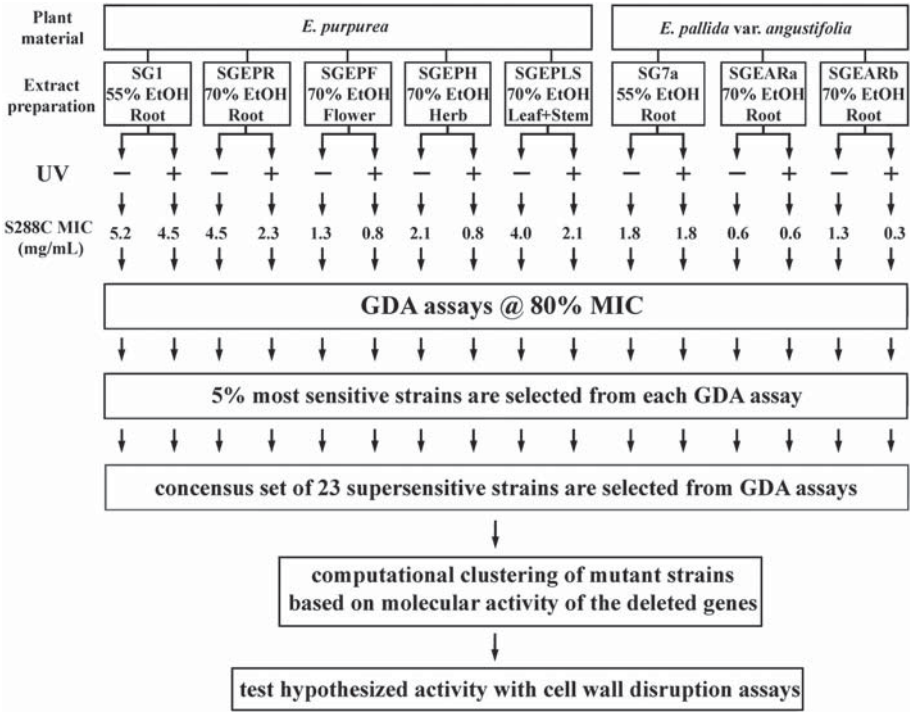

Fig. 1 Flow diagram of investigation into antifungal mode of action by *Echinacea* extracts.

Following UV light irradiation (as described above) or dark treatments, the plates were incubated for 1–2 days at 30°C, photographed with a high-resolution digital camera (Hewlett Packard PhotoSmart 735 digital camera), and analyzed as described in detail by Memarian *et al*. [27]. Briefly, for both experimental and control plates, the size of each colony was digitally determined and compared to the average colony size for all colonies on the plate. Mutant colonies that are sensitive to a given treatment are smaller than the average of colony sizes on the experimental plate but are not smaller than the average of colony sizes on the control plate. After this normalization for mutation-associated colony size differences and overall growth inhibition due to the treatment, we obtained for each extract a ranked list of most to least sensitive mutants. The top 5% (~230) most sensitive mutants were then selected from each of the 16 treatments (light and dark treatments with each of the 8 extracts). We then selected the subset of mutants that were supersensitive to five or more extract treatments to look for an overall mode of antifungal action by *Echinacea*.

Cell wall disruption assays

Two variations of a sonication-based assay [28] were used to test whether *Echinacea* exposure compromises the structure/function of yeast cell walls. The first involved a relatively long (overnight) exposure to relatively high concentrations of *Echinacea* extract. *S. cerevisiae* strains S288C (wild-type) and YLR338W (deletion of OPI9, Table 1), and *C. neoformans* were each grown overnight at 30°C with shaking in 1 ml YPD broth with *E. purpurea* 70% EtOH root extract (experimental) or with an equivalent volume of 70% EtOH (control). The extract concentrations used was ~50% of the MIC values for the respective strains. The overnight cultures were diluted by addition of 4 ml of YPD+EtOH (control) or YPD+extract (experimental) and incubated for an additional 1 h at 30°C. Cultures were adjusted to 1.0×10^7 cells/ml, aliquoted into 1.5 ml epitubes and placed on ice. Aliquots (1 ml) of control and experimental cultures were sonicated using an intensity setting of 3 and a 3 mm microtip (Vibra-Cell VC250, Sonics & Materials, Connecticut, USA) for either 0 or 2.15 minutes at room temperature and then placed back on ice. Cultures were vortexed briefly and then 10-times serial diluted to ~10^3 cells/ml. Ten µl of each dilution was then spotted onto YPD agar plates followed by 48 h incubation at 30°C before colony counts were performed.

A second variation of this sonication-based assay used milder *Echinacea* exposure conditions and investigated possible dose response effects of *Echinacea* extracts on yeast cell walls. For this assay, strain S288C was incubated at 30°C at 150 rpm to an early log phase (OD600 = 0.2). At this point, a 70% EtOH extract of *E. purpurea* roots was added to two aliquots of cells at 10% and 20% of the MIC concentration. A carrier control was also established by incubating an aliquot of cells with an equivalent volume of

Table 1 Yeast mutants identified as supersensitive to five or more *Echinacea* treatments[1].

Gene standard Name/alias	Gene ID	Gene function description
Cell wall functions		
1) *GAS1*/CWH52, GGP1	YMR307W	1,3-β-glucanosyltransferase activity[2,3], cell wall assembly[3], deletion leads to increase chitin & calcofluor white sensitivity[3]
2) *KRE6*/CWH48	YPR159W	β-1,6-glucan biosynthesis[2,3], integral to membrane[3]
3) *MNN10*/BED1, SLC2, REC41	YDR245W	α-1,6-mannosyltransferase activity[2,3], deletion leads to increase chitin & calcofluor white sensitivity[3]
4) *HOC1*	YJR075W	α-1,6-mannosyltransferase activity[2,3], cell wall mannon integrity[2]
5) *BEM2*/IPL2, SUP9, TSL1	YER155C	Rho GTPase activator activity[3], required for bud emergence[2,3] and cell cycle for cytoskeletal organization[2]
6) OPI3/PEM2	YJR073C	Phospholipid methyltransferase[2,3], deletion leads to calcofluor white sensitivity[3]
7) CAX4/CWH8	YGR036C	Generation of cell wall mannoprotein[2], Pyrophosphatase activity intermediate synthesis and protein N-glycosylation[3]
8) URM1	YIL008W	Ubiquitin[2], protein tagging activity and required for normal growth[3]
9) AXL2	YIL140W	Unknown, required for axial budding in haploid cells membrane[2,3]
10) OPI9	YLR338W	Unknown[2,3], deletion leads to increase chitin and calcofluor white sensitivity[3]
Unknown functions		
11) YPL264C	YPL264C	Unknown[2,3], integral to membrane[3]
12) YPR071W	YPR071W	Unknown[2,3], integral to membrane[3]
13) YDR455C	YDR455C	Unknown[2,3]
14) YL¬R402W	YLR402W	Unknown[2,3]
15) YPL182C	YPL182C	Unknown[2,3]
16) WSS1	YHR134W	Unknown[2,3], UV-sensitive mutant phenotype and possible DNA damage response element[3]
17) YAL056C-A/YAL058C-A	YAL056C-A	Unknown[2,3]
Other functions		
18) ARF1	YDL192W	GTPase activity, Golgi trafficking
19) SPT20/ADA5	YOL148C	Subunit of the SAGA transcriptional regulatory complex[2,3], transcription cofactor activity[3]
20) PIG2	YIL045W	Protein phosphatase type 1 regulator activity[2,3]
21) SIN4/BEL2, GAL22, SDI3	YNL236W	RNA polymerase II transcription mediator activity[2,3]
22) PHO2/BAS2, GRF10	YDL106C	Transcription factor of phosphate metabolism[2,3]
23) PRO2	YOR323C	Catalyzes the second step in proline biosynthesis[2,3]

[1]Treatments are described in Fig. 1.
[2]Source = Yeast Proteome Database (YPD) [50].
[3]Source = *Saccharomyces* Genome Database (SGD) [51].

70% EtOH. The cultures were then incubated for 4.5 h at 30°C with shaking, after which aliquots of each sample were placed on ice before sonication for times ranging from 40–85 s (60% amplitude, 3 mm microtip, Vibra-Cell VCX130). A drop plate assay was then performed as described above. The sensitivity of each sample was expressed as a cell survival percentage (#cells surviving with sonication treatment/# cells surviving no-sonication treatment × 100).

The effects of *Echinacea* extract on yeast cell walls was also examined by fluorescence microscopy. *S. cerevisiae* S288C was grown to mid-log phase in SCM and a 70% EtOH extract of *E. purpurea* roots was added at 40% of the MIC. A carrier control was likewise established. The cells were incubated for 4 h at 30°C with shaking prior to a mild sonication treatment on ice (30 s, 60% amplitude, Vibra-Cell VCX130, 3 mm microtip). Cultures were stained with 0.2 μg/μl of calcofluor white (American Cyanamid, Bound Brook, NJ) and immediately viewed by fluorescence microscopy (Axio Imager, Carl Zeiss, Toronto, ON). At least 200 cells were examined for each treatment from ≥5 randomly selected fields and evaluated for cell wall damage.

Results

Twenty-three yeast mutants exhibit high Echinacea sensitivity

Antifungal activity that is enhanced by near-UV exposure is well-established for *Echinacea* extracts [11,23]. The specific active compound(s) and their mode(s) of action in these extracts are largely unknown. To investigate antifungal mode of action of *Echinacea*, we looked for patterns among chemical-genomic interaction profiles obtained by subjecting an array of haploid yeast deletion mutants to inhibitory *Echinacea* treatments. We selected diverse treatments (Fig. 1; different EtOH extracts, plant parts, light and dark activity, and two traditionally relevant *Echinacea* species) in an attempt to identify over-riding patterns of *Echinacea* antifungal activities.

All *Echinacea* extracts tested had antifungal activity against the wild type *S. cerevisiae* S288C. MIC values ranged from 0.3–5.0 mg/ml depending on source species, plant part and extract preparation (Fig. 1). Overall, 70% EtOH extracts were more effective than 55% EtOH extracts at inhibiting the growth of yeasts (means of 1.6 and 3.3 mg/ml, respectively; $P = 0.02$, paired t-test). In concordance with previous studies (reviewed in [11]), light-mediated antifungal activity (phototoxicity) was evident as lower MIC values with near-UV light treatments (300–400 nm) as compared to dark treatments (means of 1.7 and 2.6 mg/ml, respectively; $P = 0.01$, paired t-test). Previous studies indicate that the phototoxicity of *Echinacea* ethanol extracts is likely associated with the presence of polyacetylenes and alkamide compounds, some of which are known to also have dark activity [11,25]. These compounds may be prone to oxidative degradation depending on the storage conditions and extract composition [29]. To control for the possibility of degradation of antifungal compounds and for variation in inhibitory characteristics of different extracts, we used dried or freshly prepared extracts, and adjusted extract concentrations for subsequent experiments based on inhibitory levels of a given fungal strain and extract combination (e.g., 80% of MIC value).

To examine chemical-genetic interactions that may provide insights into *Echinacea* antifungal mode of action, genetic profiles for each of the 16 different treatments were generated. For this, the effect of each treatment on gene deletion strains was determined by digital imaging and comparison of colony sizes on media with and without extract added at ~80% of the MIC (Fig. 1). Examples of three supersensitive mutant colonies from GDA experiments are shown in Fig. 2. Mutants were subsequently ordered based on percent reduction in colony size and 5% of the ~4600 mutants that showed greatest reduction in growth on experimental plates as compared to carrier control plates were designated as supersensitive mutant strains for each treatment (see Supplementary Table S1 in the online version of the paper). We next compared these most sensitive mutant strains from each treatment and selected ones that were among the supersensitive in five or more treatments. Based on binomial proportions, it is unlikely ($P < 0.05$) that a given mutant within this most sensitive 5% group would occur in five or more treatments by chance. From this we obtained the consensus set of 23 *Echinacea*-sensitive mutants listed in Table 1.

Based on subsequent MIC determinations with one of the extracts, *E. purpurea* 70% EtOH root extract, this group of 23 mutant strains was significantly more sensitive than was the S288C wild-type strain (Fig. 3; 1-tailed t-test P values of 0.01 {+UV} and 0.004 {dark}). MIC values were also correlated with colony size reductions obtained from GDA analyses in that mutants with relatively smaller colony sizes in GDA experiments were also most sensitive to *Echinacea* based on MIC determinations (data not shown). These MIC determinations validated the quantitative results of our GDA experiments.

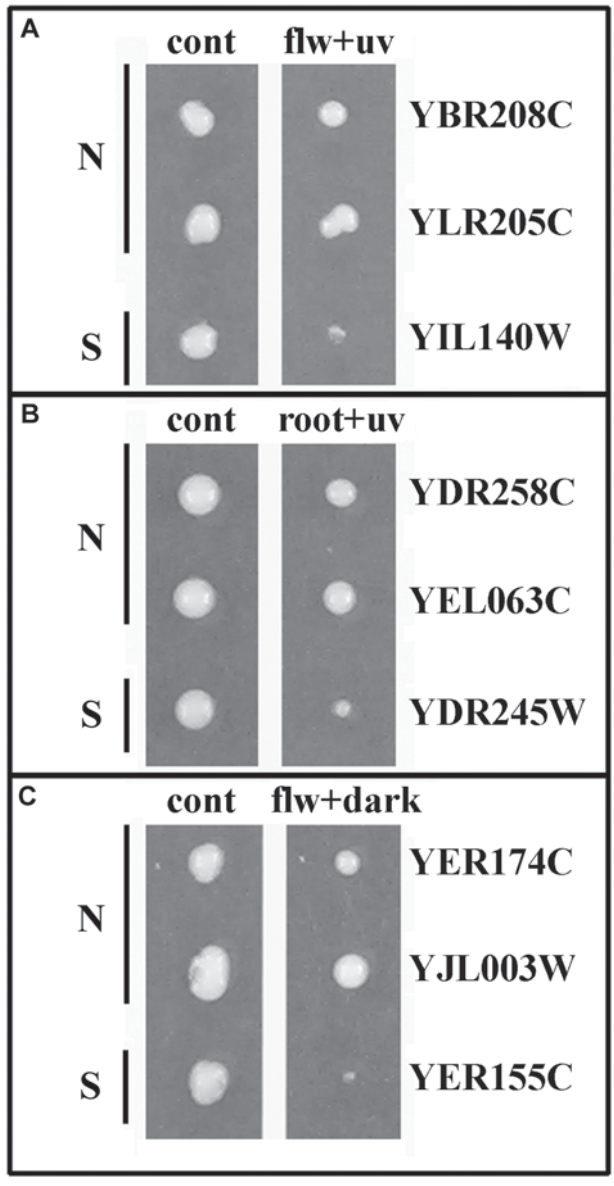

Fig. 2 Examples of supersensitive mutants from GDA experiments. In each panel, three strains are shown from control plate (cont) and experimental plate for treatments with (A) 70% EtOH extract of *Echinacea purpurea* flowers with UV exposure (flw+uv), B) (70% EtOH extract of *Echinacea angustifolia* roots with UV exposure (root+uv), and (C) 70% ethanol extract of *Echinacea purpurea* flowers with no UV (flw+dark). On average, colonies on experimental plates are slightly smaller than on control plates because *Echinacea* treatments were set up to be partially inhibitory. The top two strains in each panel are not sensitive (N), while the lower strain in each panel is supersensitive (S), as evident from the relatively small colony size on the experimental plate. Gene IDs for strains are given at the right.

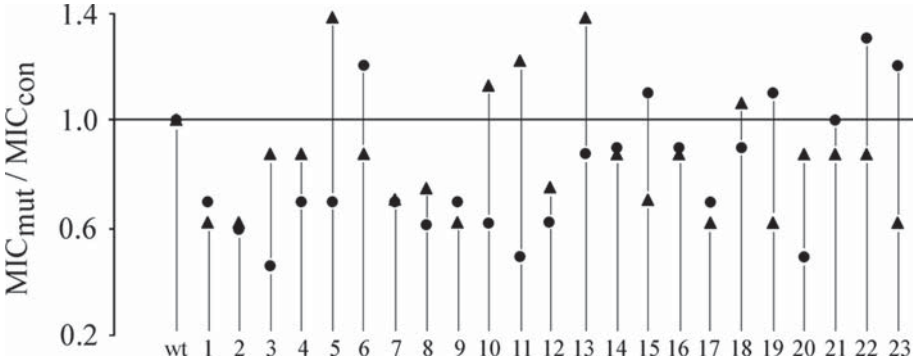

Fig. 3 Relative sensitivity of the 23 supersensitive mutant strains to one of the extracts used in GDA screens. MIC values were determined with the 70% EtOH *E. purpurea* root extract. Strains are numbered as in Table 1 and the wildtype S288C strain is at left. MICs were determined with (triangles) and without (dots) exposure to UV light. Plotted for each strain is the value, MIC_{mut}/MIC_{con}, where MIC_{mut} and MIC_{con} are the MIC values of mutant strain and S288C, respectively. Mutant strains sensitive to this extract, relative to S288C, are positioned below the horizontal line.

GDA analysis suggests Echinacea extracts disrupt cell wall functions

Of the 23 *Echinacea*-sensitive mutants, 16 have known functional defects in cell wall, transcription, protein tagging or amino acid biosynthesis, and seven are in genes of unknown function. The significant pattern to emerge from this analysis is that of the 16 known function mutations, 10 are involved in cell wall integrity/structure. We used the Yeast Features (YF) software tool [30] to assess the statistical significance of shared features among the set of 23 yeast proteins. Based on YF, the probability that these common cell wall features would occur together in our data set by chance is unlikely (*P* values are less than 4×10^{-3} for all five cell wall-related features; Table 2). Overall, these data indicate that *Echinacea* extracts primarily form chemical interactions with cell wall-associated genes or gene products, either directly or indirectly, and exert their antifungal activity by affecting cell wall function(s). This hypothesis was further tested using sonication-based assays.

Table 2 Cell wall functions of selected deletion strains supersensitive to *Echinacea* extracts. Identified functions include cell wall organization and biogenesis, decreased resistance to calcofluor white, α-1,6-mannosyltransferase complex, increased levels of chitin, and cell budding.

Gene ID	Cell wall org & syn	Calc Wt sensitive	Mannosyl transferase	Chitin	Budding
YPR159W	+				
YER155C	+				
YJR075W	+		+		
YDR245W	+	+	+	+	
YMR307W	+	+		+	
YLR338W		+		+	
YJR073C		+			
YIL140W					+
YIL008W					+
P-value	6.9×10^{-5}	8.2×10^{-5}	2.2×10^{-4}	1.6×10^{-3}	4.2×10^{-3}
P-value rank	1	2	3	5	8

Combination of sonication with Echinacea extracts greatly increases yeast cell death

We assessed whether treatment of *S. cerevisiae* and *Cryptococcus neoformans* with 70% EtOH extracts of *E. purpurea* roots perturbs cell wall function using sonication assays. The basis of these assays is that a strain that carries a cell wall defect, or that is exposed to a chemical that interferes with cell wall biogenesis, will be more likely to sustain cell wall damage and lyse during exposure to ultrasound [28]. Table 3 shows results from sonication experiments performed using yeast wild type S288C and strain YLR338W that has a mutation inferred to compromise cell wall function (Table 1), along with an isolate of *C. neoformans*, a basidiomycete yeast. The fungal strains were cultured without or with 70% EtOH extract of *E. purpurea* roots at ~50% of the MIC, a concentration that resulted in ~20% growth rate reduction of the respective strains. While some sensitivity to sonication was evident when each strain was grown in the absence of *Echinacea* extract, the combination of sonication and *Echinacea* treatments resulted in at least a 200-fold reduction in colony forming units (CFUs), indicating that exposure to *Echinacea* extract significantly sensitized yeast cells to disruption by sonication. The sensitivity to *Echinacea* extract was most pronounced in the cell wall mutant, YLR338W (>2000× reduction in CFU, Table 3). This, and that both ascomycete and basidiomycete yeasts had increased sonication sensitivities following extract exposure, suggests that a general mode of *Echinacea* antifungal activity is through perturbation of cell wall function.

Fig. 4 shows similar trends when *S. cerevisiae is* exposed to milder *Echinacea* extract treatments. Extract

Table 3 Changes in fungal CFU with and without exposure to 70% EtOH *Echinacea purpurea* root extract and/or sonication.

Strain	*Echinacea* extract[1]	CFU/ml ($\times 10^6$) at specified sonication times		CFU fold reduction[2]
		0 min	2.15 min	
S. cerevisiae S288C	–	11.0	2.0	5.5
S. cerevisiae S288C	+	2.0	0.01	200
S. cerevisiae YLR338W	–	3.0	0.2	15
S. cerevisiae YLR338W	+	2.0	<0.001	>2000
C. neoformans	–	2.0	0.2	10
C. neoformans	+	1.0	0.002	500

[1]'+' extract present, '–' carrier control with no *Echinacea* extract.
[2]Calculated as the CFU after 0 min sonication divided by CFU after 2.15 min sonication.

concentrations used in these experiments were set to 10% and 20% of the MIC values and exposure was over a 4.5 h period. The figure plots percent survival of sonicated cells over non-sonicated cells with and without the addition of *Echinacea* extract, and provides evidence of a dose-dependent response whereby increasing concentrations of *Echinacea* result in greater sensitivity to cell wall disruption by sonication. The results of the above sonication experiments supported the hypothesis that yeast cell wall functions are compromised by exposure to *Echinacea* extracts.

Finally, direct microscopic examination of *S. cerevisiae* cells provided further support for the above hypothesis (Fig. 5). A significantly greater frequency of cells had cell wall lesions evident when treated with *Echinacea* extract prior to mild sonication, in comparison to cells that were not exposed to the extract ($P \leq 0.001$, ttest of arcsine square root transformed frequencies).

Discussion

Using an ordered set of *S. cerevisiae* gene deletion mutants we explored the molecular mechanism of *Echinacea* extracts antifungal activity in terms of potential target proteins and pathways in yeast cells. The significant trend emerging from the analysis of the 23 deletion mutants that were supersensitive to 5 or more *Echinacea* treatments was one of impaired cell wall functions. Previously, Towers *et al*. [31] suggested that the increased near-UV-light-mediated inhibitory activity of phototoxic alkamides and polyacetelenes could be attributed to the production of single oxygen and peroxidation of cell membranes in the target organism. This proposed mechanism is intriguing given the results of our GDA analysis and the functional association of the cell membrane in fungal cell wall biogenesis.

Table 2 lists five cell wall functions that are compromised in mutants found to be most sensitive to *Echinacea* extracts. The associated mutations likely perturb cell wall-associated processes such as the organization and synthesis of the β-1,6-mannosyltransferase complex. For example, the deletion strain *KRE6* was shown to have significantly reduced growth in response to six of the 16 *Echinacea* treatments (Supplementary Table S1). Strains with a Kre6p deficiency show reduction in β-1,6-glucan synthase

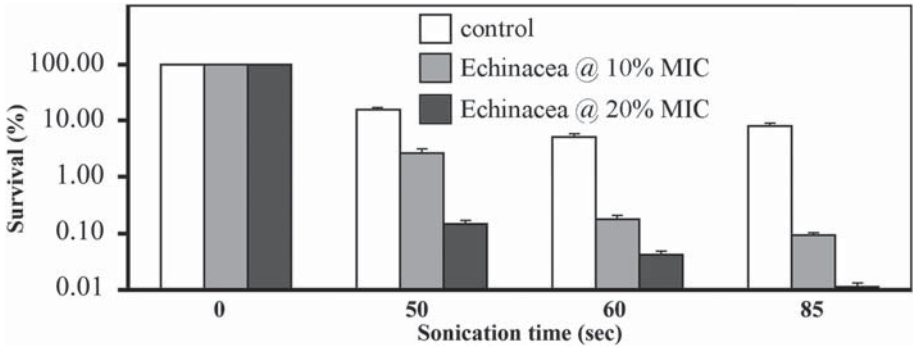

Fig. 4 Enhanced sensitivity to sonication in yeast with relatively mild treatments of 70% EtOH extract of *Echinacea purpurea* roots. Percentage of surviving S288C cells (mean + SD, $n > 4$) in sonicated samples relative to non-sonicated samples (t = 0 sec) is plotted on a logarithmic scale against sonication time, where white bar = carrier control, gray bar = *E. purpurea* at 10% of the MIC concentration and black bar = *E. purpurea* at 20% of the MIC. The results indicate that there is a dose response relationship between extract concentration and cell survival associated with exposure of S288C yeast cells to *Echinacea* extract. Yeast cells treated with this *Echinacea* extract at 10% and 20% of the MIC concentrations for 4.5 h are respectively about 100 and 1000 times more susceptible than untreated cells to 85 second sonication.

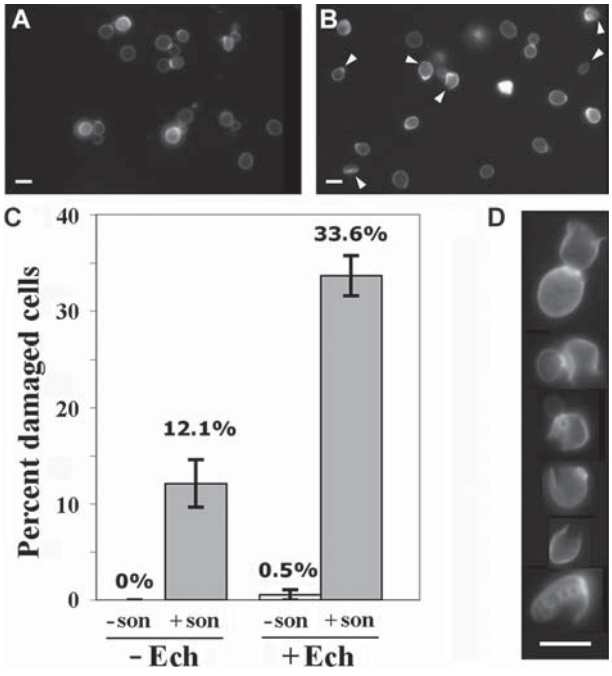

Fig. 5 Effect of exposure to 70% EtOH *Echinacea purpurea* root extract on sonication-associated cell wall damage. Fluorescence microscopy images (40×, bar = 5 µm) of sonicated *S. cerevisiae* cells that were (A) not treated, or (B) treated with Echinacea extract. White arrows indicate obvious cell wall lesions. (C) Percent (mean + SE, $n > 200$ cells) of *S. cerevisiae* cells with cell wall lesions evident without (-son) and with (+son) sonication, without (-Ech) and with (+Ech) 4 h of 70% EtOH *E. purpurea* extract exposure. (D) Fluorescent micrographs (100×, bar = 5 µm) of cells showing detail of wall damage associated with *Echinacea* exposure. For panels A, B and D, cell walls were stained with calcofluor white immediately before microscopy.

and decreased levels of alkaline soluble proteins in the *S. cerevisiae* cell wall [32]. In addition, it was shown that mutations affecting Kre6p cause synthetic lethality when expressed in gas1 mutant cells [33]. Note that the *GAS1* deletion strain was also identified in our set of 23 mutants (Table 1). *GAS1* encodes a glycosylphosphatidylinositol (GPI)-anchored protein that is located in yeast plasma membrane [34]. The disruption in *GAS1* causes leakage of ß-1,3-glucan into the medium, hypersensitivity to calcofluor white and an increased chitin content in the cell wall [34–36]. Our identification of both *GAS1* and *KRE6* mutants among the supersensitive mutants suggests that a parallel cell wall-associated pathway is negatively affected by *Echinacea* extracts.

The identification of the two genes *MNN10* and *HOC1* among the most sensitive strains in the present study provides additional evidence that *Echinacea* compounds interfere with fungal cell wall processes. Deletion of Mnn10p results in defective mannan biosynthesis *in vivo* and upregulation of other cell wall components, especially chitin [37–39]. It has also been shown that *HOC1* encodes a subunit of a Golgi-localized complex in mannosyltranferase and Hoc1p has a regulatory role in determining mannan polymer size [40].

Cell wall integrity is likely to be critical during budding. Previous studies suggested that Bem2p, also listed in Table 1, is important for cytoskeleton organization as well as cell wall maintenance in yeast [41]. In yeast, genes encoding Rho GTPases such as *RHO1*, which is activated by Bem2p, play an essential role in the regulation of cell wall synthesis and cytoskeleton organization including bud emergence and growth [42,43]. Interestingly, *BEM2* is involved in bud emergence and there is also a direct genetic interaction between *BEM2* and *RHO1* in the same pathway that regulates microfilament-mediated polarized cell growth [41]. Another important consideration is that *Echinacea* extracts affect some other functions that may indirectly cause alterations in cell wall synthesis. Of particular interest is the finding that deletion of the ubiquitin gene, *URM1*, results in increased sensitivity to *Echinacea* extracts, as ubiquitin marks transmembrane proteins for removal from the membrane [44,45].

Sonication is used to physically disturb yeast cell walls and membranes by ultrasound-induced cavitation [28,46]. Sonication assays provided further evidence that *Echinacea* extracts perturb fungal cell wall functions. Viability assays and direct microscopic examination indicated that there is a significant increase in sonication-associated cell death and lysed cell frequencies in samples exposed to *E. purpurea* root extracts. Similar cell wall disruption assays may identify additional genes that contribute to cell wall function in the set of nine unknown function mutants listed in Table 1.

Fungi are recognized as a sister taxon to animals and share many biochemical and structural cellular features with plants. Therefore, the development of antifungal compounds that inhibit fungal growth without harming the plant or animal host is a challenge. However, one of the defining characteristics of fungi is the structure and makeup of the cell wall. As a result, the cell wall and the cell wall integrity pathways are among the most desirable targets in the development of new, highly specific, antifungal drugs [47–49]. This study provides compelling evidence that the fungal cell wall is a major target of *Echinacea* extracts and may thus explain the utility of this phytomedicine in traditional treatments of mycoses.

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Supplementary material available online

Supplementary Table1.