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***Neurospora crassa* transcriptomics reveals oxidative stress and plasma membrane homeostasis biology genes as key targets in response to chitosan**

Federico Lopez-Moya¹, David Kowbel², Ma José Nueda³, Javier Palma-Guerrero^{2,4}, N. Louise Glass², and Luis Vicente Lopez-Llorca¹

¹Laboratory of Plant Pathology, Multidisciplinary Institute for Environmental Studies (MIES) Ramon Margalef, Department of Marine Sciences and Applied Biology, University of Alicante, E-03080 Alicante, Spain.

²Department of Plant and Microbial Biology, University of California, Berkeley CA, 94720-3120 USA.

³Statistics and Operation Research Department, University of Alicante, E-03080 Alicante, Spain.

Abstract

Chitosan is a natural polymer with antimicrobial activity. Chitosan causes plasma membrane permeabilization and induction of intracellular reactive oxygen species (ROS) in *Neurospora crassa*. We have determined the transcriptional profile of *N. crassa* to chitosan and identified the main gene targets involved in the cellular response to this compound. Global network analyses showed membrane, transport and oxidoreductase activity as key nodes affected by chitosan. Activation of oxidative metabolism indicates the importance of ROS and cell energy together with plasma membrane homeostasis in *N. crassa* response to chitosan. Deletion strain analysis of chitosan susceptibility pointed, NCU03639 encoding a class 3 lipase, involved in plasma membrane repair by lipid replacement and NCU04537 a MFS monosaccharide transporter related with assimilation of simple sugars, as main gene targets of chitosan. NCU10521, a glutathione S-transferase-4 involved in the generation of reducing power for scavenging intracellular ROS is also a determinant chitosan gene target. Ca²⁺ increased tolerance to chitosan in *N. crassa*. Growth of NCU10610 (*fig 1* domain) and SYT1 (a synaptotagmin) deletion strains was significantly increased by Ca²⁺ in presence of chitosan. Both genes play a determinant role in *N. crassa* membrane homeostasis. Our results are of paramount importance for developing chitosan as antifungal.

Keywords

Drug targets; time-series analysis; ROS; membrane remodeling; calcium

Corresponding author: federico.lopez@ua.es.

⁴Current address: Department of Environmental Systems Science, ETH Zurich, Zurich, Switzerland.

djkowbel@berkeley.edu

mj.nueda@ua.es

javier.palma@usys.ethz.ch

lglass@berkeley.edu

lv.lopez@ua.es

Introduction

Chitosan is a polymer obtained by partial chitin N-deacetylation¹ which has antifungal activity.² Chitosan inhibits growth of filamentous fungi and yeast human pathogens.^{3,4} To develop chitosan as an antifungal treatment, a full understanding of its mode of action is necessary. In *Saccharomyces cerevisiae*, the response to chitooligosaccharides is mediated by proteins associated with plasma membrane, respiration, ATP production and mitochondrial organization.⁵ Five genes (*arl1*, *bck2*, *erg24*, *msg5* and *rba50*) were characterized that provided chitosan resistance when overexpressed or increased sensitivity as a deletion strains. These genes have important roles in signaling pathways, cell membrane integrity and transcription regulation.⁵ Other transcriptional studies in *S. cerevisiae* revealed the relevance of oxidative respiration, mitochondrial biogenesis and transport in the response to chitosan.⁶ Previous physiological studies in *N. crassa* demonstrated that chitosan causes plasma membrane permeabilization.⁷ Membrane fluidity is a key factor determining chitosan sensitivity in fungi.⁸ Cell energy and mitochondrial activity have also an important role in moderating the antifungal activity of chitosan.⁷ The transcriptional response of filamentous fungi to this antifungal remains unknown.

Membrane damage caused by currently used antifungals (eg. azoles) is associated with the induction of intracellular reactive oxygen species (ROS).^{9,10} We have recently shown that low chitosan concentration increased intracellular ROS levels in *N. crassa* leading to partial membrane permeabilization.⁴ Increasing chitosan dose dramatically raised ROS levels causing full membrane permeabilization and subsequent cell death. Oxidative stress by chitosan is mediated by the energetic status of the cell. A reduction in cell energy by blocking the electron transport chain protected *N. crassa* from chitosan damage.⁷ The plasma membrane of *N. crassa* contains high levels of polyunsaturated free fatty acids (FFA), this fact is directly associated with its susceptibility to chitosan.⁸ Fungal plasma membrane lipids could be easily oxidized by an induction of intracellular oxidative stress generated by chitosan as found for other antifungals.^{10,11} This fact would link ROS and membrane homeostasis biology in the mode of action of chitosan.

Ca²⁺ is known to be involved in plasma membrane repair.¹² Previous molecular studies revealed SYT1, a synaptotagmin, involved in membrane repair in several organisms¹³ including *N. crassa*.¹⁴ Moreover, Ca²⁺ plays a role in the response to oxidative stress and programmed cell death in *N. crassa*.¹⁵ PRM1 and FIG1 are key proteins in calcium-dependent plasma membrane remodeling during membrane fusion in *S. cerevisiae* and *N. crassa*.¹⁶⁻¹⁹ In *N. crassa*, two additional proteins, LFD1 and LFD2 are also involved in Ca²⁺-dependent plasma membrane repair during cell fusion.^{14,20} It is currently unknown, however, how fungi repair membrane damage caused by chitosan.

In this work, we analyzed the transcriptional response of *N. crassa* germinating conidia and determined the main gene functions related with the exposure to chitosan. We applied temporal series analysis (Next-maSigPro²¹ and ASCA-genes²²) and a network analysis approach (Cytoscape)²³ to understand the dynamics of functions and gene targets involved in *N. crassa* response to chitosan. This study has pointed mitochondrion (ROS) and

membrane homeostasis as the main functions in the response of *N. crassa* to chitosan and has identified key gene targets. Deletion strains of these key genes were evaluated for fitness and growth. We further demonstrated that extracellular calcium protects fungal cells from damage caused by chitosan. These studies are a key step for improving the knowledge on the mode of action of chitosan, which is essential for its future development as antifungal.

Results and Discussion

Chitosan causes an early activation and late repression of *N. crassa* genes

The experimental conditions for analyzing the effect of chitosan on *N. crassa* germination and development are shown in Fig. 1. Time-course of *N. crassa* conidia germination is included in Figure 1A. Germination defects were quantified after 8h exposure of *N. crassa* conidia to 0.5 $\mu\text{g ml}^{-1}$ chitosan (Fig. 1B; IC₅₀) which showed an approximately 50% reduction in germination. This chitosan concentration was used for high throughput transcriptomic study.

To identify transcriptional changes caused by exposure of *N. crassa* to chitosan a 3-stage time-course (4, 8 and 16h post-inoculation) was performed. A total of 523 *N. crassa* genes were considered differentially expressed (p -value < 0.05), with a fold change ≥ 2 (lower fold change values were considered non-significant), in response to chitosan (Fig. 2A). Of these, 55.6% (291 genes) were down-regulated and 45.3% (237 genes) up-regulated. Our time-course experiment showed a progressive reduction in the number of genes whose expression increased upon exposure to chitosan (142 induced genes at 4h, 119 at 8h and 45 at 16h; Fig. 2B). In contrast, exposure to chitosan resulted in an increase in the number of genes whose expression levels decreased over time (79, 93 and 207 genes down-regulated at 4, 8 and 16h, respectively; Fig. 2C). A subset of 22 genes was differentially expressed consistently (p -value < 0.05; log₂foldchange ≥ 2) throughout the whole time-course (Fig. 2A). Most of these genes (19) were down-regulated, two genes were up-regulated and only one gene of this set (NCU05018) had an early (4 and 8h) induction and a late (16h) gene repression (Fig. 2D).

Expression of 10 *N. crassa* genes representative of functional categories that were differentially expressed by exposure to chitosan were selected to validate our RNA-seq analysis. Gene expression was evaluated by qRT-PCR following an 8h exposure to chitosan (Fig S1). These genes were NCU05134, NCU06123, NCU07610, NCU01382 and NCU05712 (involved in response to oxidative stress), NCU02363 (involved in response to chemical compounds), NCU05018, NCU3494 *pin-c* (related with heterokaryon incompatibility and membrane biology), NCU05764 (a sam-dependent methyltransferase) and a transcription factor with a zinc-finger domain (NCU05767). All genes analyzed by qRT-PCR showed an expression pattern consistent with that derived from RNA-seq data analysis (Fig. S1).

N. crassa main gene functions differentially expressed with chitosan are oxidoreductase activity, membrane homeostasis and microtubule organization

A gene ontology (GO) functional annotation of *N. crassa* genes differentially expressed in response to chitosan was carried out using Blast2GO (Fig. 3 and Figs. S2 and S3). All GO-

domains (molecular function, MF; biological process, BP; cell component, CC) and times were considered together for a complete functional gene expression analysis (Fig. 3A). Oxidoreductase activity (70 genes), membrane (57 genes) and transport (44 genes) were the most enriched GO-terms.

Using maSigFun software for RNA-seq data time series analysis combined with GO annotation, we generated the time-course of functional gene expression for the most significantly enriched GO-terms representing *N. crassa* response to chitosan (Fig. 3B). The analysis identified 12 significant GO-terms using FDR=0.05 and $R^2=0.4$ levels, as suggested in previous studies²⁴. Chitosan modified patterns of expression of ROS-related GO terms mitochondrion and peroxisome organization (Fig. 3B). Mitochondrion genes increased expression through time reaching maximum values of expression at 16h, suggesting that chitosan enhances synthesis/turnover of mitochondrion components (respiration). Genes associated with peroxisome organization, involved in ROS degradation and catabolism of free fatty acids, were first highly expressed (4h) then completely repressed (16h). Likewise, chitosan modified patterns of expression of GO categories related with membrane structure and biology. Exposure to chitosan was associated with repression at 16h of genes involved in cell cortex, vesicle organization and conjugation (Fig. 3B). Moreover, G-protein coupled receptor signaling were compromised by chitosan during all the time-course study (Fig. 3B). These features indicate that chitosan significantly compromised both structure and signaling associated with cell membrane homeostasis. Genes associated with GO-terms related to cell growth such as microtubule organizing center and motor activity had decreased expression values by chitosan through time (Fig. 3B). This behavior suggests the importance of cytoskeleton in the antifungal action of chitosan. Conversely, chitosan increased expression of genes associated with GO terms involved in protein synthesis (ribosome and ribosome biogenesis, Fig. 3B). This would support the increasing expression of genes and synthesis of proteins related to oxidoreductase activity by chitosan (Fig. 3A). In a similar way, nucleolus and structural molecule activity (Fig. 3B) genes were also late activated by chitosan.

Potential gene targets of *N. crassa* to chitosan and their dynamics of expression

Initial time-course analysis showed 5% of *N. crassa* genes significantly expressed in response of *N. crassa* to chitosan. A subset of 33 genes with a relevant change (p -value < 0.05; $\log_2$ fold-change > ± 4.5) of expression is shown in Table S1. A restrictive cut-off was applied with the aim of detect the genes with large change in expression in response to chitosan. This subset included the 22 genes found in the differential gene expression analysis (Fig. 2) plus genes highly expressed (at early or late steps) associated with enriched GO-terms after chitosan exposure.

When applying ASCA-genes method we focused on submodel (b+ab) that represents 67.18% of total variation. Two components were selected, explaining 93% of this variability (52.38% and 40.62%, respectively; Fig. S4). They, therefore, represented the main gene expression in response to chitosan. First component identified a gene expression difference between chitosan and control constant through time (Fig. S4A). Second component identified expression pattern characterized by a clear interaction through time (Fig. S4B). The analysis of the squared prediction error (SPE) and leverage, determined a cut-off using

gamma method, revealed 410 genes which followed the selected components (which explained 93% of variation) and 474 with a behaviour not identified in these (Fig. S5). Comparisons between ASCA and the fold-change gene selection methods (523 genes in total) revealed 447 genes in common (Fig. S4C). Summarizes graphically this comparison where is observed a high overlap between fold-change gene selection and genes with high leverage (also scores can be observed). Moreover 33 genes with a relevant change (listed in Table S1) were also identified showing high scores for the two components identified after PCA (Fig.S4C).

To inspect ASCA gene selection time series two cluster analyses were applied: one to the well-modelled genes (M) and another to the bad-modelled genes (NM) obtaining 4 and 6 clusters respectively (Fig 4 and S6). Both analyses were performed with the hierarchical method. Cluster 1M (Fig. 4A) contained genes associated with an early response to chitosan including two dioxygenases: NCU01849, the most highly expressed gene in response to chitosan (11.16 fold-induction) and NCU01071 a predicted 2OG-Fe dioxygenase, both involved in response to oxidative stress.

We also found a set of genes mainly associated with plasma membrane, signaling and response to chemical compound (NCU02363; RTA1-like protein). In addition, a plasma membrane protein (*het* domain) associated with intracellular oxidative stress (NCU07840), hypothetical protein with a C-terminal homeodomain (NCU00733) and hypothetical protein with a peroxisome membrane anchored protein conserved region (NCU04555) which strongly decreased in expression levels in *N. crassa* conidia treated with chitosan. Cluster 4M showed a steady increase of gene expression (Fig. 4D). Genes in this cluster were involved in cell response to oxidative stress (NCU05134 and NCU08907) and a monosaccharide transporter perhaps involved in chitosan assimilation or detoxification (NCU04537, fold-induction 9.27 after 16h growing with chitosan). Besides, other genes related with sugars assimilation were also induces in presence of chitosan such as NCU01633 (*hxt13*; Table S2). Clusters 2M and 3M, had gene expression changes in the control but not in the chitosan treatment (Figs. 4B and 4C). Genes in these clusters were mainly related with fungal reproduction and development and response to oxidative stress. Cluster 2M included two genes associated with membrane homeostasis: NCU03494 (*pin-c*) essential for non-self-recognition and NCU10610, a protein with a *fig 1* domain (Ca²⁺ regulator and membrane fusion) related with cell fusion.

Genes which did not fit the model (NM), with high SPE and leverage in the ASCA analysis, were grouped in 6 clusters including 474 genes (Fig. S6). Cluster 5NM which showed a late activation in presence of chitosan, included genes such as NCU10521 (fold-induction 8.16 at 16h) a glutathione S-transferase-4 possibly involved in the generation of reducing power for scavenging intracellular ROS. Other genes involved in ROS assimilation were also induced at 16h such as NCU05780 (*gst-1*; Table S2). Cluster 3NM included expression of genes such as NCU08770 a hypothetical protein with a histone chaperone domain with slight changes of expression in presence of chitosan (Fig. S6). Cluster 4NM included genes with an early induction (4-8h) and then a reduction of gene expression such as NCU03639, a lipase class 3 involved in lipid, fatty acids and isoprenoid metabolism. The overexpression of this gene suggests its role in plasma membrane homeostasis during chitosan damage.

Other significantly expressed (more than 6 fold-change expression, Table S2) genes in response to chitosan related with the main functions described previously included NCU03213 encoding a predicted mannosyl-phosphorylation protein related with phosphocholine metabolism (lipid modification). Early induction of other genes related with predicted roles in lipid metabolism such as NCU16960 (geranyl reductase) putative involved in the biosynthesis of plasma membrane lipids were also detected.

***N. crassa* deletion strains involved in membrane homeostasis and ROS detoxification showed increased sensitivity to chitosan**

Fifteen deletion strains of genes highly expressed and associated with enriched GO-terms in response to chitosan were evaluated to identify gene targets in *N. crassa*. Five deletion strains showed increased sensitivity to chitosan (Fig. 5 and Fig. S7). NCU03639 (lipase) and NCU04537 (monosaccharide transporter) were the most sensitive. These deletion strains exhibited a minimal inhibitory concentration (MIC, 3 $\mu\text{g ml}^{-1}$) lower than the WT (MIC 6 $\mu\text{g ml}^{-1}$; Fig. 5A). They also showed a 6-8h delay in the start of the exponential growth phase at 2 $\mu\text{g ml}^{-1}$ of chitosan in comparison to the WT (Figs. 5B-5D). Furthermore, NCU10521 (glutathione S-transferase), NCU08907 Clock controller gene 13 (*cgc-13*) and NCU07840 (plasma membrane protein with a *het* domain) were moderately (MIC at 4 $\mu\text{g ml}^{-1}$) sensitive to chitosan (Fig. 5A). These strains showed a 6-12h delay in the start of exponential growth phase with respect to WT at 3 $\mu\text{g ml}^{-1}$ of chitosan (Fig. S7).

NCU10610 (Ca^{2+} regulator with *fig 1* domain) showed the same MIC as WT (6 $\mu\text{g ml}^{-1}$), but had a delay (8h) in the start of exponential growth phase at 4 $\mu\text{g ml}^{-1}$ chitosan (Fig. S7 and Table S3). Conversely, NCU02363 (RTA1 like-protein) and NCU05134 (hypothetical protein) with the same MIC as the WT, started their exponential phases 7 and 16h earlier than WT (Fig. S7 and Table S3) indicating moderate tolerance of chitosan respect to WT. NCU08770 (hypothetical protein with a histone chaperone domain CHZ) had increased resistance to chitosan (MIC > 6 $\mu\text{g ml}^{-1}$; Fig. 5E). The start of the exponential growth phase in this deletion strain was 15h earlier than WT at 4 $\mu\text{g ml}^{-1}$ of chitosan (Table S3).

Thirteen deletion strains (mating type a) were crossed to WT (mating type A) to assess meiotic segregation of chitosan sensitivity phenotype with the hygromycin marker.

Segregants of each mutant showed similar chitosan sensitivity than the original deletion strain. In four chitosan gene targets (NCU03639, NCU04537, NCU07840 and

NCU10521), segregants showed the same chitosan antifungal phenotype (MIC) and hygromycin resistance than the original deletion strains.

Ca^{2+} protects *N. crassa* conidia from chitosan damage

Ca^{2+} increased tolerance to chitosan in *N. crassa* (Fig. 6). The WT strain at 0.68 mM CaCl_2 with 0.5 $\mu\text{g ml}^{-1}$ chitosan resumed growth 4h earlier than without Ca^{2+} (Figs. 6A). A higher level of CaCl_2 (2.72 mM) in the presence of 0.5 $\mu\text{g ml}^{-1}$ chitosan further improved fungal growth with a 7h advance in the start of the exponential phase with respect to *N. crassa* with chitosan and no calcium (Fig. 6A). Increasing CaCl_2 concentrations with no chitosan did not affect fungal growth (data not shown).

Conidia in calcium-free medium treated with chitosan ($0.5 \mu\text{g ml}^{-1}$) were stained (Fig. 6B) with the vital dye propidium iodide (PI) indicating cell mortality. On the contrary, conidia treated with both chitosan ($0.5 \mu\text{g ml}^{-1}$) and calcium chloride (0.68 mM), this showed no staining remaining alive (Fig. 6C). Similar results were found when increasing chitosan concentrations (Fig. S8). In particular, 0.5 , 2.5 and $5 \mu\text{g ml}^{-1}$ chitosan and CaCl_2 treated cells had significantly ($p\text{-value} < 0.05$) lower mortality than conidia treated with chitosan but no calcium.

Treatment with Ca^{2+} also reduced chitosan damage in deletion strain in the locus

NCU10610 with a *fig 1* domain and NCU03263 (*syt-1*), both associated with plasma membrane remodeling (Figs. 6D and 6E). Increasing CaCl_2 concentration (10 mM to 20 mM) significantly improved growth of WT, NCU10610 and NCU03263 strains in a medium amended with a high amount of chitosan ($4 \mu\text{g ml}^{-1}$; Figs. 6D and 6E). With less concentration of Ca^{2+} in the medium (0.68 mM), chitosan completely inhibited fungal growth. NCU10610 showed more tolerance to chitosan respect to WT, this strain started exponential phase at 27h, whereas WT strain did so 3h later under the same conditions.

NCU03263 was most sensitive to chitosan with high amount of calcium, starting the exponential phase after 35h, with slower growth than WT and NCU10610. When $[\text{CaCl}_2]$ was increased (20 mM) all strains tested showed higher resistance to chitosan (Fig. 6E). This was especially relevant for NCU03263 which showed a ca. 2 fold growth increase under these conditions (Fig. 6E).

We found in this work that chitosan significantly induced changes of expression of 5% of *N. crassa* genes in the genome. A global Cytoscape network showed membrane and transport as key nodes grouping genes affected by chitosan (Fig. 7). Plasma membrane was connected with cell vesicles and cell wall suggesting the importance of these outer structures and their dynamics in presence of chitosan. Oxidoreductase enhanced node indicated the importance of ROS and cell energy in *N. crassa* response to chitosan.^{4,7} Several nodes related with cytoskeleton dynamics indicate that chitosan also affects cell growth (Fig. 7). Other transcriptional studies, using *S. cerevisiae* mutant collections determined genes associated with plasma membrane, respiration, ATP production and mitochondrial organization as main targets of chitoooligosaccharides.^{5,6}

In this study, we demonstrated that exposure to chitosan increased the expression of genes involved in plasma membrane dynamics such as lipases. Imidazoles and triazoles (e.g. fluconazole, voriconazol and others) mode of action is based on the ergosterol biosynthesis inhibition,^{25,26} thereby altering plasma membrane fluidity. Chitosan is also an antifungal affecting plasma membrane. Fungi with enriched unsaturated free fatty acids in their plasma membrane (increased fluidity) are sensitive to chitosan (e.g. *N. crassa*). In contrast, fungi with less unsaturated free fatty acids in their membranes (low fluidity) such as the nematophagus fungus *Pochonia chlamydosporia*, are resistant to chitosan.^{8,27} In our work, we show that chitosan activates genes related with plasma membrane homeostasis such as the class 3 lipase NCU03639 (Fig. 8). The increase on chitosan sensitivity of NCU03639 deletion strain and the induction of genes related with free fatty acid plasma membrane remodeling such as NCU16960 (geranyl reductase), suggests their role in lipid replacement. This group of genes is mainly associated with plasma membrane stabilization by changes in

free fatty acid composition caused by other abiotic stresses.²⁸ Furthermore, chitosan also activated genes related with vesicular transport, which is associated with lipid transfer.²⁹

Moreover, chitosan also induced expression of *N. crassa* genes related with movement of molecules through plasma membrane such as MFS transporters. The activation of a monosaccharide transporter and other genes related with exchange of molecules is one of the general responses of *N. crassa* to chitosan. Transport activation is a widely described response of several filamentous fungi and yeast in response to antifungals.³⁰ *C. albicans* activates genes involved in transport and molecule trafficking in presence of ketoconazole.³¹ Susceptibility to azoles has been likely found due to a reduced efflux activity of pumps.³² Likewise, amphotericin B induces expression of high-affinity glucose transporters (MFS transporters) and permeases encoding genes in *S. cerevisiae*.³⁰ In our study, *N. crassa* NCU04537 deletion strain, encoding a monosaccharide transporter, showed an increase in chitosan sensitivity, suggesting a determinant role of this protein in the assimilation of glucosamine and N-acetyl glucosamine monomers.³³

Currently used antifungals, as well as chitosan, induce intracellular oxidative stress affecting plasma membrane permeability. This may be associated with an imbalance of intracellular redox state.^{4,10} An increase in the intracellular ROS is a general response to several antifungals and antimicrobial peptides which target the plasma membrane.^{9,34} We have also recently demonstrated that chitosan elicited a rise in ROS coincident with the start of plasma membrane permeabilization.⁴ In this paper we have demonstrated that chitosan induced the expression of genes encoding mono- and dioxygenases and other proteins related with ROS homeostasis. Other antifungals (e.g. rotenone and staurosporine) also increase levels of intracellular oxidative stress associated with subsequent cellular death.^{35,36} Increase in associated ROS by chitosan could induce plasma membrane free fatty acid oxidation and formation of oxylipins.³⁷ These would damage plasma membrane and cause its subsequent permeabilization.³⁸ In our study, when NCU10521, encoding a glutathione S-transferase (GST), was eliminated sensitivity of *N. crassa* to chitosan increased. GST is known to deaden ROS by-products such as peroxidized lipids.³⁹ This suggests a link between ROS and membrane damage in the mode of action of chitosan (Fig. 8). Other antifungals also induce glutathione enzymes to reduce intracellular ROS levels in *N. crassa*.⁴⁰

We have discovered that chitosan inhibits gene functions related with cytoskeleton dynamics such as microtubule organization and motor activity. Increased levels of intracellular ROS in *Magnaporthe oryzae* caused F-actin depolymerization affecting hyphal polar growth.⁴¹ In *N. crassa* deletion of a NOX gene encoding a NADPH oxidase results in reduction of hyphal growth.⁴² These observations support the hypothesis that an increase in intracellular ROS causes an abnormal distribution of F-actin. Cytoskeleton disorganization could then be one of the mechanisms by which chitosan inhibits fungal growth. The oxidative stress and associated phenomena such as free fatty acid peroxidation or F-actin polymerization could be directly involved in chitosan antifungal activity.

It is known that the balance between Ca^{2+} and ROS affects intracellular signaling and cell homeostasis.⁴³ We have demonstrated that Ca^{2+} is involved in *N. crassa* tolerance to chitosan. Ca^{2+} is also involved in the increasing threshold of *N. crassa* to antifungals such as

staurosporine.⁴⁴ Calcium plays a role in the mechanisms of plasma membrane remodeling in *S. cerevisiae* budding⁴⁵ and during cell fusion in *N. crassa*.^{14,17} In this work, we report NCU10610 (Ca²⁺ regulator with *fig 1* domain) significantly repressed by chitosan. The presence of a *fig 1* domain suggests its role as Ca²⁺ regulator in cell fusion. In view of the relevance of this phenomenon in plasma membrane remodeling, we have also evaluated the role of SYT1 in the mechanisms of plasma membrane remodeling mediated by Ca²⁺. SYT1 may be involved in membrane damage restored during fusion of germlings in *N. crassa*.¹⁴ In our study *sytl* had increased sensitivity to chitosan. When *sytl* was exposed to chitosan together with Ca²⁺ (10 mM) we found increased sensitivity of this deletion strain to chitosan respect to WT. This would be associated with the capability of this gene to trigger mechanisms of plasma membrane damage repair mediated by Ca²⁺. Besides, high levels of extracellular Ca²⁺ (20 mM), highly reduced chitosan damage in *sytl*. This deletion strain grow with the same fitness that the WT under these conditions. This would be associated with the activation of other *N. crassa* genes involved in plasma membrane remodeling mediated by Ca²⁺. Our results would suggest the importance of Ca²⁺ on the mechanisms of plasma membrane remodeling after chitosan damages.

Conclusion and outlook

This work provides the first study of the gene expression response of a filamentous fungus (*N. crassa*) to chitosan. Transcriptomics revealed oxidoreductase activity, membrane homeostasis and microtubule organization as the main gene functions differentially expressed. We identified a class 3 lipase, a MFS monosaccharide transporter and a glutathione transferase as main gene targets of chitosan in *N. crassa*. Our study opens new possibilities to study gene pathways involved in membrane remodeling after chitosan damage with a relevant role of Ca²⁺. These studies are a key step to develop chitosan as antifungal drug in the future. Our results could help to identify the main gene targets of chitosan in medical important fungi.

Methods

Growth conditions

Neurospora crassa wild-type strain was 74-OR23-IVA (FGSC2489) and the deletion strains were generated by the *Neurospora* Genome Project^{46,47} and kindly provided by the Fungal Genetics Stock Center (FGSC, Kansas, USA)⁴⁸ are shown in Table S1. Strains were grown on Vogel's minimal medium agar (VMMA) (1x Vogel's salts, 2% sucrose and 1.5% technical agar).

Chitosan

A medium molecular weight chitosan (70 kDa) with an 82.5% deacetylation degree (T8s; Marine BioProducts GmbH; Bremerhaven, Germany) was used. Chitosan was prepared as described in Palma-Guerrero *et al.*, 2008.²⁷

Germinating conditions and time-course of *N. crassa* sensitivity to chitosan

To determine the optimal medium to assess the behavior of *N. crassa* exposed to chitosan, three variants of the Vogel's minimal medium were evaluated (VMM). These media were standard VMM (1x salts, 2% sucrose), VMM salts diluted 100 times with 2% sucrose and VMM salts diluted 100 times and 0.02% sucrose. We finally adopted the second one because chitosan precipitated with some salts included in standard VMM. Time-course experiments of germination were assessed every 2h for 24h under continuous light, shaking at 200 rpm and 25°C.

N. crassa conidia sensitivity to chitosan was evaluated using selected media, with sub-lethal concentrations of chitosan (0.1-1 µg ml⁻¹). The percentage of *N. crassa* conidial germination with chitosan for 2, 4, 6, 8, 10, 12 and 16h after inoculation was measured. We selected a chitosan dose that resulted in a 50% inhibition of germination respect to the control (IC₅₀).

RNA extraction and cDNA synthesis

From *N. crassa* cultures in contact with chitosan and controls (without chitosan) for 4, 8 and 16h total RNA was isolated using TRIzol reagent (Life Tech) according to the manufacturer's instructions. RNA was then treated with DNase (Turbo DNA-free, Ambion) to eliminate DNA remains. For poly (A+) RNA purification, 10 µg of total RNA was bound to dynal oligo (dT) magnetic beads (Invitrogen) twice, using the manufacturer's instructions. Purified poly (A+) RNA was fragmented by metal-ion catalysis (Ambion) followed by precipitation with 1/10 vol 3M sodium acetate and 3× vol 100% ethanol. Precipitated RNA was 70% ethanol washed and then resuspended into 10.5 µl nuclease free water. For first strand cDNA synthesis, the fragmented poly (A+) RNA was incubated with 3 µg random hexamers (Invitrogen), incubated at 65°C for 5 min and then transferred to ice. First strand buffer (4 µL; Invitrogen), Dithiothreitol (DTT), dNTPs and RNaseOUT (Invitrogen) were added to a final concentration of 1×, 10 mM, 200 µM and 1U/µL, respectively in a final volume of 20 µl and the samples were incubated at 25°C for 2 minutes. Superscript II (200 U; Invitrogen) were added and the samples were incubated at 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. For second strand synthesis, 51 µL of H₂O, 20 µL of 5× second strand buffer (Invitrogen), and dNTPs (10 mM) were added to the first cDNA strand synthesis mix and incubated on ice for 5 min. RNaseH (2 U; Invitrogen), DNA pol I (50 U; Invitrogen) were then added and the mixture was incubated at 16°C for 2.5h.

Library construction and sequencing

End-repair was performed by adding 45 µL of H₂O, T4 DNA ligase buffer with 10 mM ATP (NEB; 10 µL), dNTP mix (10 mM), T4 DNA polymerase (15 U; NEB), Klenow DNA polymerase (5 U; NEB), and T4 PNK (50 U; NEB) to the sample and incubating for 30 min at 20°C. A single base was added each to cDNA fragment by adding Klenow buffer (NEB), dATP (1 mM), and Klenow 3' to 5' exo- (15 U; NEB). The mixture was then incubated at 37°C for 30 min. Standard Illumina adapters (FC) were ligated to the cDNA fragments using 2× DNA ligase buffer (Enzymatics), 1 µL of adapter oligo mix and DNA ligase (5 U; Enzymatics). The sample was incubated at 25°C for 15 min. The sample was purified in a 2% low-melting point agarose gel, and a slice of gel containing 200-bp fragments was

removed and the DNA purified. The polymerase chain reaction (PCR) was used to enrich the sequencing library. A 10 µL aliquot of purified cDNA library was amplified by PCR using the pfx DNA polymerase (2 U; Invitrogen) and with 1 µl of genomic primers 1.1 and 2.1 (Illumina). PCR cycling conditions included a denaturing step at 98°C for 30 sec, 12 cycles of 98°C for 10 sec, 65°C for 30 sec, 68°C for 30 sec, and a final extension at 68°C for 5 min. All libraries were sequenced on a HiSeq 2000 platform to a depth of over 190 million 50 bp reads using standard Illumina operating procedures.

Transcript abundance, annotation and functional analysis

Sequenced libraries were mapped against predicted transcripts from the *Neurospora crassa* OR74A genome (v10) with TopHat (v2.0.4)⁴⁹ and the short sequence aligner Bowtie (v2.0.0.6).⁵⁰ Transcript abundance measured as FPKMs (Fragments Per Kilobase transcript model per Million fragments mapped) was calculated with Cufflinks (v 2.0.2) using counts that exclusively mapped to predicted transcripts to estimate the FPKM denominator. Genes which had a differential expression cut-off of p -value < 0.05 (we adjusted p -value as the Benjamini Hochberg filter; q value in TopHat; to adjust for the false discovery rate) between control and sample were used for further analysis. In the fold change analysis a $\log_2$ foldchange ≥ 2 was adopted to characterize the main gene functions and genes involved in the response of *N. crassa* to chitosan. The project of *N. crassa* gene expression profile in response to chitosan has been deposited in NCBI's Gene Expression Omnibus⁵¹ and is accessible through GEO Series accession number GSE75293 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75293>).

N. crassa transcript sequences were re-annotated using Blast2GO software (Version 2.7.1) to improve the standard annotation provided by the Broad *N. crassa* genome (<http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html>), a consensus set of transcripts were functionally annotated (gene ontology, GO) using Blast2GO (<http://www.blast2go.com/b2ghome>).⁵² Gene families were established using the InterPro (<http://www.ebi.ac.uk/interpro>) and KEGG databases (<http://www.genome.jp/kegg/pathway.html>). For *N. crassa* gene annotation we also used several tools, HMMER⁵³ including Pfam, TIGRFAM, Gene 3D and Superfamily databases. In addition, Wolf PSORT⁵⁴ was used to obtain information about domains and cellular gene localizations. Gene annotations were finally examined using BLASTp.⁵⁵

RNA-seq time-series data analysis

Significant differential gene expression changes over time were assessed by applying the maSigPro R package²¹ to the groups of genes included in each functional GO category. This approach was described, as an adaptation of maSigPro⁵⁶ named maSigFun²⁴ for microarray data. This algorithm has been updated for RNA-seq data in this work. The maSigPro method follows a two-stage regression strategy to identify genes with significant changes in expression over time. False discovery rate (FDR) and R^2 level as measure of the good of fit of the regression model are the factors for gene selection. Finally the package includes several clustering algorithms and visualization tools available to group and display the selected gene-profiles.

Transcriptional responses of interest were detected with the application of ASCA-genes method.²² Considering an experiment with 2 factors (a and b, usually time and the experimental group, in our case chitosan treatment), data can be collected in a data matrix X , where rows represent samples and columns represent genes. ASCA first decomposes X into matrices (X_a , X_b and X_{ab}) with the estimates of the ANOVA (Analysis of Variance) parameters: X_a contains the time effects, X_b treatment effects and X_{ab} the interactions, obtained gene by gene. When the main interest of a study is the identification of genes with differences in the experimental groups, X_b is joined to X_{ab} . Principal Component Analysis (PCA) is then applied on each of these matrices to summarize the information of each source of variation and giving as a result two PCA analyses that are called submodels. ASCA-genes compute the main patterns of variation and two statistics for each gene in each submodel: leverage and the squared-prediction error (SPE). Leverage indicates the importance of a gene in the main behavior discovered. SPE quantifies the variability of a gene that is not detected for the model. Focusing on these measures, ASCA-genes provides two lists of genes: the first one with genes that follow the main general patterns. The second one including genes with odd behaviors or outlier data. To obtain this gene selection the gamma method⁵⁷ was applied.

Real time quantitative PCR for RNA-seq validation

cDNA was synthesized with a retro-transcriptase RevertAid (Thermo) using oligo dT (Thermo). Gene expression was quantified using real-time reverse transcription PCR (qRT-PCR), SYBR Green with ROX (Roche) were used following the manufacturer's instructions. Gene quantifications were performed in a Step One Plus real-time PCR system (Applied Biosystems). Relative gene expression was estimated with the C_t methodology,⁵⁸ with three technical replicates per condition. Primers used to quantify the expression of genes related with *N. crassa* response to chitosan are shown in Table S4. Expression of the TATA-binding protein (NCU04770) and transcription elongation factor S-II (NCU02563) were used as endogenous controls for all experiments, since these genes showed C_t stability for all conditions tested.

Evaluation of selected deletion strains to determine the genes involved in the response of *N. crassa* to chitosan

Experiments in liquid media were set to evaluate growth kinetics of *N. crassa* (WT) and selected homokaryons deletion strains (Table S1). *N. crassa* conidia were obtained from 8-10 day-old sporulated cultures, by adding 2 ml of distilled water. The resulting conidial suspensions were then filtered through Miracloth (Calbiochem) to remove hyphal fragments. Conidial suspensions were adjusted to a final concentration of 10^6 conidia ml^{-1} with 1/100 VMM salts and 2% sucrose.

Chitosan ($1\text{--}6 \mu\text{g ml}^{-1}$) was added to the medium and 200 μL per well were dispensed into 96 well microtiter plates (Sterillin Ltd., Newport, UK). Plates were inoculated with *N. crassa* conidia (2×10^5 conidia per well) and then incubated at 25°C during 48h in a GENios™ multiwell spectrophotometer (Tecan, Männedorf, Switzerland) in the dark. The chitosan effect on growth of *N. crassa* strains was evaluated by measuring optical density at 490 nm (OD_{490}).⁴ In order to identify the antifungal activity of chitosan on *N. crassa* strains,

we applied a spot assay in SFG medium (2% sorbose, 0.05% glucose and fructose and 1.5% agar).⁵⁹

Growth in presence of the same concentration of deletion strains (mating type a) to chitosan was confirmed by segregation analysis.⁶⁰ Ascospore progeny were selected from crosses with FGSC 2489 (mating type A). Segregants were tested both for chitosan and hygromycin sensitivity. The latter was tested in all deletion strains used in this work. Segregants had the same chitosan sensitivity than the original deletion strain and were hygromycin (200 µg ml⁻¹) resistant.

Evaluation of the effect of Ca²⁺ in the response of *N. crassa* to chitosan

To evaluate the effect of Ca²⁺ on conidia treated with chitosan, we exposed *N. crassa* conidia (10⁶ conidia ml⁻¹) to chitosan (0.5 µg ml⁻¹) with either 0.17; 0.34; 0.68; 1.36 or 2.72 mM CaCl₂. Growth kinetics was evaluated in a 96-multiwell microplate by measuring optical density at 490 nm for 48h, as described above.

Viability of conidia was determined using propidium iodide (PI; Sigma)⁷ after exposure to chitosan (0.5 µg ml⁻¹), and CaCl₂ at 0.68 mM, conidia without CaCl₂ were used as a controls for this compound. *N. crassa* conidia were treated with chitosan for 2 h and then labeled with 2 µg ml⁻¹ PI to evaluate cell viability. Fluorescence in conidia was assessed using an Olympus BH-2 fluorescence microscope with 488 nm and 560 nm as excitation and detection wavelengths, respectively, and then photographed with a Leica DFC480 digital camera (Leica Microsystems, Wetzlar, Germany).

The effect of higher concentrations of Ca²⁺ (10 and 20 mM) on WT and two deletion strains, NCU10610 (Ca²⁺ regulator with *fig 1* domain) and NCU03263 (*syt1*) when combined with chitosan (4 µg ml⁻¹) was also determined.

Cytoscape network of functional gene annotation of *N. crassa* gene response to chitosan

For this analysis, we performed functional enrichment analysis with GSEA (Gene Set Enrichment Analysis).⁶¹ The enrichment maps were generated with Enrichment Map Plugin v1.1⁶² developed for Cytoscape.²³ Nodes in the maps were clustered with the Markov clustering algorithm, using an overlap coefficient computed by the plugin as the similarity metric (coefficient < 0.5 were set to zero) and an inflation parameter with value of 2. For each cluster, the leading edge was computed as in Subramanian *et al.* (2005)⁶¹ for each member of a node. A complete functional gene network map of *N. crassa* in response to chitosan was finally generated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kumar RMNV. *React. Funct. Polym.* 2000; 46(1):1–27.
2. Allan CR, Hadwiger LA. *Exp. Mycol.* 1979; 3(3):285–287.
3. Kulikov SN, Lisovskaya SA, Zelenikhin PV, Bezrodnykh EA, Shakirova DR, Blagodatskikh IV, Tikhonov VE. *Eur. J. Med. Chem.* 2014; 74:169–178. [PubMed: 24462847]
4. Lopez-Moya F, Colom-Valiente MF, Martinez-Peinado P, Martinez-Lopez JE, Puelles E, Sempere-Ortells JM, Lopez-Llorca LV. *Fun. Biol.* 2015; 119(2-3):154–169.
5. Jaime MDLA, Lopez-Llorca LV, Conesa A, Lee AY, Proctor M, Heisler LE, Gebbia M, Giaever G, Westwood JT, Nislow C. *BMC Genomics.* 2012; 13(1):267. [PubMed: 22727066]
6. Zakrzewska A, Boorsma A, Brul S, Hellingwerf KJ, Klis FM. *Eukaryot. Cell.* 2005; 4(4):703–715. [PubMed: 15821130]
7. Palma-Guerrero J, Huang IC, Jansson H-B, Salinas J, Lopez-Llorca LV, Read ND. *Fungal Genet. Biol.* 2009; 46(8):585–594. [PubMed: 19389478]
8. Palma-Guerrero J, Lopez-Jimenez JA, Pérez-Berná AJ, Huang I, Jansson H-B, Salinas J, Villalaín J, Read ND, Lopez-Llorca LV. *Mol. Microbiol.* 2010; 75(4):1021–1032. [PubMed: 20487294]
9. Ghannoum MA, Rice LB. *Clin. Microbiol. Rev.* 1999; 12(4):501–517. [PubMed: 10515900]
10. François IEJA, Cammue BPA, Borgers M, Ausma J, Dispersyn GD, Thevissen K. *Antimicrob. Agents Med. Chem.* 2006; 5(1):3–13.
11. Delattin N, Cammue BP, Thevissen K. *Future Med. Chem.* 2014; 6(1):77–90. [PubMed: 24358949]
12. Andrews NW, Almeida PE, Corrotte M. *Trends Cell Biol.* 2014; 24(12):734–742. [PubMed: 25150593]
13. Brewer KD, Bacaj T, Cavalli A, Camilloni C, Swarbrick JD, Liu J, Zhou A, Zhou P, Barlow N, Xu J, Seven AB, Prinslow EA, Voleti R, Häussinger D, Bonvin AM, Tomchick DR, Vendruscolo M, Graham B, Südhof TC, Rizo J. *Nat. Struct. Mol. Biol.* 2015; 22(7):555–564. [PubMed: 26030874]
14. Palma-Guerrero J, Leeder AC, Welch J, Glass NL. *Mol. Microbiol.* 2014; 92(1):164–182. [PubMed: 24673848]
15. Gonçalves AP, Cordeiro JM, Monteiro J, Lucchi C, Correia-de-Sá P, Videira A. *Biochim. Biophys. Acta.* 2015; 1847(10):1064–1074. [PubMed: 26004627]
16. Muller EM, Mackin NA, Erdman SE, Cunningham KW. *J. Biol. Chem.* 2003; 278(40):38461–38469. [PubMed: 12878605]
17. Fleissner A, Diamond S, Glass NL. *Genetics.* 2009; 181(2):497–510. [PubMed: 19064710]
18. Cavinder F B. Trail, *Eukaryot. Cell.* 2012; 11(8):978–988. [PubMed: 22635922]
19. Curto MÁ, Sharifmoghdam MR, Calpena E, De León N, Hoya M, Doncel C, Leatherwood J, Valdivieso MH. *Genetics.* 2014; 196(4):1059–1076. [PubMed: 24514900]
20. Palma-Guerrero J, Zhao J, Gonçalves AP, Starr TL, Glass NL. *Eukaryot. Cell.* 2015; 14(3):265–277. [PubMed: 25595444]
21. Nueda MJ, Tarazona S, Conesa A. *Bioinformatics.* 2014; 30(18):2598–2602. [PubMed: 24894503]
22. Nueda MJ, Conesa A, Westerhuis JA, Hoefsloot HCJ, Smilde AK, Talón M, Ferrer A. *Bioinformatics.* 2007; 23(14):1792–1800. [PubMed: 17519250]
23. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. *Bioinformatics.* 2011; 27(3):431–432. [PubMed: 21149340]
24. Nueda MJ, Sebastián P, Tarazona S, García-García F, Dopazo J, Ferrer A, Conesa A. *BMC-Bioinfo.* 2009; 10(6):S9.
25. Odds FC, Brown AJ, Gow NA. *Trends Microbiol.* 2003; 11:272–279(6)
26. Carrillo-Muñoz AJ, Giusiano G, Ezkurra PA, Quindós G. *Rev. Esp. Quimioter.* 2006; 19(2):130–139. [PubMed: 16964330]

27. Palma-Guerrero J, Jansson H-B, Salinas J, Lopez-Llorca LV. *J. Appl. Microbiol.* 2008; 104(2): 541–553. [PubMed: 17927761]
28. Nguyen LN, Gacser A, Nosanchuk JD. *Virulence.* 2011; 2(6):538–541. [PubMed: 22030857]
29. Schauder CM, Wu X, Saheki Y, Narayanaswamy P, Torta F, Wenk MR, De Camilli P, Reinisch KM. *Nature.* 2014; 510(7506):552–555. [PubMed: 24847877]
30. Agarwal AK, Rogers PD, Baerson SR, Jacob MR, Barker KS, Cleary JD, Walker LA, Nagle DG, Clark AM. *J. Biol. Chem.* 2003; 278(37):34998–5015. [PubMed: 12824174]
31. Hoehamer CF, Cummings ED, Hilliard GM, Rogers PD. *Antimicrob. Agents. Chemother.* 2010; 54(5):1655–1664. [PubMed: 20145080]
32. Thomas E, Roman E, Claypool S, Manzoor N, Pla J, Panwar SL. *Antimicrob. Agents. Chemother.* 2013; 57(11):5580–99. [PubMed: 23979757]
33. Mobley HL, Doyle RJ, Streips UN, Langemeier SO, Bacteriol J. 1982; 150(1):8–15.
34. Hayes BM, Anderson MA, Traven A, van der Weerden NL, Bleackley MR. *Cell. Mol. Life Sci.* 2014; 71(14):2651–2666. [PubMed: 24526056]
35. Castro A, Lemos C, Falcão A, Fernandes AS, Glass NL, Videira A. *Eukaryot. Cell.* 2010; 9(6): 906–914. [PubMed: 20435699]
36. Gonçalves AP, Hall C, Kowbel DJ, Glass NL, Videira A. *G3 (Bethesda).* 2014; 4(6):1091–102. [PubMed: 24717808]
37. Brodhun F, Feussner I. *FEBS J.* 2011; 278(7):1047–1063. [PubMed: 21281447]
38. Singh A, Del Poeta M. *Cell Microbiol.* 2011; 13(2):177–85. [PubMed: 21091925]
39. Yang Y, Cheng JZ, Singhal SS, Saini M, Pandya U, Awasthi S, Awasthi YC. *J. Biol. Chem.* 2010; 276(22):19220–19230. [PubMed: 11279091]
40. Fernandes AS, Castro A, Videira A. *Apoptosis.* 2013; 18(8):940–8. [PubMed: 23666236]
41. Ryder LS, Dagdas YF, Mentlak TA, Kershaw MJ, Thornton CR, Schuster M, Chen J, Wang Z, Talbot NJ. *Proc. Natl. Acad. Sci. USA.* 2013; 110(8):3179–3184. [PubMed: 23382235]
42. Cano-Domínguez N, Álvarez-Delfín K, Hansberg W, Aguirre J. *Eukaryot. Cell.* 2008; 7(8):1352–1361. [PubMed: 18567788]
43. Yan Y, Wei CL, Zhang WR, Cheng HP, Liu J. *Acta Pharmacol. Sin.* 2006; 200627(7):821–826. [PubMed: 16787564]
44. Gonçalves AP, Monteiro J, Lucchi C, Kowbel DJ, Cordeiro JM, Correia-de-Sá P, Rigden DJ, Glass NL, Videira A. *Microbial Cell.* 2014; 1(9):289–302.
45. D'hooge P, Coun C, Van Eyck V, Faes L, Ghillebert R, Mariën L, Winderickx J, Callewaert G. *Cell Calcium.* 2015; 58(2):226–235. [PubMed: 26055636]
46. Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC. *Proc. Natl. Acad. Sci. USA.* 2006; 103(27):10352–10357. [PubMed: 16801547]
47. Dunlap JC, Borkovich KA, Henn MR, Turner GE, Sachs MS, Glass NL, McCluskey K, Plamann M, Galagan JE, Birren BW, Weiss RL, Townsend JP, Loros JJ, Nelson MA, Lambregts R, Colot HV, Park G, Collopy P, Ringelberg C, Crew C, Litvinkova L, DeCaprio D, Hood HM, Curilla S, Shi M, Crawford M, Koerhsen M, Montgomery P, Larson L, Pearson M, Kasuga T, Tian C, Ba türkmen M, Altamirano L, Xu J. *Adv. Genet.* 2007; 57:49–96. [PubMed: 17352902]
48. McCluskey K. *Adv. Appl. Microbiol.* 2003; 52:245–262. [PubMed: 12964247]
49. Trapnell C, Pachter L, Salzberg SL. *Bioinformatics.* 2009; 25(9):1105–1111. [PubMed: 19289445]
50. Langmead B, Trapnell C, Pop M, Salzberg SL. *Genome Biol.* 2009; 10(3):R25. [PubMed: 19261174]
51. Conesa A, Götz S. *Int. J. Plant Genomics.* 2008; 2008:619832. [PubMed: 18483572]
52. Edgar R, Domrachev M, Lash AE. *Nucl. Ac. Res.* 2002; 30(1):207–210.
53. Finn RD, Clements J, Eddy SR. *Nucl. Ac. Res.* 2011; 39:29–37.
54. Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K. *Nucleic. Acids. Res.* 2007; W585–587
55. Gish W, States DJ. *Nature Genet.* 1993; (3):266–272. [PubMed: 8485583]
56. Conesa A, Nueda MJ, Ferrer A, Talon M. *Bioinformatics.* 2006; 22(9):1096–1102. [PubMed: 16481333]

57. Tarazona S, Prado-López S, Dopazo J, Ferrer A, Conesa A. *Chemometr. Intell. Lab. Syst.* 2012; 100:113–122.
58. Livak KJ, Schmittgen TD. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
59. Brockman HE, de Serres FJ. *Genetics.* 1963; 48(4):597–604. [PubMed: 17248163]
60. Leeder AC, Jonkers W, Li J, Glass NL. *Genetics.* 2013; 195(3):883–898. [PubMed: 24037267]
61. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. *Proc. Natl. Acad. Sci. USA.* 2005; 102(43): 15545–15550. [PubMed: 16199517]
62. Merico D, Isserlin R, Stueker O, Emili A, Bader GD. *PLoS One.* 2010; 5(11):e13984. [PubMed: 21085593]

Figure 1.

Time-course effect of chitosan on *N. crassa* conidia germination. **(A)** *N. crassa* germination started prior to 4h then conidia develop a germ tube (6-8h) and established a young mycelium before 16h. **(B)** Effect of chitosan on conidia germination at 8h, IC_{50} (50% germination) was found at $0.5 \mu\text{g ml}^{-1}$ chitosan. IC_{50} : half maximal inhibitory concentration.

Figure 2.

Venn diagram of differential gene expression of *N. crassa* in response to chitosan. **(A)** Complete differential gene expression (DGE) including induced and repressed genes in the 4-16h time-course. **(B)** Increased DGE, up-regulated genes. **(C)** Decreased DGE, down-regulated genes. **(D)** Fold-change of 22 genes significantly differentially expressed in response to chitosan during the whole time-course experiment.

Figure 3.

Gene Ontology (GO) functional annotation of *N. crassa* genes differentially expressed in response to chitosan. **(A)** Global GO annotation of significantly expressed genes. **(B)** Selected GO-terms time-series with maSigFun represented as the average expression profile of the associated genes to each GO.

Figure 4.

(A-D) Time-series analysis of genes associated with the response of *N. crassa* to chitosan by ASCA-genes. Graphs represent gene expression average trend of four clusters of genes that follow the discovered general patterns of the ASCA model. Genes that are well represented by the PC obtained with the ASCA model.

A Bar chart showing the Minimum Inhibitory Concentration (MIC) in $\mu\text{g ml}^{-1}$ for 12 *N. crassa* strains. The y-axis ranges from 0 to 8. The x-axis lists the strains: WT, ΔnuaA , ΔnuaB , ΔnuaC , ΔnuaD , ΔnuaE , ΔnuaF , ΔnuaG , ΔnuaH , ΔnuaI , ΔnuaJ , and ΔnuaK . The MIC values are approximately: WT (6), ΔnuaA (6), ΔnuaB (6), ΔnuaC (6), ΔnuaD (2.5), ΔnuaE (2.5), ΔnuaF (6), ΔnuaG (6), ΔnuaH (4), ΔnuaI (4), ΔnuaJ (7), and ΔnuaK (6).

B Growth curve for WT *N. crassa* showing optical density (OD_{600}) over 50 hours. The y-axis ranges from 0.05 to 0.40. The x-axis ranges from 0 to 50 hours. The legend indicates: Control (red circles), 1 $\mu\text{g ml}^{-1}$ (orange squares), 2 $\mu\text{g ml}^{-1}$ (yellow triangles), 3 $\mu\text{g ml}^{-1}$ (green diamonds), 4 $\mu\text{g ml}^{-1}$ (blue inverted triangles), and 6 $\mu\text{g ml}^{-1}$ (dark blue circles). The control shows a rapid increase in optical density, reaching approximately 0.35 by 25 hours. The growth is inhibited in a dose-dependent manner by the treatment.

C Growth curve for $\Delta\text{NCU03639} *N. crassa* showing optical density (OD_{600}) over 50 hours. The y-axis ranges from 0.10 to 0.45. The x-axis ranges from 0 to 50 hours. The legend is the same as in B. The control shows a rapid increase in optical density, reaching approximately 0.40 by 45 hours. The growth is inhibited in a dose-dependent manner by the treatment.$

D Growth curve for $\Delta\text{NCU04537} *N. crassa* showing optical density (OD_{600}) over 50 hours. The y-axis ranges from 0.10 to 0.45. The x-axis ranges from 0 to 50 hours. The legend is the same as in B. The control shows a rapid increase in optical density, reaching approximately 0.38 by 45 hours. The growth is inhibited in a dose-dependent manner by the treatment.$

E Growth curve for $\Delta\text{NCU08770} *N. crassa* showing optical density (OD_{600}) over 50 hours. The y-axis ranges from 0.10 to 0.50. The x-axis ranges from 0 to 50 hours. The legend is the same as in B. The control shows a rapid increase in optical density, reaching approximately 0.45 by 45 hours. The growth is inhibited in a dose-dependent manner by the treatment.$

Figure 6.

Effect of Ca²⁺ on chitosan antifungal activity to *N. crassa* WT and deletion strains from membrane remodeling genes (NCU10610 and NCU03263-*syt1*). (A) *N. crassa* WT growth in response to chitosan (0.5 µg ml⁻¹) under several Ca²⁺ concentrations. (B) Nuclear damage after treatment of conidia of a strain in which PI has been targeted to the nuclei. Conidia treated with chitosan and stained with 2 µg ml⁻¹ propidium iodide (PI). Fluorescence images right and DIC images of same conidia on the left. Bar = 5 µm. (C) Evaluation of conidia viability treated with chitosan and Ca²⁺ stained with PI.

Figure 7. Cytoscape network of functional gene annotation of *N. crassa* gene response to chitosan. Large font titles represents a summary of GO-terms found enriched in clusters. Node size correlates to the number of genes annotated to that functional category. Each node represents a gene function significantly enriched (FDR 0.1).

Figure 8.

Key genes associated with *N. crassa* response to chitosan. In this model, NCU03639 would increase membrane permeability by altering mechanisms of plasma membrane remodeling and fluidity. NCU10610 (Ca^{2+} regulator with *fig 1* domain) would be associated with the mechanisms of plasma membrane remodeling mediated by Ca^{2+} . NCU04534 (MFS transporter) could be involved in mechanisms of assimilation or detoxification monosaccharides (e.g. monomers of N-acetyl glucosamine). NCU10521 (glutathione transferase), NCU01849 and NCU01071 (dioxygenases) would be related with the response

of the fungus to the oxidative stress, the key response of *N. crassa* to chitosan. Genes involved in mechanisms associated with protein synthesis (NCU04555) and resistance to chemical compounds (NCU02363) are also differentially expressed in response to chitosan.

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