Exploring plasma membrane stress response in Saccharomyces cerevisiae with functional genomics

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Exploring plasma membrane stress response in *Saccharomyces cerevisiae* with functional genomics

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Dla Moich Rodziców

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General Introduction

1. GENERAL INTRODUCTION

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1.1. *Saccharomyces cerevisiae* as a model system

Saccharomyces cerevisiae, also known as bakers' yeast, is a simple, unicellular eukaryotic organism that belongs to the phylum Ascomycota. It was one of the first microorganisms to be domesticated by humans, as it was, and still is, used for brewing alcoholic beverages and producing bread in ancient Sumeria and Egypt. The genome of *S. cerevisiae* is relatively small, 12.8 Mb; it comprises 16 chromosomes, encoding approximately 6000 ORFs. Its rapid growth, simple growth requirements and easy genetic manipulation have made it an ideal model organism for studies in fundamental biology and biotechnology. In fact, over the past 30 years, this organism was used by researchers to reveal much of what we know about how the eukaryotic cell works (Hughes *et al.*, 2004). Importantly, most of the cellular processes occurring in bakers' yeast are conserved in other Eukaryotes. Furthermore, a large number of pathogenic and food spoilage fungi belong to the phylum Ascomycota, and share many characteristics with *S. cerevisiae*.

Recently, it was discovered that both food spoilage and pathogenic fungi are developing increased resistance to the antifungal compounds that have been used by mankind for many years. This has led to a concerted research effort of industry and science to develop new antifungal strategies that will be safe for humans. The fact that *S. cerevisiae* is so similar to these fungi, along with the availability of a large number of modern genome-wide techniques for this organism, makes *S. cerevisiae* an excellent model system for studies of the effects of antifungal drugs and development of novel antifungal compounds.

1.2. Functional genomics studies in *Saccharomyces cerevisiae*

S. cerevisiae was the first eukaryotic organism, whose genome was completely sequenced (Goffeau *et al.*, 1996). Consequently, it became the key model organism for functional genomics studies (Grunenfelder and Winzeler, 2002). The availability of the entire genome sequence of bakers' yeast has made it possible to ask new kinds of research questions and has led to the development of various large-scale tools for studies in functional genomics (Table 1) (Dolinski and Botstein, 2005). The ultimate goal of functional genomics is to understand and model how gene activities are controlled, how genetic networks operate, how gene products act and are regulated, and how cellular homeostasis is maintained over the entire life cycle and over the range of environmental conditions experienced by the organism (Dolinski and Botstein, 2005).

1.2.1. Global expression analysis a.k.a. transcriptome analysis

DNA microarrays were developed in the mid to late 1990s and allow for simultaneous assessment of gene expression levels for the entire yeast genome. Whole-genome expression profiling provides a direct and simple way to identify genes whose expression alters in response to environmental stimuli and stresses. The key step in the analysis of genome-wide expression data was the development of clustering analysis, which

uses standard statistical algorithms to group genes according to similarity in their expression pattern and has a simple visual output (Eisen *et al.*, 1998).

Important examples, where genes with characteristic expression changes were systematically identified include transcriptome analysis of sporulation, the cell cycle, and identification of the environmental stress response (ESR) program, an expression program activated in the majority of stress conditions in yeast (Cho *et al.*, 1998; Chu *et al.*, 1998; Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002; Spellman *et al.*, 1998). In addition, Hughes *et al* (Hughes *et al.*, 2000) described the use of a large compendium of ~300 gene expression profiles to identify genes and pathways affected by bioactive compounds by means of two-dimensional hierarchical clustering. Finally, expression studies coupled with statistical methods allow for linking global transcription mechanisms to physiology. For example, a recent study reported large-scale modulation of transcriptional networks of mitochondrial and cytoplasmic ribosomal proteins that is connected to the capacity for anaerobic growth of *S. cerevisiae* (Ihmels *et al.*, 2005).

Table1. Functional Genomics Toolbox in *Saccharomyces cerevisiae.*
The literature references for these tools can be found in the text.

Tool	Usage
DNA microarrays	Assessment of gene expression levels over the entire genome
ChIP-chip microarrays	Analysis of *in vivo* binding of transcriptional regulators to the promoters of all yeast genes
Altered gene dosage collections	Testing the effect of gene dosage on phenotype in various environmental contexts
Homozygous and haploid non-essential deletion mutants	Determination of fitness of deletion mutants of all non-essential genes
Heterozygous strains of all genes	Testing fitness of strains where gene dosage of individual genes is reduced by 50%
Overexpression library	Investigating the effect of increased gene dosage on phenotype
SGA	Mapping synthetic genetic interactions among genes (both non-essential and essential)
Other	
GFP-tagged proteins library	Examination of localization and trafficking of all yeast proteins
Metabolic flux analysis	Quantification of flux responses to determine flexibility and optimal performance of the yeast metabolic network
Proteomics	Analysis of protein levels over the entire genome

1.2.2. Transcriptional regulatory networks

Recognition of specific promoter sequences by transcriptional regulatory proteins is crucial for induction or repression of gene expression programs. Recently, a method for

microarray analysis of promoter binding *in vivo* was established, named the ChIP-chip. This method involves chromatin immunoprecipitation of tagged transcriptional regulators bound to DNA fragments containing target promoters, followed by hybridization of the amplified DNA fragments to a microarray of intergenic regions (Iyer *et al.*, 2001; Ren *et al.*, 2000). This method allows for *in vivo* determination of transcription factor binding to promoter regions across the genome. In two major studies the binding of all known yeast transcriptional regulators to promoter regions was investigated for cells grown in rich medium and under 12 different stress conditions (Harbison *et al.*, 2004; Lee *et al.*, 2002). Several crucial findings were obtained; (a) individual yeast genes were found to be frequently regulated by a combination of transcription factors rather than just one transcription factor, (b) six various models of transcription regulation were identified, e.g. feed-back loop (c) environment-dependent activity of several transcription factors was described, (d) the architecture of yeast promoter regions was characterized, (e) and a number of specific sequence features for binding-sites for transcriptional regulators were discovered. These data facilitate the construction of global transcriptional regulatory networks in *S. cerevisiae*.

1.2.3. Genome-wide fitness analysis and gene dosage effects

The standard strategy in research of gene function is to analyze the phenotype resulting from gene perturbation, either by gene deletion or overexpression (Sopko *et al.*, 2006). In most cases the effect of gene dosage alteration can only be observed in a specific environmental context, in other words under suboptimal growth conditions.

A remarkable accomplishment of the bakers' yeast research community was the construction of the whole set of gene deletion mutants, covering both the essential (heterozygous pool) and non-essential (homozygous and haploid pool) genes of the *S. cerevisiae* genome (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). A striking feature of these mutants is the presence of unique sequence identifiers for each disrupted ORF that are flanked by sequences universal for the whole deletion collection. These characteristics allow simultaneous and quantitative evaluation of condition-specific growth defects of individual mutants on high density arrays of oligonucleotides capable of hybridization with the unique sequence identifiers.

The homozygous yeast deletion collection was tested for mutants' fitness in many types of stress in at least three ways. The growth rate of individual mutants was monitored, the colony size of each mutant was determined, and finally, by amplification of unique tags for each mutant, the abundance of all mutants was scored simultaneously on microarrays (Birrell *et al.*, 2001; Birrell *et al.*, 2002; Dudley *et al.*, 2005; Ericson *et al.*, 2006; Fernandez-Ricaud *et al.*, 2005; Giaever *et al.*, 2002; Warringer *et al.*, 2003; Wu *et al.*, 2004). The results of these genome-wide fitness studies led to identification of novel genes and functions involved in response to six well-studied environmental stress conditions; growth in minimal medium, galactose, sorbitol, salt, nystatin, and high pH stress, as well as helped to understand the

DNA-damage response in yeast (Birrell *et al.*, 2001; Birrell *et al.*, 2002; Brown *et al.*, 2006; Giaever *et al.*, 2002; Wu *et al.*, 2004). In addition, the compendium of mutants' colony size analysis in response to 21 conditions enhanced our understanding of the phenomenon of pleiotropy in yeast; pleiotropy being the ability of a single deletion mutant to cause multiple phenotypes (Dudley *et al.*, 2005).

Another method, termed chemical-genetic profiling, took advantage of haploid deletion mutants of all non-essential yeast genes, which were tested for hypersensitivity to a diverse collection of bioactive compounds. The resulting data was used to generate a compendium of chemical-genetic profiles (by clustering), which help to identify functions and pathways that buffer the cell against the toxic effects of these drugs and to interpret cellular effects of novel drugs (Parsons *et al.*, 2004; Parsons *et al.*, 2006).

The heterozygous deletion collection, including all essential genes, i.e. a collection that contains strains with a decreased copy number of each respective gene, was established as a tool for drug-induced haploinsufficiency screening. This method, based on the idea that lowering the gene dosage from two to one in a heterozygote results in hypersensitivity to a compound specifically targeting that gene, has been successfully used to confirm known targets of compounds and to identify the target genes of novel compounds (Giaever *et al.*, 1999; Lum *et al.*, 2004).

A recent study, that reported the construction of a genome-wide overexpression library of *S.cerevisiae*, revealed that ~ 15% of all yeast genes are toxic to the cell when overexpressed, as they cause an evident slow growth phenotype (Sopko *et al.*, 2006). Interestingly, the increase in gene dosage was found to be most toxic for genes involved in functions such as transport, organization of the cytoskeleton, secretion, the cell cycle, and transcriptional regulation. Finally, hierarchical clustering analysis of genome-wide fitness datasets of 51 diverse treatments was used to provide cues for the function of unknown genes (Brown *et al.*, 2006).

1.2.4. Synthetic genetic interactions

The effect of gene dosage can be studied not only in an environmental but also in a genetic context. The development of a high-throughput method, called SGA (synthetic genetic analysis), which allows for combining different gene deletions or loss-of-function alleles within a single cell has enabled large-scale mapping of genetic interactions. In such a screen, a deletion mutant of a query gene is crossed into an array of viable gene deletions, which generates an array of double mutants that can be scored for synthetic lethal or sick (reduced fitness) phenotypes. Such genetic interactions, where the combination of two mutants in one cell leads to death or decreased fitness, enables discovery of genes that participate in the same biological process or in two processes that functionally compensate for or buffer the defects of each other (Tong *et al.*, 2001). The study of Tong *et al.* presents results of 132 SGA experiments performed on mutants of non-essential genes, where chosen query genes

are involved in functions such as actin-based polarity, cell wall biosynthesis, DNA synthesis and repair, and microtubule-based chromosome segregation (Tong *et al.*, 2004).

Recently, a large-scale mapping of genetic interactions of essential genes was attempted using conditional temperature-sensitive alleles and conditional expression alleles (using tetracycline (tet)-controlled promoters) of essential genes (Davierwala *et al.*, 2005). Thirty query genes were crossed with an array containing tet-promoter mutants of ~50% of all of yeast essential genes. The key findings were: (a) synthetic genetic interactions of essential genes were biased toward functionally related genes, (b) the essential genes showed about five times the number of genetic interactions as compared with non-essential genes, indicating a much higher interaction density of the essential genes interaction network.

The SGA analysis provides insights into biological functions that are linked within the cell, helps to characterize unknown genes, and can be utilized in the analysis of other large-scale datasets such as studies of genome-wide mutant fitness in response to drugs (Parsons *et al.*, 2004; Parsons *et al.*, 2006).

1.2.5. Other functional genomics tools

The community of yeast researchers has developed a number of other functional genomics tools that allow for high-throughput studies of various biological aspects. For example, a global protein analysis, where a *S. cerevisiae* fusion library was created such that each ORF was tagged with a high-affinity epitope was used to measure absolute levels of most of the yeast proteins during log-phase growth. The key findings were that about 80% of the proteome is expressed during optimal growth, and the abundance of proteins ranges from fewer than 50 to more than 10^6 per cell (Ghaemmaghami *et al.*, 2003).

Further, Huh *et al.* (Huh *et al.*, 2003) constructed and analyzed a collection of yeast fusion strains where individual proteins were tagged with green fluorescent protein. This study classified 75% of all yeast proteins into distinct subcellular localization categories, including 70% of the proteins with previously unknown localization. Many efforts were also made to identify yeast protein-protein interactions using tandem affinity purification and mass spectrometry (Gavin *et al.*, 2002; Ho *et al.*, 2002; Krogan *et al.*, 2006).

Finally, the emerging field of metabolomics, that is the global analysis of cellular metabolites, uses ^{13}C- or ^{15}N- labeling followed by mass spectrometry to quantify absolute levels of metabolites and determine changes in metabolic fluxes in various environmental or genetic contexts (Blank *et al.*, 2005; Lafaye *et al.*, 2005).

1.2.6. Taking the next step: The challenge of large-scale data integration

Large-scale functional genomics datasets provide a vast amount of information that allows comprehensive and global observations to be made, that were impossible previously, when only a few individual genes or proteins were investigated per study (Grunenfelder and Winzeler, 2002). The current challenge is to develop computational methods for integrating

genome-wide datasets from various platforms (transcriptome, metabolome, fitness, or proteome). Some attempts to integrate data from different platforms that describe response to the same environmental condition have already been made in *S.cerevisiae*, including metabolic and transcriptional changes upon sudden relief from glucose limitation (Kresnowati *et al.*, 2006), or the comparison of global fitness and transcription profiles during yeast exposure to arsenic (Haugen *et al.*, 2004), or to fatty acids (Smith *et al.*, 2006).

In our lab we have developed T-profiler, a simple and intuitive bioinformatics tool based on t-statistics (Boorsma *et al.*, 2005), that allows for cross-platform comparison and integration of datasets obtained from various functional genomics experiments. We took advantage of this tool to explore the response of yeast to plasma membrane stress on the level of transcription and genome-wide fitness.

1.3. The plasma membrane of *S. cerevisiae*

1.3.1. Structure

The plasma membrane is an essential structure of any unicellular organism, forming the border that separates the inside of the living cell from the external environment. Lipids of the yeast plasma membrane are distributed asymmetrically between its two leaflets. The external leaflet of the plasma membrane in *S. cerevisiae* is enriched in phosphatidylcholine (PC), ergosterol and sphingolipids, whereas the internal leaflet is enriched in phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) (van der Rest *et al.*, 1995).

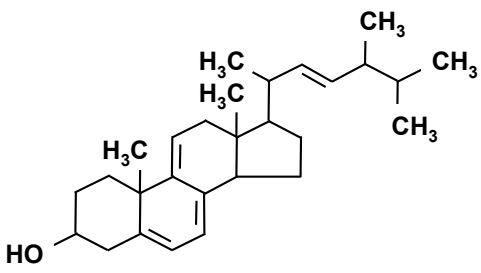


Figure 1. Structure of ergosterol.

Ergosterol plays a crucial role in maintaining plasma membrane rigidity, and is suggested to affect the activity of plasma membrane proteins (Figure 1). Phospholipids (PC, PE, PS, PI, and sphingolipids) possess a specific head group and two fatty acyl chains, whose length and degree of saturation largely determine membrane fluidity (Figure 2). PS is a key intermediate in the biosynthesis of other important membrane lipids, i.e. PE and PC. Additionally, exposure of PS on the cell surface is one of the markers of apoptosis. The essential membrane lipid PI is involved not only in membrane architecture but also in several other processes in the cell, such as, synthesis of phosphoinosides and inositol polyphosphates, as well as complex sphingolipids, signal transduction and vesicle trafficking. The functions and maintenance of lipid asymmetry in the plasma membrane of eukaryotic cells has been reviewed recently elsewhere (Ikeda *et al.*, 2006).

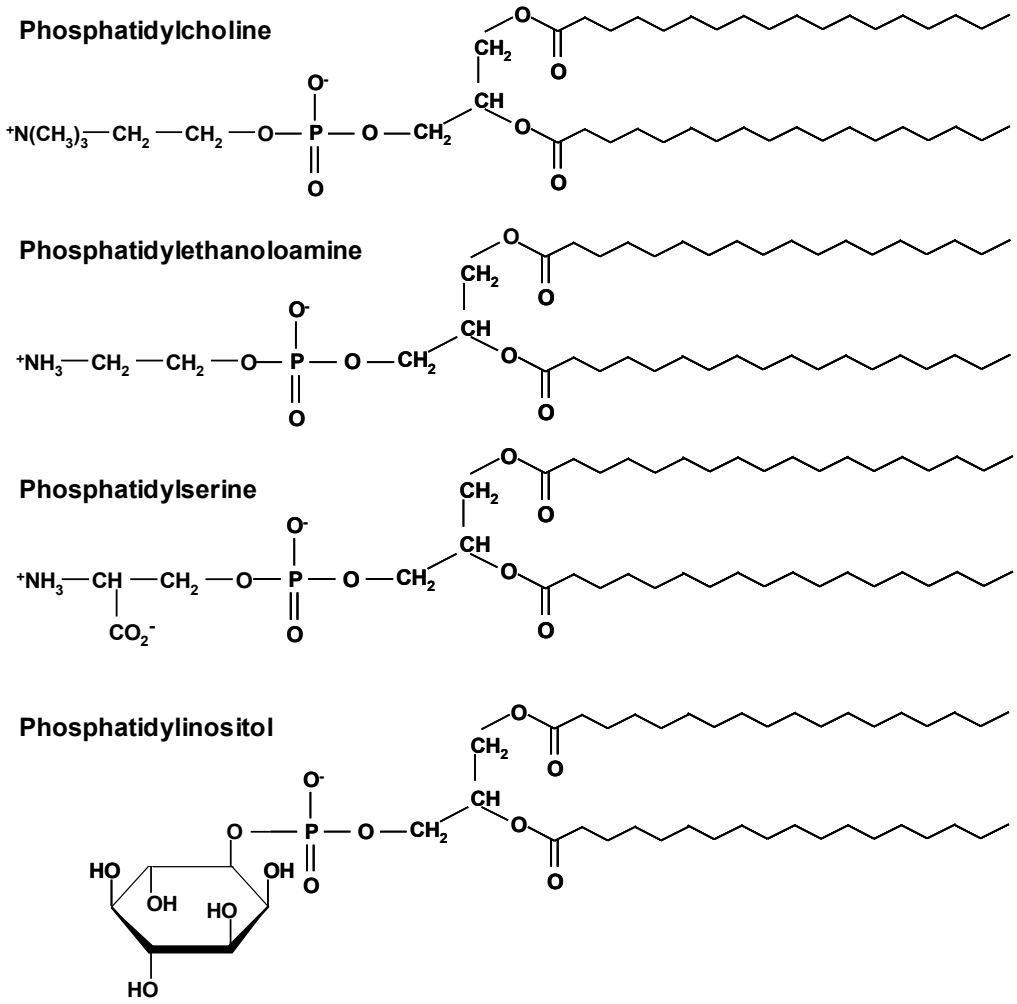


Figure 2. Structure of the major plasma membrane phospholipids of *Saccharomyces cerevisiae*.

Sphingolipids are the most abundant class of negatively charged components of the plasma membrane of yeast (Figure 3) (Dickson and Lester, 2002). In yeast they possess an inositol phosphate moiety, which is absent in animal sphingolipids (Daum *et al.*, 1998). $M(IP)_2C$ (mannosyl-diinositolphosphate-ceramide) is the most abundant yeast sphingolipid, which contains two inositol phosphates with a mannose unit attached to one of the inositols (van der Rest *et al.*, 1995).

Sphingolipid LCBs (long-chain bases) play important roles in the heat stress response and in signaling (Dickson *et al.*, 1997a; Jenkins *et al.*, 1997; Jenkins, 2003), as well as in endocytosis and actin cytoskeleton organization (Friant *et al.*, 2001; Zanolari *et al.*, 2000).

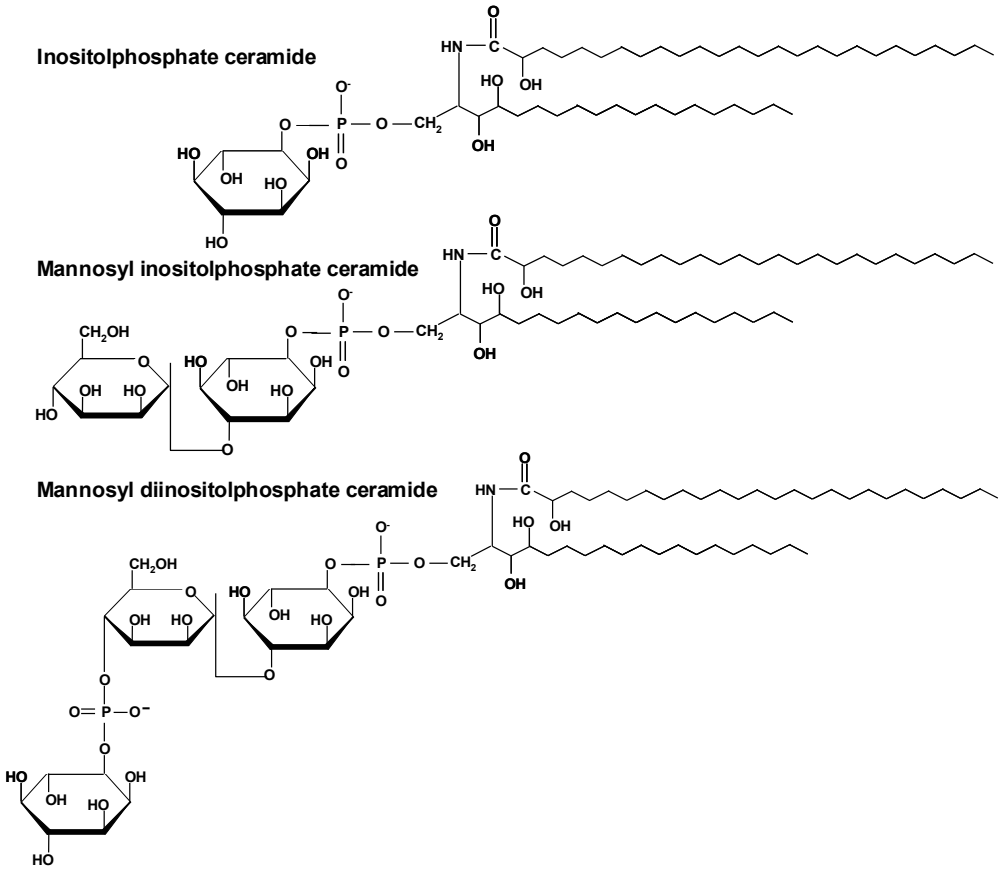


Figure 3. Structure of the sphingolipids found in the plasma membrane of *Saccharomyces cerevisiae*.

As a final point, we will briefly discuss the concept of lipid rafts. Lipid rafts are detergent-insoluble membrane domains (Kubler *et al.*, 1996), that contain ergosterol and sphingolipids, as well as proteins (Bagnat *et al.*, 2000). In *S. cerevisiae*, formation of lipid rafts is disrupted in an ergosterol-deficient mutant, in cells treated with the ergosterol-binding drug nystatin, and in a mutant defective in sphingolipid biosynthesis. In addition, the delivery of two membrane proteins, Pma1p and Gas1p, to the cell surface is dependent on their association with lipid rafts, indicating a role for lipid rafts in surface delivery of these proteins and, possibly, other proteins in yeast as well (Bagnat *et al.*, 2000; Bagnat *et al.*, 2001). Finally,

polarization of lipid rafts to the leading edge of growth in *C. albicans* plays an important role in hyphal morphogenesis, and reduction of the content of ergosterol or sphingolipids disrupts the interaction of these lipids and formation of lipid rafts, which leads to hypersensitivity to many drugs (Martin and Konopka, 2004; Mukhopadhyay *et al.*, 2004).

1.3.2. Function

Apart from its lipid components, the yeast plasma membrane contains a number of proteins involved in various cellular processes, such as solute transport, nutrient uptake, signal transduction, cell wall biosynthesis and organization of the cytoskeleton. The collaboration of protein and lipid components of the plasma membrane is required for proper execution of these functions.

The main function of the plasma membrane is to manage what enters or leaves the cell. The transport of ions, amino acids, and several disacharides through the plasma membrane is facilitated by maintaining an electrochemical proton gradient, which is also involved in the regulation of the intracellular pH. *PMA1* encodes the major plasma membrane H^+-ATPase, which accounts for 15 to 20% of total plasma membrane protein and is essential for viability (Ambesi *et al.*, 2000; Serrano *et al.*, 1986). Pma1p is a crucial proton pump responsible for generation of the electrochemical proton gradient across the plasma membrane. The transport of the main carbon source, glucose, occurs mostly by facilitated diffusion, which carries molecules down a concentration gradient with the assistance of transport proteins.

The yeast plasma membrane also has a crucial function in the synthesis and transport of cell wall precursors, in signaling of cell wall stress and in cell wall biogenesis. During growth on glucose, β-1,3-glucan, the main component of the cell wall, is synthesized by the enzyme Fks1p, the catalytic subunit of the β-1,3-glucan synthase complex, that resides in the plasma membrane. β-1,3-glucan synthesis is regulated by Rho1p, which is also localized in the membrane. The enzyme thought to be responsible for elongation of β-1,3-glucan and for connecting the emergent chain to the existing β-1,3-glucan, Gas1p, has been found to be associated with lipid rafts, regions of plasma membrane enriched in sphingolipids and ergosterol. Chs1p, a chitin synthase, also resides in the yeast plasma membrane. Finally, the main receptors of the cell wall integrity pathway, Wsc1-4, Mid2, Mtl1, are also integral membrane proteins.

Finally, the plasma membrane is the site of actin cytoskeleton anchoring. The actin cytoskeleton and endocytosis jointly participate in maintaining a functional plasma membrane. Actin patches colocalize with sites of endocytosis, associate with endosomes and their movement is mediated by polarized actin cables (Huckaba *et al.*, 2004). Endocytosis in *S.cerevisiae* is essential for several plasma membrane-associated functions, because it is required for recycling of plasma membrane components, uptake of nutrients, and regulation of cell-surface signaling receptors (Engqvist-Goldstein and Drubin, 2003; Walther *et al.*, 2006).

1.3.3. Biosynthesis of membrane components

Here we will briefly discuss the pathways that are involved in the synthesis of ergosterol and sphingolipids. The biosynthesis of phospholipids is discussed in detail elsewhere (Carman and Zeimetz, 1996; Carman and Henry, 1999).

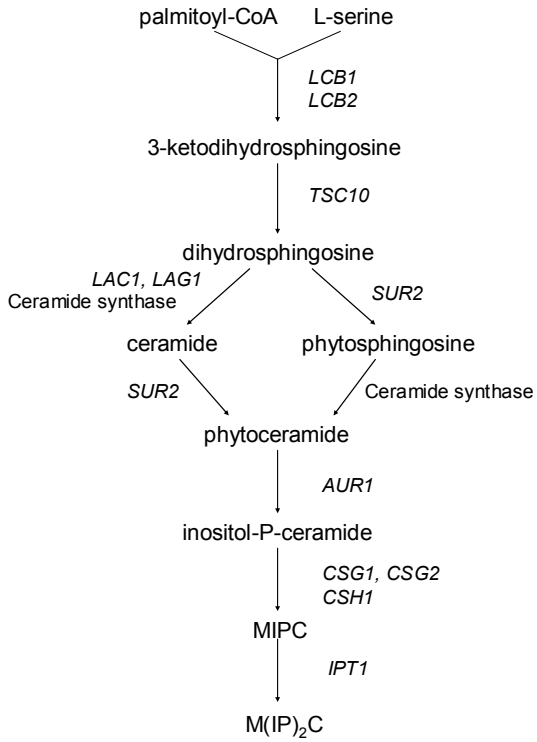
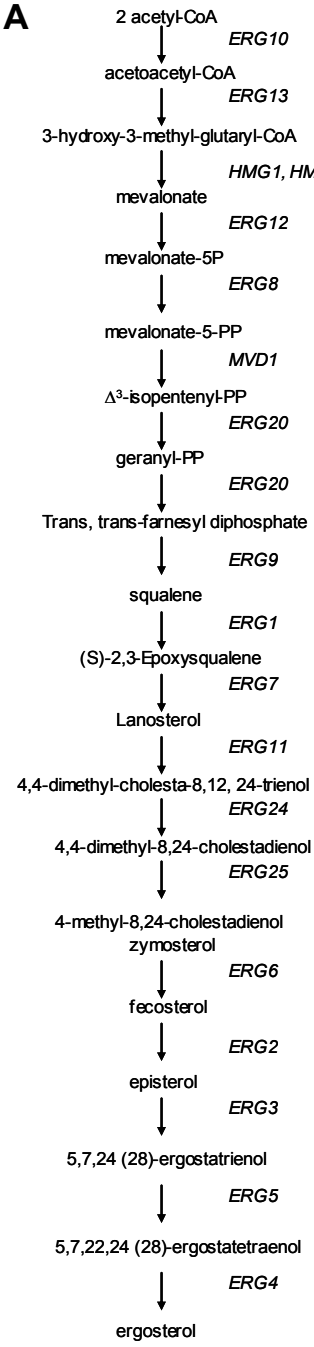


Figure 4. Scheme of sphingolipid biosynthesis in *Saccharomyces cerevisiae*.
Genes encoding the enzymes responsible for each step are shown in italics. MIPC-mannosyl-inositolphosphate ceramide, M(IP)2C-mannosyl-diinositolphosphate ceramide.

All yeast sphingolipids contain a long-chain base (LCB), a fatty acid and a polar head group (Dickson *et al.*, 2006). The sphingolipid biosynthesis pathway begins with condensation of palmitoyl-CoA and serine to form 3-ketodihydrosphingosine, a reaction that occurs in the ER (endoplasmic reticulum) and is catalyzed by serine palmitoyltransferase, encoded by two genes *LCB1* and *LCB2* (Nagiec *et al.*, 1994) (Figure 4). The next few steps also take place in the ER and lead to formation of ceramide, which forms the backbone of the sphingolipids (Guillas *et al.*, 2001; Haak *et al.*, 1997; Schorling *et al.*, 2001). The final three reactions, which are believed to occur in the Golgi, lead to formation of three types of complex sphingolipids. Inositol phosphoceramide (IPC), the first complex sphingolipid, is synthesized

A



B

Gene	Localization of the gene product
ERG10	cytosol
ERG13	ER, mitochondrion
HMG1	ER membrane, nuclear envelope, mitochondrial matrix
HMG2	As *HMG1*
ERG12	cytosol
ERG8	cytosol
MVD1	cytosol
ERG20	cytosol
ERG9	ER, mitochondrial outer membrane
ERG1	ER, lipid particle (target of terbinafine)
ERG7	ER, lipid particle, plasma membrane
ERG11	ER, (target of azoles)
ERG24	ER
ERG25	ER membrane, PM
ERG6	ER, lipid particle, mitochondrial outer membrane
ERG2	ER
ERG3	ER
ERG5	ER
ERG4	ER

Figure 5. Ergosterol biosynthesis in *Saccharomyces cerevisiae*

(A) Scheme of the ergosterol biosynthesis pathway

(B) Localization of gene products participating in ergosterol biosynthesis

by transfer of inositol phosphate from phosphatidyloinositol to ceramide, and this reaction is catalyzed by IPC synthase, which in yeast is encoded by the essential gene *AUR1* (Nagiec *et al.*, 1997). Next, three proteins, encoded by *CSG1*, *CSG2*, and *CSH1* participate in the synthesis of mannosyl-inositol-phosphoceramide (MIPC) by transfer of mannose from GDP-mannose to the inositol group of IPC. Finally, the most complex sphingolipid, mannosyl-(inositol-P)$_2$-ceramide (M(IP)$_2$C) is synthesized by the product of the *IPT1* gene, by transfer of a second inositol phosphate from phosphatidylinositol to MIPC (Dickson *et al.*, 1997b). A recent and detailed review of sphingolipid biosynthesis and metabolism can be found elsewhere (Dickson *et al.*, 2006). Recently, a modeling approach has also been undertaken to gain a better insight into sphingolipid metabolism (Alvarez-Vasquez *et al.*, 2005).

The synthesis of ergosterol begins with acetyl-CoA. The first step in ergosterol biosynthesis requires the gene *ERG10*, whose product transfers an acetyl group from one acetyl-CoA molecule to another, leading to the formation of acetoacetyl-CoA in the cytosol (Figure 5). Next, HMG-CoA is formed from acetyl-CoA and acetoacetyl-CoA, a reaction catalyzed in the ER by the product of the gene *ERG13*. The conversion of HMG-CoA to mevalonate, which is the rate-limiting step in sterol biosynthesis, is catalyzed by HMG-CoA reductase that comprises two isoenzymes coded by *HMG1* and *HMG2*. The next six steps lead to the formation of squalene, which is then oxidized by squalene epoxidase, which is encoded by the gene *ERG1* and is essential for growth under aerobic conditions. Subsequently, lanosterol synthase, encoded by the essential gene *ERG7*, catalyzes the synthesis of lanosterol. The essential gene *ERG11* codes for the enzyme that catalyzes the C-14 demethylation of lanosterol and is a member of the cytochrome P450 family, so it requires molecular oxygen and binds heme. Then follow seven steps that lead to formation of ergosterol, via intermediates such as zymosterol, fecosterol and episterol, with the final step in ergosterol biosynthesis being catalyzed by sterol C-24(28) reductase, encoded by *ERG4*. Importantly, yeast cannot synthesize ergosterol without oxygen and therefore requires supplementation of ergosterol to the medium for optimal growth under anaerobic conditions.

1.4. Plasma membrane stress

The fungal plasma membrane has been a preferred drug target for many years, yet a proper description and understanding of the cellular effects and the response of yeast to plasma membrane stress are still lacking. The main focus of this work is the plasma membrane stress caused by chemical or genetic perturbation of its lipid components. There are two main categories of drugs that can be distinguished on the basis of their effect on the plasma membrane. The first class of compounds has an indirect effect on the membrane, by inhibiting membrane lipid biosynthesis. The second category of compounds share a common characteristic in that their toxic effect is related to their ability to directly interact with lipid components of the plasma membrane Many of these compounds lead to alterations in plasma membrane integrity and composition. Finally, we discuss deletions or mutations in genes

involved in biosynthesis of plasma membrane lipids that lead to change in membrane lipid composition and membrane integrity, thus are also considered to cause plasma membrane stress (Dickson *et al.*, 1997b; Mukhopadhyay *et al.*, 2002; Mukhopadhyay *et al.*, 2004; Thevissen *et al.*, 2000a; Valachovic *et al.*, 2006).

1.4.1. Indirect plasma membrane perturbation

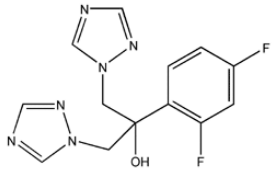
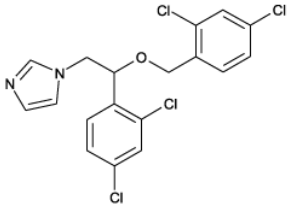
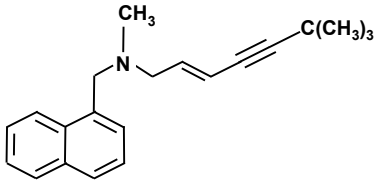
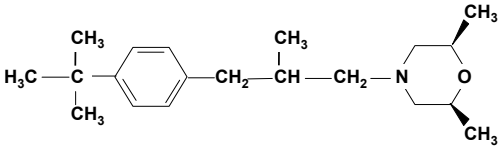
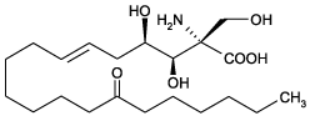
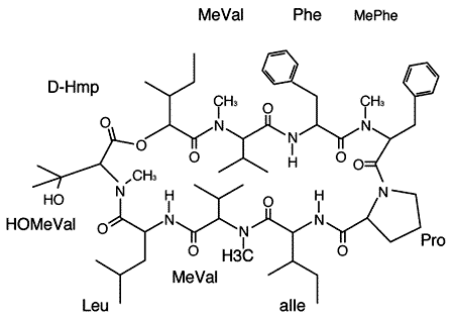
The category of indirect perturbants of the plasma membrane is represented by ergosterol biosynthesis inhibitors, such as azoles, allylamines (terbinafine), and morpholines (fenpropimorph), and the sphingolipid inhibitors, myriocin and aureobasidin A (Table 2).

The response to azoles, the key antifungal compounds that inhibit ergosterol biosynthesis, is well documented as they are commonly used against the opportunistic human pathogen, *Candida albicans*. Azoles are characterized by possession of an azole ring, which is a ring structure containing two heteroatoms in addition to carbon, one N and one or more of N,S,O. Azoles target the cytochrome P-450-dependent C14 lanosterol demethylase, encoded by *ERG11* in *S.cerevisiae*. The azole ring of triazoles binds to the heme group in the active site of the enzyme, thus blocking the binding of the substrate (Bhat *et al.*, 1977; Xiao *et al.*, 2004). The triazoles are fungistatic rather than fungicidal. Their fungistatic effect is proposed to be the result of ergosterol depletion in the plasma membrane and accumulation of toxic 14α-methylated sterol precursor. Unfortunately, fungal pathogens have become increasingly resistant to azoles. Recent studies have shown that this resistance is linked to the overexpression of multidrug resistance transporters that reduces drug intake and increases their efflux; to amplification and/or modification of the drug target Erg11p; and/or to mutations in other ergosterol biosynthesis genes that upon mutation lead to accumulation of nontoxic intermediates.

Among the triazoles fluconazole is most frequently used. Fluconazole has a synergistic inhibitory effect on growth of *C. albicans* when combined with the calcineurin inhibitors cyclosporin A or FK506. In addition, the *cnb1/cnb1* deletion mutant of the gene coding for calcineurin B and the deletion strain lacking the transcription factor Crz1p are hypersensitive to fluconazole, suggesting that the Ca^{2+}/ calcineurin pathway is involved in response to plasma membrane stress caused by fluconazole. Similar to fluconazole synergistic effects with CsA and FK506, and deletion of *CNB1* were observed when other azoles, such as ketoconazole, itraconazole, posoconazole, and voriconazole were used (Cruz *et al.*, 2002; Marchetti *et al.*, 2000; Santos and de Larrinoa, 2005).

Miconazole inhibits growth of many *Candida* species, including the most common opportunistic fungal pathogen, *Candida albicans*. Exposure of *C. albicans* cells to relatively high (15-120 μg/ml) concentrations of miconazole induces leakage of cellular components as measured at 260nm. Interestingly, this effect of miconazole is reduced by addition of divalent cations, such as Ca^{2+} and Mg^{2+} (Sreedhara Swamy *et al.*, 1974).

Table 2. Antifungal compounds that cause indirect plasma membrane stress in *Saccharomyces cerevisiae*.

Drug name	Structure	Specific target
Fluconazole		*ERG11*
Miconazole		*ERG11*
Terbinafine		*ERG1*
Fenpropimorph		*ERG2, ERG24*
Myriocin		*LCB1, LCB2*
Aureobasidin A		*AUR1*

In addition, it has been shown that the MIC (minimal inhibitory concentration) of miconazole increased when lipid extracts were added to *C. albicans* treated with miconazole, whereas the addition of defatted membranes did not change the MIC, suggesting that miconazole interacts with the lipid components of the plasma membrane and that this interaction is partially responsible for its antifungal ability. Further study of the antagonistic effect of particular lipid fractions revealed that unsaturated phospholipids and triglycerides but not sterols, were responsible for the increased MIC of miconazole (Yamaguchi, 1977). Treatment of *C. albicans* with miconazole results in the inhibition of plasma membrane-residing enzymes, for example H^+-ATPase and glucan synthase (Surarit and Shepherd, 1987). Importantly, miconazole possesses a positively charged imidazole ring that could potentially react with negatively charged lipid components in the cell membrane. Finally, in *S. cerevisiae*, the antifungal activity of miconazole is reduced in a mutant, in which the Ca^{2+}/ calcineurin pathway is constitutively activated, and the antifungal activity is increased when combination of miconazole with the specific inhibitor of that pathway, FK506, is used. Additionally, the deletant strains of genes in this pathway (*CNB1*, *CNA1*, and *CRZ1*) are hypersensitive to miconazole (Edlind *et al.*, 2002). Similarly, the *C. albicans* deletion mutant of the *CRZ1* gene shows increased sensitivity to this compound (Santos and de Larrinoa, 2005).

Two other types of drugs targeted at the ergosterol biosynthesis pathway have been developed, namely allylamines and morpholines. Terbinafine is a representative of allylamines that target the enzyme squalene epoxidase, which in *S. cerevisiae* is encoded by the gene *ERG1*. Terbinafine has a fungicidal effect against many fungi. It leads to ergosterol deficiency, and accumulation of squalene, which can cause cell membrane disruption (Balfour and Faulds, 1992). In *S. cerevisiae*, the effect of terbinafine is also reduced by addition of extracellular Ca^{2+}, and enhanced by addition of the Ca^{2+} chelator EGTA. A mutant with constitutive activation of the Ca^{2+}/ calcineurin pathway is more resistant to terbinafine, and also overexpression of the transcription factor of that pathway, *CRZ1*, leads to increased resistance to terbinafine (Edlind *et al.*, 2002). The combination of terbinafine with either of the two azoles, itraconazole and fluconazole, or amphotericin B, against clinical isolates of *C. albicans* leads to an enhanced growth inhibitory effect (Barchiesi *et al.*, 1998).

The morpholine, fenpropimorph, targets products of two *S. cerevisiae* genes, namely *ERG2*, which codes for sterol C8-C7 isomerase, and *ERG24*, which codes for a sterol reductase. In bakers' yeast the inhibitory effect of fenpropimorph was suggested to be a consequence of ergosterol deficiency as well as of the accumulation of the ergosterol precursor - ignosterol (Lorenz and Parks, 1991; Marcireau *et al.*, 1990). Fenpropimorph was also shown to affect membrane functions by affecting uracil uptake in *S. cerevisiae* (Crowley *et al.*, 1994). Finally, fenpropimorph and terbinafine were synergistic against *C. albicans* when combined with CsA or FK506, specific inhibitors of the Ca^{2+}/ calcineurin pathway, and the *cnb1/cnb1* deletion mutant showed hypersensitivity to both drugs (Onyewu *et al.*, 2003).

Myriocin is a fungal metabolite isolated from *Myriococcum albomyces*, *Isaria sinclairi*, and *Mycelia sterilia*. Myriocin (ISP-1) is a potent inhibitor of serine palmitoyltransferase, encoded by *LCB1* and *LCB2*. Serine palmitoyltransferase is a key enzyme in *de novo* synthesis of sphingolipids that catalyzes the first committed step in sphingolipid biosynthesis, which is the condensation of serine and palmitoyl-CoA (Miyake *et al.*, 1995; Sun *et al.*, 2000). In *S. cerevisiae*, the product of the gene *SLI1* confers resistance to myriocin by converting it into N-acetyl-myriocin (Momoi *et al.*, 2004). In *C. albicans*, inhibition of sphingolipid biosynthesis with myriocin leads to disruption of lipid rafts, their decreased polarization to the hyphal tip and to abnormal hyphal morphogenesis (Martin and Konopka, 2004).

Aureobasidin A, a cyclic depsipeptide produced by *Aureobasidium pullulans* R106, is highly toxic to fungi, including *S. cerevisiae*. Mutations in the essential gene *AUR1* confer resistance to this drug (Hashida-Okado *et al.*, 1996; Heidler and Radding, 1995). Aureobasidin A is a potent inhibitor of IPC synthase, encoded by *AUR1*, which catalyzes the first step in the synthesis of complex sphingolipids (Nagiec *et al.*, 1997). Finally, overexpression of the gene *YOR1*, which encodes a plasma membrane transporter of the ATP-binding cassette (ABC) family, confers resistance to aureobasidin A in yeast (Ogawa *et al.*, 1998).

1.4.2. Direct perturbation of the plasma membrane

The second class of plasma membrane stress-inducing compounds share a common characteristic in that their effect is related to their ability to directly interact with lipid components of the plasma membrane (Table 3). This category comprises such agents as ergosterol – binding antimicrobials, anionic detergents (LAS-sodium n-dodecyl benzene sulfonate, SDS-sodium dodecyl sulfate), chlorpromazine, naturally occurring polycationic peptides (defensins), and chitosan.

The compounds that bind to ergosterol are the polyene macrolide antibiotics, such as amphotericin B, nystatin, and natamycin. Their mode of action is not yet completely resolved, yet the most prominent hypothesis is that the binding of polyenes to ergosterol leads to formation of pores, and consequently to leakage of cellular components (see also below). Polyenes are poly-unsaturated organic compounds that contain one or more conjugated double carbon-carbon bonds. This series of conjugated double bonds typically absorbs strongly in the ultraviolet to visible region of the electromagnetic spectrum, often resulting in the polyene antibiotics having an intense yellow color.

Amphotericin B is a medically important antifungal agent, first isolated by Gold *et al* from a Gram-positive, filamentous bacterium, *Streptomyces nodosus*, in 1955 in Venezuela. Amphotericin B is one of two polyketides synthesized by *S. nodosus* through its polyketide synthesis pathway (Caffrey *et al.*, 2001). Amphotericin B is poorly soluble in water and is toxic towards fungal cells because of its high affinity for ergosterol, the predominant sterol in fungal cell membranes. It disrupts the eukaryotic cell membrane by complexing with sterols to form

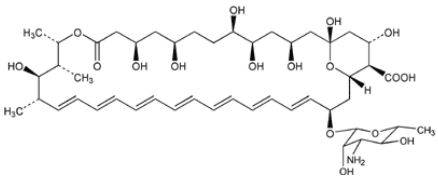
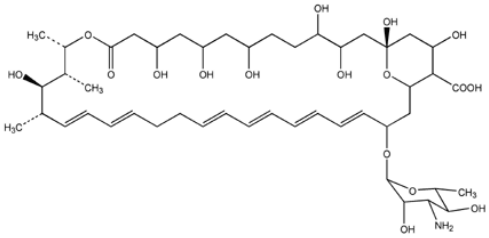
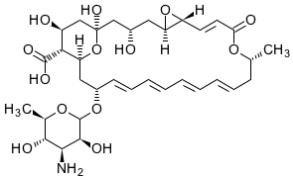
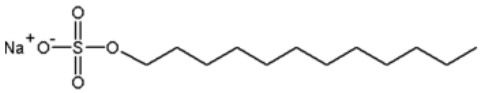
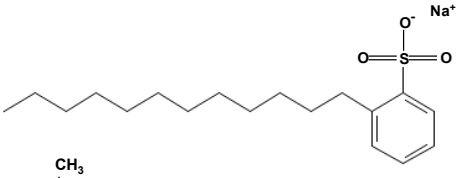
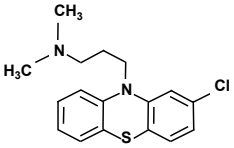
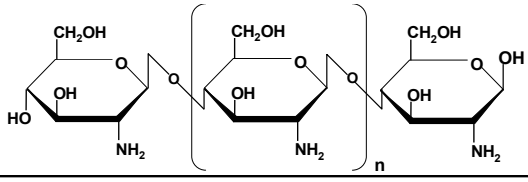
channels that lead to leakage of intracellular molecules and ions, resulting in loss of membrane potential and eventually causes cell death (Bolard, 1986; Peter *et al.*, 2005). To a lesser extent, amphotericin B also interacts with cholesterol in mammalian cell membranes and because of this it shows many severe side-effects, especially nephrotoxicity, when used as antifungal in mammals (Fanos and Cataldi, 2000). Despite its harmful side-effects, amphotericin B is still one of the principal antimicrobials for the treatment of life-threatening systemic fungal infections in humans.

Interestingly, amphotericin B has the potential to inhibit infection of cultured human cells by the human immunodeficiency virus (HIV) (Pontani *et al.*, 1989; Schaffner *et al.*, 1986), which could be due to a higher cholesterol:phospholipid ratio of the membrane envelopes of these virus particles than found in the host cell membranes (Aloia *et al.*, 1993). In addition, amphotericin B is active against *Leishmania*, a protozoic parasite that contains ergosterol precursors in its membranes (Hartsel and Bolard, 1996). Amphotericin B can also interfere with the formation of abnormal isoforms of prion proteins during intracellular trafficking of sterol-rich membrane domains (lipid-rafts) that contain these proteins, which can lead to delay of the onset of prion disease symptoms in animal models (Mange *et al.*, 2000; Pocchiari *et al.*, 1987). Finally, amphotericin B was shown to have an enhanced growth-inhibitory effect when combined with the inhibitor of cell wall glucan synthesis, caspofungin, against *C. glabrata* in an *in vivo* murine candidemia model (Barchiesi *et al.*, 2005).

A similar mechanism of action on the ergosterol-containing membranes as amphotericin B has been proposed for nystatin and natamycin. Nystatin was isolated from *Streptomyces noursei* found in a soil sample in 1950 (Brown *et al.*, 1953). Natamycin is produced by *Streptomyces natalensis*, and is widely used in the food industry in order to prevent mould contamination of cheese and other non-sterile foods (*i.e.* sausages, ham, etc.). Both nystatin and amphotericin B lead to inhibition of the activity of enzymes localized in the plasma membrane of *C. albicans*, for example the H^+-ATPase and glucan synthase (Surarit and Shepherd, 1987). Although nystatin and amphotericin B are thought to share the same mode of antifungal action, involving the binding to ergosterol in the plasma membrane, recently two different mutants in *S. cerevisiae* have been isolated that show selective resistance to these two drugs, implicating that their cellular effects are not entirely the same (Hapala *et al.*, 2005), and thus require further studies.

Sodium dodecyl sulfate (SDS), also known as sodium lauryl sulfate, and sodium n-dodecyl benzene sulfonate (LAS) are anionic surfactants that, together with several derivatives are commonly used in household products such as toothpastes, shampoos, shaving foams and bubble baths and dish-washing liquids due to their ability to interact with lipids (Sirisattha *et al.*, 2004). SDS is also widely used by life scientists for cell membrane permeabilization, for preparing cell extracts, and in gel electrophoresis of denatured proteins. In *C. albicans*, SDS is toxic to WT cells when combined with the specific inhibitors of calcineurin, CsA and KF506 (Cruz *et al.*, 2002).

Table 3. Compounds that cause direct membrane perturbation in *Saccharomyces cerevisiae*. SDS stands for sodium dodecyl sulfate, LAS stands for sodium n-dodecyl benzene sulfonate. In case of defensin, DmAMP1, the amino acid sequence is shown.

Drug name	Structure	Target
Amphotericin B		Ergosterol in the plasma membrane
Nystatin		Ergosterol in the plasma membrane
Natamycin		Ergosterol in the plasma membrane
SDS		Plasma membrane lipids
LAS		Plasma membrane lipids
Chlorpromazine		Plasma membrane
DmAMP1	ELCEKASKTWSGNCGNTGHCDNQCKSWEGAAHGACHVRNGKHMCFCYFNC	Plasma membrane lipids
Chitosan		Plasma membrane?

Additionally, the deletion mutants of genes of the Ca^{2+}/ calcineurin pathway, namely *CRZ1*, *CNA1*, and *CNB1*, are hypersensitive to SDS, indicating that this pathway is crucial for adaptation of *C. albicans* to treatment with this compound (Cruz *et al.*, 2002; Karababa *et al.*, 2006; Sanglard *et al.*, 2003; Santos and de Larrinoa, 2005).

Finally, in *S.cerevisiae*, SDS causes depolarization of the actin cytoskeleton and consequently, delocalization of membrane-localized Fks1p, the enzyme responsible for synthesis of main polymer of the cell wall, β-1,3-glucan (Delley and Hall, 1999). LAS, which shows structural similarities to SDS, is not so well-studied in fungi, yet its effect on lipids is similar to that of SDS.

Chlorpromazine belongs to the phenothiazines, a class of compounds that has been widely used as tranquilizers and antipsychotic drugs. Chlorpromazine induces Ca^{2+} influx and a significant efflux of cellular K^+ in *S. cerevisiae* (Eilam, 1983). In addition, it depolarizes the yeast membrane, measured as a decrease in the accumulation of the lipophilic cation TPP^+ (Eilam, 1984). The accumulation of TPP^+ by cells treated with chlorpromazine is dependent on the presence of glucose. Interestingly, another phenothiazine, trifluoperazine, inhibits the plasma membrane H^+-ATPase (Eilam, 1984).

Chlorpromazine has a growth inhibitory effect on five common pathogenic yeasts, *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *Cryptococcus neoformans*. Further, chlorpromazine shows an enhanced inhibitory effect when used in combination with the ergosterol-binding compound - amphotericin B, and the ergosterol biosynthesis inhibitor – ketoconazole (Ben-Gigi *et al.*, 1988; Eilam *et al.*, 1987; Wood and Nugent, 1985). Finally, chlorpromazine, which causes plasma membrane stretch by insertion into the plasma membrane, induces depolarization of the actin cytoskeleton as well as activates the cell wall integrity pathway, in an Slt2p-dependent manner (Delley and Hall, 1999; Kamada *et al.*, 1995).

Defensins are antimicrobial cationic peptides that participate in innate immunity, which is the most ancient non-specific defense mechanism that can protect host organisms against pathogenic microorganisms (Brown and Hancock, 2006). The properties of cationic antimicrobial peptides have been extensively reviewed (Devine and Hancock, 2002; Ganz, 2003; Hancock, 2001; Hancock and Rozek, 2002). Here, we will focus on the plant defensins. They are small, consisting of up to 50 amino acids, generally basic, cysteine-rich, and with a net-positive charge. Plant defensins are able to inhibit the growth of many fungi, but appear to be nontoxic to mammalian and plant cells. Their antifungal activity seems to require specific binding to targets in the fungal plasma membrane (Thomma *et al.*, 2002).

DmAMP1, a plant defensin isolated from the seeds of *Dahlia merckii*, induces Ca^{2+} influx and K^+ efflux, changes in membrane potential, and increased uptake of fluorescent dyes in fungi (Thevissen *et al.*, 1996; Thevissen *et al.*, 1999). In addition, a deletion mutant of the gene *IPT1*, which codes for the enzyme that catalyzes the final step in the synthesis of $M(IP)_2C$, is significantly more resistant to DmAMP1 than the wild type strain. Finally,

DmAMP1 has specific binding sites on the fungal plasma membrane, which have been shown to correspond to the sphingolipid type $M(IP)_2C$ of the plasma membrane of *S.cerevisiae* and this interaction is enhanced in the presence of ergosterol (Im *et al.*, 2003; Thevissen *et al.*, 2000a; Thevissen *et al.*, 2000b; Thevissen *et al.*, 2003b). In *Neurospora crassa*, DmAMP1 causes dosage-dependent membrane permeabilization, measured as uptake of fluorescent dye. Ineterstingly, he degree of permeabilization at high concentrations of defensin is decreased in the presence of cations in the medium (Thevissen *et al.*, 1999). Another plant defensin, RsAFP2, isolated from seeds of *Raphanus sativus*, is highly effective in inhibiting growth of *C.albicans* and *P.pastoris* wild type strains; yet the deletants of genes responsible for the final step in the biosynthesis of plasma membrane glucosylceramides in these species are resistant to this compound. In *P.pastoris*, RsAFP2 induces membrane permeabilization, and interacts with membrane glucosylceramides. Interestingly, it does not interact with glucosylceramides of soyabean and human, and *S.cerevisiae*, which lacks glucosylceramides is resistant to this defensin (Thevissen *et al.*, 2004). Finally, addition of PnAMP1, a defensin from *Pharbitis nil*, leads to rapid depolarization of the actin cytoskeleton both in *S. cerevisiae* and in *C. albicans*, and some of the mutants in the yeast cell wall integrity pathway are hypersensitive to this compound (Koo et al., 2004).

Chitosan is a linear β-1,4-D-glucosamine polymer. It can be obtained by deacetylation of chitin. It has growth-inhibitory activity against several filamentous fungi, spoilage yeasts and bacteria, but seems to have no or much less toxic effect on mammalian cells (Rhoades and Roller, 2000; Roller and Covill, 1999). The antifungal activity of chitosan depends on the degree of deacetylation and polymerization, such that chitosan molecules with lower polymer chain lengths are more active than native chitosan in inhibiting fungal growth (Rhoades and Roller, 2000). As chitosan has a pK_a value of around 6.3, at lower pH values the majority of the glucosamine residues in chitosan molecules become cationic, due to protonation of their amino groups, which enables them to interact with anionic components of the cell surface. Importantly, chitosan is less effective in the presence of divalent cations (Helander *et al.*, 2001), which suggests that the cationic nature of chitosan is part of its mode of action. A recent study of the effect of chitosan on bacterial membranes showed that chitosan interacts with negatively charged phospholipids, with the NH_3^+ groups being responsible for this interaction (Liu *et al.*, 2004). Finally, low molecular weight chitosan induces formation of mass-transfer channels in artificially created lipid bilayers, thus providing further evidence for the disturbing effect of chitosan on cell membranes (Yang *et al.*, 2002).

1.4.3. Genetic perturbation of plasma membrane lipid biosynthesis

The general method of investigating plasma membrane stress in yeast is to study the responses of yeast cells to compounds that directly perturb the membrane or affect the biosynthesis of lipid components of the membrane. However, deletion or mutation of genes

whose products are involved in the synthesis of plasma membrane lipids also results in plasma membrane stress, and as such is shortly discussed here.

Ergosterol is responsible for the rigidity and stability of the plasma membrane. Most of the deletions of genes involved in the early steps of ergosterol biosynthesis are inviable, and the mutant strains are ergosterol-auxotrophic. The deletion mutants involved in later biosynthetic steps such as *erg2*Δ, *erg3*Δ, *erg4*Δ, and *erg6*Δ are viable, but lack ergosterol, and instead accumulate various ergosterol intermediates. Except for *erg4*Δ, these mutants show an increase in membrane fluidity and a higher level of passive diffusion, which results in higher sensitivity to a number of drugs (Mukhopadhyay *et al.*, 2002). Further, the mutant *erg6*Δ shows defective conjugation, reduced transformation capacity, and defective uptake of tryptophan, indicating that the deletion of *ERG6* influences membrane permeability and function (Gaber *et al.*, 1989). The mutants *erg2*Δ, *erg3*Δ, and *erg6*Δ also show changes in the levels of individual phospholipids in the plasma membrane, and have a lower sterol/ phospholipid molar ratio than the wild type (Sharma, 2006). In *C. albicans*, the deletion mutants of *ERG2* and *ERG16* both lack ergosterol, and this is accompanied by higher membrane fluidity and increased passive diffusion. Further, deletion of these genes disrupts the efflux activity of Cdr1p, the multidrug ABC transporter, which is suggested to reside in lipid rafts (Mukhopadhyay *et al.*, 2004). Finally, the function of Cdr1p is also disturbed in a *C. albicans* heterozygous *ERG1/erg1*Δ strain, which is deficient in ergosterol, exhibits hypersensitivity to various drugs, and shows defects in hyphae formation (Pasrija *et al.*, 2005).

Mutants in the *LCB1* gene, whose product catalyzes the first step in sphingolipid biosynthesis, are hypersensitive to high salt concentrations, low pH, and elevated temperature, and they show defects in proton extrusion at low pH, indicating that this gene product participates in proper plasma membrane functioning under non-optimal conditions (Patton *et al.*, 1992).

The deletant strain of gene *IPT1*, which is involved in the last step of sphingolipid biosynthesis in *S.cerevisiae*, lacks $M(IP)_2C$, and instead accumulates increased amounts of its precursor (Dickson *et al.*, 1997b). In addition, the function and localization of Cdr1p is affected in a *C.albicans ipt1*Δ *ipt1*Δ strain, which lacks $M(IP)_2C$ and instead accumulates MIPC, has increased sensitivity to many drugs, and is unable to form proper hyphae (Pasrija *et al.*, 2005; Prasad *et al.*, 2005).

Finally, the formation and proper functioning of plasma membrane lipid rafts in the delivery of cell surface proteins, Gas1p and Pma1p, is disrupted in a lcb1-100 mutant (Bagnat *et al.*, 2000; Bagnat *et al.*, 2001), again pointing to the crucial role of sphingolipids in normal functioning of the plasma membrane.

1.5. A final word

There are many other conditions that may be viewed as plasma membrane stress, due to their effect on membrane integrity. For example, conditions such as a hypo- or

hyperosmotic environment, and elevated or decreased temperatures lead to changes in membrane fluidity and permeability. However, they are discussed elsewhere (Hayashi and Maeda, 2006; Mager and Varela, 1993; Panadero *et al.*, 2006; Piper, 1995). Interestingly, previous studies have shown that membrane lipid composition plays an important role in yeast tolerance to various stress conditions (Swan and Watson, 1997, 1998, 1999).

To conclude, we are still far from completely understanding how the yeast plasma membrane functions, and how it interacts with other cellular components. In this thesis we present work that sheds new light on several aspects of the cellular response to plasma membrane stress, including transcriptional programs and the identification of previously unknown functions that are involved in counteracting loss of plasma membrane integrity.

1.6. Outline of this thesis

The main goal of the work presented here is to gain a better understanding of the general and specific responses of the model microorganism *Saccharomyces cerevisiae* to various plasma membrane stresses by using large-scale genomics tools.

Chapter 1 is a general introduction and comprises three parts: (i) part one describes the genome-wide tools available for functional genomic studies in bakers' yeast, (ii) part two provides an overview of plasma membrane composition, structure and biosynthesis, and finally (iii) in part three the types of plasma membrane stress that can be imposed on an *S. cerevisiae* cell are discussed.

In Chapter 2 the time-dependent transcriptional response of *S.cerevisiae* to mild concentration of antifungal compound, chitosan, is characterized. The yeast temporal expression patterns are analyzed with the statistical tool developed in our lab, namely T-profiler. The yeast transcription program in response to plasma membrane stress caused by chitosan is identified.

Chapter 3 provides evidence for a common transcriptional response program induced in *S. cerevisiae* subjected to plasma membrane stress that is also similar to response to cell wall stress on the transcriptional level.

Chapter 4 describes the cellular functions required by bakers' yeast to counteract chitosan stress. These functions are identified by using the genome-wide fitness profiling with the whole yeast deletion collection treated with mild concentration of chitosan. Results are obtained with T-profiler, a tool adapted from analysis of expression datasets.

In Chapter 5 a functional genomics approach is undertaken, where vast numbers of transcription and fitness experiments are subjected to integrative analysis using predefined functional classes, which yields new insights into crucial functions required by yeast in multi-stress responses, and into the multi-level regulation of these cellular functions.

Finally, Chapter 6 discusses the findings of this study in the context of the available and recent literature on yeast stress responses and functional genomics.

Chapter 2

The transcriptional response of yeast to the plasma membrane perturbing compound chitosan.

This chapter has been published previously:
Zakrzewska, A., Boorsma, A., Brul, S., Hellingwerf, K.J., and Klis, F.M. (2005), Eukaryot. Cell, Vol.4, p:703-715

Abstract

Chitosan is a plasma membrane perturbing compound consisting of linear chains of β-1,4-linked glucosamine residues, which at acidic pHs become positively charged. It is extensively used as an antimicrobial compound, yet its mode of action is still unresolved. Chitosan strongly affected the growth of the yeast *Saccharomyces cerevisiae*, the food spoilage yeast *Zygosaccharomyces bailii*, and two human pathogenic yeasts, namely, *Candida albicans* and *Candida glabrata*. Microarray analysis of yeast cells treated with sublethal concentrations of chitosan revealed induction of the environmental stress response and three more major transcriptional responses: (1) A rapid and stable Cin5p-mediated response. Cin5p/Yap4p is a transcription factor involved in various stress responses. Deletion of *CIN5* led to increased chitosan sensitivity. (2) A Crz1p-mediated response, which is delayed compared to the Cin5p-response. Crz1p is a transcription factor of the calcineurin pathway. Cells deleted for *CRZ1* or treated with the calcineurin inhibitor FK506 became hypersensitive to chitosan, supporting the notion that the Crz1p-controlled response offers protection against chitosan. (3) A strong Rlm1p-mediated response, which ran parallel in time with the Crz1p-regulated response. Rlm1p is a transcription factor of the cell wall integrity pathway, which is activated by cell wall stress. Importantly, chitosan-treated cells became more resistant to β-1,3-glucanase, which is a well-known response to cell wall stress. We propose that the transcriptional response to chitosan may be representative for other plasma membrane perturbing compounds.

Keywords: *Saccharomyces cerevisiae, Candida albicans, Candida glabrata, Zygosaccharomyces bailii*, cell wall integrity pathway, calcineurin, antimicrobial peptides

Introduction

Chitosan, a linear β-1,4-D-glucosamine polymer, is a deacetylated derivative of chitin, and is non-toxic and biodegradable. Chitosan inhibits growth of several filamentous fungi, spoilage yeasts and bacteria, but has no effect on mammalian cells (Rhoades and Roller, 2000; Roller and Covill, 1999). The antifungal activity of chitosan depends on the degree of deacetylation and polymerization. Mildly hydrolysed chitosan was shown to be more active in inhibiting fungal growth than native chitosan (Rhoades and Roller, 2000). Chitosan has a pK_a value of around 6.3. Thus at lower pH values the majority of the glucosamine residues in chitosan molecules are cationic, due to protonation of amino groups, which enables them to interact with anionic components of the cell surface.

One of the well-known defense strategies of mammalian, insect and plant cells against pathogenic fungi and bacteria involves the production of cationic antimicrobial peptides (AMPs) (Thevissen *et al.*, 2004). An extensively studied class of the AMPs is formed by the plant defensins. It has been shown that they interact with the anionic components of the fungal plasma membrane and that this is the basis of their antifungal activity (Thevissen *et al.*, 2003a; Thevissen *et al.*, 2003b). The *S. cerevisiae* plasma membrane forms a lipid bilayer, whose external leaflet is enriched in phosphatidylcholine (PC), ergosterol and sphingolipids. Sphingolipids comprise the most abundant class of negatively charged components of the plasma membrane (Dickson and Lester, 2002). In yeast they possess an inositol phosphate moiety, which is absent in animal sphingolipids (Daum *et al.*, 1998). The most abundant yeast sphingolipid is $M(IP)_2C$ (mannosyl-diinositolphosphate-ceramide), containing two inositol phosphates with a mannose unit attached to one of the inositols (van der Rest *et al.*, 1995). Interestingly, many fungal species such as *S. cerevisiae*, *N. crassa*, *C. albicans* and *P. pastoris* have been found sensitive to cationic plant defensins in a sphingolipid-dependent manner (Ferket *et al.*, 2003; Thevissen *et al.*, 1999; Thevissen *et al.*, 2003b; Thevissen *et al.*, 2004).

To protect themselves against various types of stress including the presence of cationic antimicrobial peptides, fungi have developed a number of mechanisms that enable them to survive. *S. cerevisiae* is an excellent model organism to study stress responses among fungi. The ability of baker's yeast to adapt to and survive a large variety of environmental conditions depends on cooperation among the limited number of known pathways in mounting a response specific to a given kind of stress. The recently described environmental stress response (ESR) is a common expression program of ~900 genes, whose transcription is altered upon shifting of cells to various stressful conditions. The environmental stress response comprises two groups of genes with distinct transcriptional patterns of up- and down-regulation. The expression of many genes induced in the ESR is largely mediated by the transcription factors Msn2p and Msn4p (Gasch *et al.*, 2000; Moskvina *et al.*, 1999). The downregulated cluster consists of genes encoding ribosomal proteins and genes involved in various aspects of RNA metabolism. Though the ESR program is initiated in

response to many diverse conditions, the precise levels and timing of the gene expression may change suggesting a very sensitive, condition-specific regulation of this program, involving participation of other signaling pathways.

An example of a condition-specific response is seen in the recent study of a number of cell wall deletion mutants, where the “cell wall compensatory cluster” was shown to involve three pathways: the aforementioned ESR, the Ca^{2+}/calcineurin-dependent pathway, and the cell wall integrity pathway (Lagorce *et al.*, 2003). The Ca^{2+}/calcineurin-dependent pathway is implicated in a variety of stresses, including cell wall perturbation, high extracellular levels of Ca^{2+} and Na^{+}, increased temperature and prolonged exposure to α-factor (Yoshimoto *et al.*, 2002). This pathway operates via the Crz1p transcription factor and leads to induction of a number of genes involved in maintaining cell ion homeostasis. The Ca^{2+}/calcineurin-pathway was found to act together with the cell wall integrity pathway, which operates through the Rlm1p transcription factor (Lagorce *et al.*, 2003). The cell wall integrity pathway is also activated upon treatment with cell wall perturbing compounds such as Calcofluor White, Congo Red and caspofungin (Boorsma *et al.*, 2004; Garcia *et al.*, 2004; Reinoso-Martin *et al.*, 2003). The activation of the cell wall integrity pathway leads to induction of genes involved in cell wall biogenesis and maintenance, and in turn to cell wall remodeling (Smits *et al.*, 2001). Two main enzymes involved in cell wall maintenance namely Fks1, a β-1,3-glucan synthase, and Chs1, a chitin synthase, reside in the yeast plasma membrane. Interestingly, it has been shown that treatment with chlorpromazine, a compound causing plasma membrane stretch, can lead to activation of the cell wall integrity pathway (Kamada *et al.*, 1995), thus suggesting that stress targeted at the plasma membrane can be compensated on the cell wall level.

In this study *S. cerevisiae* was used as a model organism to investigate its response to sublethal concentrations of chitosan using global transcriptome analysis. The microarray data revealed upregulation of genes involved in the core stress response. In addition, three groups of genes sharing the Cin5, Crz1 and Rlm1 regulatory motifs, respectively, were significantly induced. To validate the results obtained from the transcriptome analysis additional experiments were performed. Deletion of *CIN5* resulted in increased sensitivity to chitosan. Further, deletion of *CRZ1* or exposure of chitosan-treated cells to a low concentration of FK506, a known inhibitor of the Crz1p-mediated response, significantly aggravated the effect of chitosan, supporting the idea that the calcineurin pathway helps the cells to survive in the presence of chitosan. Chitosan treatment also resulted in increased resistance of cells to β-1,3-glucanase, which is characteristic for cell-wall stressed cells and indicative of activation of the cell wall integrity pathway. Thus, our data suggest that the cells try to cope with chitosan-induced plasma membrane stress by launching four major transcriptional pathways.

Experimental procedures

Strains and culture conditions

Saccharomyces cerevisiae strain X2180-1A (MAT**a**, *SUC2, mal, gal2, CUP1*) was used for microarray experiments. Synthetic Complete medium (SC: 0.67% Yeast Nitrogen Base without amino acids, 0.5% casamino acids, 2% glucose), pH 5.5, was used to culture the X2180-1A strain. Cells were pre-cultured overnight in YPD (1% yeast extract, 2% peptone, 2% dextrose) and inoculated in batch fermentors in SC medium to an OD_{600} of 0.2 (approximately 1.5 x 10^6 cells/ml). The cultures were grown at 30°C, with an aeration rate of 0.5 L/min and fast stirring (200 rpm). Chitosan was added after 3 hours. Growth was measured by following OD_{600}. Cell viability was established by plating appropriate dilutions of the culture samples on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). The number of colonies was counted after incubation at 30°C for two days. The cell number was determined by using a CASY cell counter according to the manufacturer's protocol.
Candida albicans strain SC5314, and *Candida glabrata* strain ATCC 9087.6 were grown in adjusted Synthetic Complete medium (SC) consisting of 0.67% Yeast Nitrogen Base without amino acids, 1% casamino acids, 2% glucose, additional amino acids (leucine, tyrosine, tryptophan and adenine), pH 5.5, to OD_{600} = 0.1, then chitosan was added and growth was followed by OD_{600} measurement. A food isolate of *Zygosaccharomyces bailii* was cultured in SC medium (as described for *S. cerevisiae*).

Chitosan preparation and characterization

Chitosan was obtained from SIGMA (crab shells, minimum 85% deacetylated). It was fragmented using nitrous deamination. Chitosan was dissolved in 10% acetic acid and incubated with sodium nitrite at a concentration of 20 mg per gram of chitosan at room temperature for 17 h. To stop the reaction, the pH of the preparation was adjusted to 5.5 with NaOH. The final concentration of chitosan was measured as glucosamine equivalents after hydrolysis in 6 M HCl for 17 h at 100°C (Popolo *et al.*, 1997). Different batches of chitosan slightly varied in their inhibitory effect.

Cell leakage assay

The leakage of cellular components was analyzed by adapting the method of De Nobel *et al.* (De Nobel *et al.*, 1990). Cells were grown to mid-log phase, harvested by centrifugation, and washed two times with 10 mM MES/NaOH, pH 5.5. Next, the cells were incubated for 30 min at 30°C in 10 mM MES/NaOH, pH 5.5, in the presence of various concentrations of chitosan. The cells were pelleted at 14,000 rpm for 2 minutes; the supernatant was collected and centrifuged again at 14,000 rpm for 2 min. Subsequently, the supernatant was filtered (Acrodisc LC 25 mm Syringe Filter with a 0.45 μm PVDF membrane, Gelman Laboratory) and the absorbance was measured at a wavelength of 260 nm (UVmini

1240, UV-VIS Spectrophotometer, SHIMADZU). Cellular leakage was defined as A_{260} sample - A_{260} buffer (with the respective concentrations of chitosan).

Preparation of total RNA and labeled cRNA

The cells were cultured in a batch fermentor for 3 hours prior to addition of chitosan. Samples for RNA isolation were collected at appropriate time points, 0 (control), 15, 30, 60, 120, and 180 minutes after addition of chitosan. They were flash-frozen in liquid nitrogen and stored at –80^{o}C. Total RNA was extracted using the method of Llinas (http://www.microarray.org/pdfs/TotalRNAIsolation.pdf). The concentration and quality of RNA were determined by measuring absorbance at 260, 280, and 230 nm on a Nanodrop spectrophotometer. The purity and integrity of the RNA samples were further validated with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies.

Total RNA was labeled according to the manufacturer's protocol (http://www.affymetrix.com/). Twenty μg of total RNA was used for first strand cDNA synthesis. This was followed by synthesis of second strand cDNA. cDNA was purified using the GeneChip Sample CleanUp Module from Qiagen. The cDNA was used for synthesis of biotin-labeled cRNA, which was performed with the ENZO BioArray HighYield RNA Transcript Labeling Kit from Affymetrix. The synthesized cRNA was purified with the GeneChip Sample CleanUp Module from Qiagen. The concentration and quality of labeled cRNA were tested using a Nanodrop spectrophotometer. Subsequently, the cRNA fragmentation reaction was carried out according to the manufacturer's protocol. The degree of fragmentation was confirmed with RNA LabChip on a 2100 Bioanalyzer from Agilent Technologies. The samples were stored at –20^{o}C prior to hybridization.

Hybridization and scanning of the DNA microarrays

The biotin-labeled cRNA samples were hybridized to the Affymetrix GeneChip® Yeast Genome S98 Array according to Affymetrix protocols (http://www.affymetrix.com/). This chip contains 25-mer oligonucleotide probes for approximately 6,400 *S. cerevisiae* ORFs. Each ORF is represented by approximately 16 probes, covering different parts of its sequence. Every probe is neighboured by a probe that is identical, except for one nucleotide in the middle of its sequence. This probe is called the 'mismatch' probe (MM), as opposed to the 'perfect match' probe (PM). The arrays were scanned with the GeneArray Scanner System on standard settings at 3 μm resolution. The data were extracted from the scanned images with MAS 5.0 (Microarray Suite 5.0).

Data processing

The raw data were analyzed in dChip. DNA-Chip Analyzer (dChip) is a software package implementing model-based expression analysis of oligonucleotide arrays (Li and Wong, 2001) and several high-level analysis procedures. The model-based approach allows

probe-level analysis on multiple arrays. By pooling information across many arrays, it is possible to assess standard errors for the expression indexes. This procedure also allows automatic probe selection in the analysis stage to reduce errors due to cross-hybridizing probes and image contamination. Fifteen datasets from three time series experiments were analyzed with dChip. The normalization procedure was used to adjust the brightness of the arrays to a similar level. The background subtraction was performed prior to calculation of expression. The model based expression was calculated with the PM (Perfect match)-only model. The PM-only model is unaffected by adverse effect of MM (Mis-Match) probes.
We used the following algorithm to calculate expression values for single probes:

$$PM_{ij}=\theta_i*\varphi_j + \varepsilon$$

θ_i is the model-based expression index (MBEI) of the target gene in array i
φ_j is the probe sensitivity index (PSI) of probe j in a probe set
ε a generic symbol for random error (Li and Wong, 2001).
Log_2 ratios were calculated, based on the obtained expression values, and used in further analysis with the T-profiler algorithm.

T-profiler analysis of DNA microarray data

To assess the contribution of the expression of genes from specific gene classes to the total gene expression, we used a modification of the Quontology algorithm (Boorsma *et al.*, 2004; Bussemaker *et al.*, 2001) namely T-profiler (Boorsma *et al.*, 2005). This algorithm uses an unpaired t-test, which gives a measure of significance to the difference between the mean of a specific class of genes and the mean of the remaining genes of the total gene expression profile:
Formula:

$$\text{T - value} = \frac{m_a - m_b}{\text{pooled SD}\sqrt{\frac{1}{N_a}+\frac{1}{N_b}}}$$

$$\text{pooled SD} = \sqrt{\frac{(N_a - 1)\times s_a^{\,2} + (N_b - 1)\times s_b^{\,2}}{N_a + N_b - 2}}$$

m_a = mean a
m_b = mean b
N_a = number of genes a
N_b = number of genes b
s_a^2 = variance a
s_b^2 = variance b
df = $N_a + N_b - 2$

To increase the robustness of the t-test, we discarded the highest and lowest gene expression value of all gene classes. This method is comparable to the 'jack knife' procedure and will discard single outliers, which might cause false positive or false negative results. An associated P-value was calculated, which was Bonferroni corrected for multiple testing of many gene classes by multiplying it by the number of gene groups that is being tested in parallel. The t-values with an assigned P-value of < 0.05 are considered to be significant.

Gene expression profiles were analyzed using two types of gene groups.

(1) Gene groups with a common regulatory motif in the 600-base pair upstream region. Regulatory motifs were (a) discovered with REDUCE by analysis of published transcript profiling data (Bussemaker *et al.*, 2001), (b) obtained from the SCPD database (http://rulai.cshl.edu/SCPD/), and (c) obtained from Kellis and co-workers, who identified them by comparing four related yeast species (Kellis *et al.*, 2003). All regulatory motifs (in total 153) used for analysis and their characteristics can be found at www.T-profiler.com . Note that T-profiler does not take into account how often a particular motif occurs in a promoter region.

(2) The Gene Ontology categories represent a second type of gene groups. We tested in total 1389 GO gene groups related to function, process or cellular localization (Ashburner *et al.*, 2000). In addition, the gene groups as defined in the MIPS database were tested (http://mips.gsf.de/genre/proj/yeast/).

Testing effects of combined treatments of chitosan and FK506

Overnight cultures of the *Saccharomyces cerevisiae* wild type strain X2180-1**a** were diluted into 60 ml SC and grown to OD_{600} = 0.1 at 30°C. Either no drug, 2 μg/ml FK506, 25 μg/ml chitosan, or a combination of both drugs were added. Cells were incubated at 30°C at 200 rpm. Growth was followed by OD_{600} measurements at 30 minute intervals. The relative growth rates were calculated by linear regression from logarithmic plots of the OD_{600} data versus time.

β 1,3- glucanase sensitivity

Strain X2180-1**a** was inoculated into SC medium, pH 5.5. Cells were cultured at 30°C in flasks (200 rpm) at a culture/air ratio of 1/5. Chitosan was added after 3 h to a concentration of 50 or 100 μg/ml. OD_{600} was measured every hour. After 1, 2, 3 or 4 hours of chitosan treatment a small volume of the culture was spun down (2 minutes at 3,500 rpm) and resuspended in 65 mM Tris-HCl, pH 7.4, to an OD_{600} of 1. β-Mercaptoethanol was added to a concentration of 40 mM. After 1 h incubation at ambient temperature without agitation, 60 units of Quantazyme/ml (Quantum Biotechnologies) were added. After vigorous shaking, the OD_{600} was measured every 10 seconds for 30 minutes in a microtiterplate-reader (Spectramax Plus^{384}, Molecular Devices).

Chitosan susceptibility

Susceptibility of the deletion mutants was tested by spotting serial dilutions of yeast cells onto SC agarose plates with or without chitosan. Agarose was used as a means to solidify the medium instead of agar, because chitosan was less effective in agar plates presumably because it bound to agar polysaccharides The yeast strains were grown overnight at 30^{o}C in SC liquid medium, cells were diluted to OD_{600} =1, and 10-fold serial dilutions of each strain were spotted onto different plates, and followed by incubation for 3 days at 30^{o}C. The plates were photographed, and without further modifications included in the figure.

Results

Chitosan disrupts the Plasma Membrane of Yeast

Chitosan is known to disrupt the outer membrane of Gram-negative bacteria (Helander et al., 2001). In addition, it has been shown that LMW (low molecular weight) chitosan induces formation of mass-transfer channels in artificially created lipid bilayers, thus providing additional evidence for the disturbing effect of chitosan on cell membranes (Yang *et al.*, 2002). To test whether chitosan disrupts the plasma membrane of the yeast *Saccharomyces cerevisiae*, we measured leakage of ultraviolet absorbing compounds (at 260 nm), which represent the nucleotide and coenzyme pools (De Nobel *et al.*, 1990).

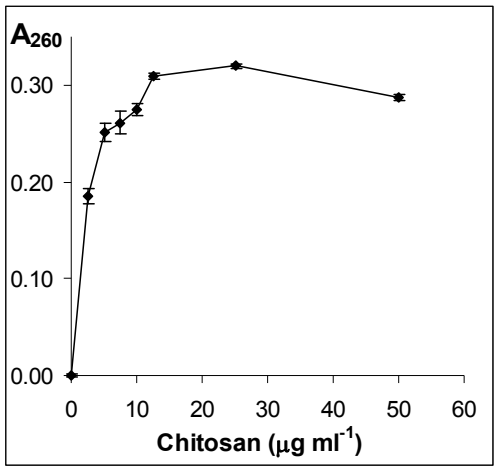


Figure 1. Chitosan causes cellular leakage in yeast.
Cellular leakage was measured at 260 nm after treatment of cells in a buffer at pH 5.5 with the indicated concentrations of chitosan as described in Materials and Methods. The values displayed are means of three independent experiments with the standard error.

Wild-type cells (X2180-1A) were grown to exponential phase, and then exposed to various concentrations of chitosan in 10 mM MES-NaOH buffer at pH 5.5. Under these conditions chitosan induced considerable leakage of cellular components at a concentration as low as 2.5 μg/ ml (Figure 1). Cell leakage reached a plateau at 10-15 μg/ml. These results indicate that chitosan perturbs the plasma membrane of yeast.

Chitosan affects Growth Rate and Viability in a Concentration-dependent Manner

When chitosan was tested in synthetic complete medium (SC) at pH 5.5 at three different concentrations (25, 50, and 100 μg/ ml), we observed almost no effect on growth at 25 μg/ ml chitosan, showing that under these conditions it was considerably less effective

than in buffer alone. This is probably due to the higher ion concentrations in the growth medium. Chitosan at 50 μg/ ml slightly inhibited growth leading to a 10-15% reduction of relative growth rate (Figure 2). However, viability measured as colony forming units was not significantly altered during the course of treatment (results not shown). At 100 μg/ ml we observed a substantial loss of viability (results not shown). For further experiments a concentration of 50 μg/ ml was chosen.

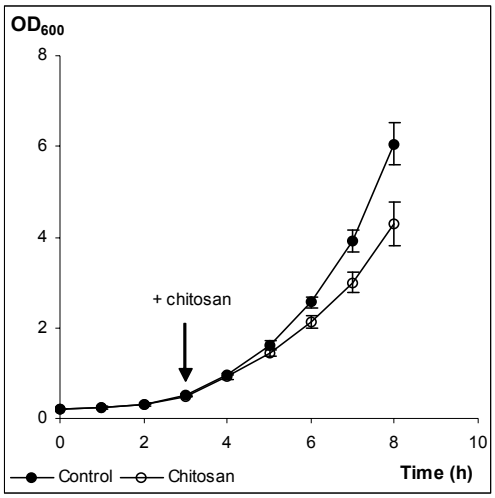


Figure 2. Chitosan inhibits growth of *Saccharomyces cerevisiae*. Strain X2180-1A was grown in batch fermentors in SC medium, pH 5.5. The chitosan concentration used was 50 μg/ ml. Chitosan was added 3 hours after the start of culturing. The displayed values are means of three independent experiments with the standard error.

We also tested growth of three other fungal species in the presence of chitosan.
The human pathogenic fungi *C. albicans* and *C. glabrata* both showed substantial reduction of growth upon exposure to this compound during exponential phase. The relative growth rate of *C. albicans* decreased approximately by 40% and 50% at 50 and 100 μg/ ml chitosan, respectively. *C. glabrata* showed higher resistance to chitosan, yet at 100 and 200 μg/ ml the reduction in relative growth rate was around 15% and 25%, respectively. Interestingly, the common food spoilage fungus *Z. bailii* showed complete growth inhibition at 50 μg/ ml chitosan (Table 1).

Table 1. Chitosan inhibits growth of various fungal species. All species were grown to exponential phase (OD_{600}= 0.1), then chitosan was added at the indicated concentrations. The relative growth rates were calculated from the time points following the addition of chitosan, for at least 3 hours (*Z. bailii*), or up to 5 hours (*C. albicans* and *C. glabrata*). The data are representative of at least two independent experiments. ND , not determined; NG, no growth.

Organism	Relative growth rate (h^{-1})			
	Chitosan (μg/ ml)			
	0	50	100	200
S. cerevisiae	0.54	0.20	ND	ND
C. albicans	0.58	0.33	0.29	ND
C. glabrata	0.65	ND	0.56	0.48
Z. bailii	0.29	NG	ND	ND

Global Expression Changes in Response to Chitosan Treatment in Yeast

To characterize how yeast responds and adapts to chitosan-induced stress, we studied the patterns of gene expression after 15, 30, 60, 120 and 180 minutes of exposure to chitosan, as compared to the zero time point. Six hundred and seventy-one genes were differentially expressed (either induced or repressed at least 1.5-fold at one time point tested). After 15 minutes of treatment with chitosan we found 46 genes that showed ≥ 1.5-fold upregulation. Among these we found *TPO2* and *TPO3* to have the highest expression levels at 15 minutes. Interestingly, genes *TPO2* and *TPO3* code for polyamine transporters that are probably localized in the plasma membrane (Albertsen *et al.*, 2003). Both these proteins have been shown to be involved in transport of spermine (Tomitori *et al.*, 2001), which -like chitosan - is a cationic molecule. There were 55 genes downregulated ≥ 1.5-fold after 15 minutes. In the following time points we observed increasing numbers of both up- and downregulated genes. After 30 minutes the transcript levels of 97 genes were up and 118 genes were down. At the 60 min time point 97 genes showed increased and 73 decreased expression. Chitosan treatment of 120 minutes led to increased expression of 234 genes and decreased expression of 176 genes. The last time point in the series revealed 432 genes induced in expression and 239 genes reduced by at least 1.5-fold.

Functional Classification of the Expression Changes Induced in Response to Chitosan

The transcriptome data were next grouped into categories according to the Gene Ontology nomenclature (1389 groups) and analyzed using T-profiler (see Experimental procedures). Within 15 minutes of chitosan treatment we observed upregulation of two gene groups belonging to the categories “Endoplasmic Reticulum” and ”Integral to Membrane”, respectively (Table 2). Both of these categories remained upregulated throughout the entire time series. The “Integral to membrane” group of genes consists of genes coding for proteins having at least one transmembrane sequence. The “Endoplasmic Reticulum” category is enriched in genes involved in biosynthesis of plasma membrane components, consistent with the plasma membrane being the target of chitosan. After 30 minutes of treatment the categories of “Cell Wall (sensu Fungi)”, and “Cell Wall Organization and Biogenesis”, showed

significant induction, thus indicating that the cell wall response was mounted. In addition, the "Vacuole" genes showed increased induction. We also observed substantial upregulation of the "Plasma Membrane" category at this time point. At 60 minutes of chitosan stress the "Cell Wall" and "Integral to Membrane" groups showed further increase, whereas other categories remained at a similar level of induction.

Table 2. Functional categories induced during chitosan stress.

The number of ORFs in each category is shown in the parentheses. All functional categories were tested (1389). The selected categories have a number of ORFs of ≤ 1000, and a t-value of ≥4 in at least one of the time points tested. Significant t-values are shown in bold. The remaining data are available as supplemental material in a database.

Functional category (no. of ORFs)	t-value at time (min):				
	15	30	60	120	180
Endoplasmic reticulum (113)	**4.7**	**4.7**	**4.9**	**5.6**	**4.7**
Integral to membrane (79)	**4.1**	**5.7**	**6.4**	**5.2**	**4.8**
Cell wall (sensu Fungi) (69)	3.2	**5.8**	**7.3**	**7.5**	**5.3**
Cell wall organization and biogenesis (99)	3.1	**4.8**	**4.6**	**5.2**	**4.1**
Vacuole (sensu Fungi) (22)	3.9	**4.9**	**4.4**	**4.9**	**4.2**
Aspartic type endopeptidase activity (4)	2.0	3.1	3.9	**4.2**	**4.2**
Response to stress (57)	3.8	3.7	2.1	**4.3**	**5.1**
Plasma membrane (151)	2.4	**4.4**	3.8	3.7	1.63

The "Response to Stress" category became significantly upregulated at 120 minutes and was further increased at the last time point. No other major changes in expression were observed at 180 minutes. Among the 1389 GO categories tested we found 18 categories to be significantly repressed at least at one time point (Table 3). Most of the downregulated categories were functionally related to rRNA processing, and ribosomes, thus suggesting that chitosan stress leads to a decrease in translational activity of the cells. A similar initial response has also been observed in case of various other types of stress (Gasch and Werner-Washburne, 2002). Overall, T-profiler analysis using Gene Ontology functional categories to define the gene groups supports the hypothesis that chitosan causes stress on the level of the plasma membrane and induces a cell wall compensatory mechanism. We have also tested the functional categories as defined in MIPS. Similar results were obtained (data not shown), suggesting that our analysis has been comprehensive.

Table 3. Functional categories repressed during chitosan stress.

The number of ORFs in each category is shown in the parentheses. All functional categories were tested (1389). The selected categories have a number of ORFs of ≤ 1000, and a t-value of ≤ -4 in at least one of the time points tested. Significant t-values are shown in bold. The remaining data are available as supplemental material in a database.

Functional category (no. of ORFs)	T-value at time (min):				
	15	30	60	120	180
Nucleolus (83)	**-9.2**	**-12.9**	**-9.4**	**-13.2**	**-13.5**
Nucleus (524)	**-7.2**	**-6.7**	**-8.0**	**-7.9**	**-7.0**
35S primary transcript processing (46)	**-5.7**	**-8.1**	**-6.0**	**-8.5**	**-9.1**
Processing of 20S pre-rRNA (40)	**-5.3**	**-8.5**	**-6.0**	**-9.0**	**-9.6**
rRNA processing (44)	**-4.8**	**-8.5**	**-5.9**	**-8.0**	**-8.1**
Small nucleolar ribonucleoprotein complex (26)	**-4.5**	**-7.0**	**-5.1**	**-7.1**	**-7.8**
snoRNA binding (19)	**-4.1**	**-6.2**	**-4.2**	**-6.7**	**-7.4**
ATP dependent RNA helicase activity (23)	**-4.2**	**-4.6**	-3.5	**-5.4**	**-5.6**
Ribosomal large subunit biogenesis (12)	-2.9	**-5.8**	**-4.2**	**-5.9**	**-5.9**
Ribosome biogenesis (12)	-3.4	**-4.7**	-3.2	**-4.7**	**-5.2**
Ribosomal large subunit assembly and maintenance (30)	-3.0	**-5.5**	-3.7	**-5.3**	**-5.8**
DNA-directed RNA polymerase activity (28)	-2.7	**-5.3**	-3.9	**-4.6**	-3.5
Small nuclear ribonucleoprotein complex (27)	-2.7	**-5.3**	-3.9	**-4.6**	-3.5
Nuclear mRNA splicing, via spliceosome (54)	-2.1	**-4.6**	-3.5	**-4.1**	-2.4
rRNA modification (13)	-2.8	**-4.0**	-3.4	-3.6	-3.9
Transcription from Pol II promoter (39)	-1.7	-3.6	-3.7	**-4.5**	-3.0
General RNA polymerase II transcription factor activity (38)	-1.9	-3.1	-3.7	**-4.1**	-2.5
Spliceosome complex (28)	-3.1	-3.9	-3.7	**-4.0**	-2.7

T-profiler Analysis of Gene Groups with Common Regulatory Elements

Using *T-profiler* (see Experimental procedures) we identified several groups of genes sharing a specific motif (i.e. regulatory element binding site) in the 600 bp of their upstream region that showed significant alteration in transcriptional response to chitosan. We found a number of gene groups sharing a regulatory element that scored significantly at least at one of the time points tested. The seven most significant groups are discussed here. Five of these are positively correlated with expression levels and two represent down-regulated groups of genes.

Chitosan induces the Environmental Stress Response

Most of the commonly known stress conditions induce an expression program called Environmental Stress Response (ESR). It refers to a specific pattern of transcription that involves a large number of genes (~900) that are either up or down regulated in response to

various environmental changes. The induced genes are mainly under control of the Msn2p and Msn4p transcription factors and contain one or more STRE elements in their promoter region. The repressed genes are involved in various aspects of RNA metabolism and ribosome biogenesis (Gasch and Werner-Washburne, 2002).

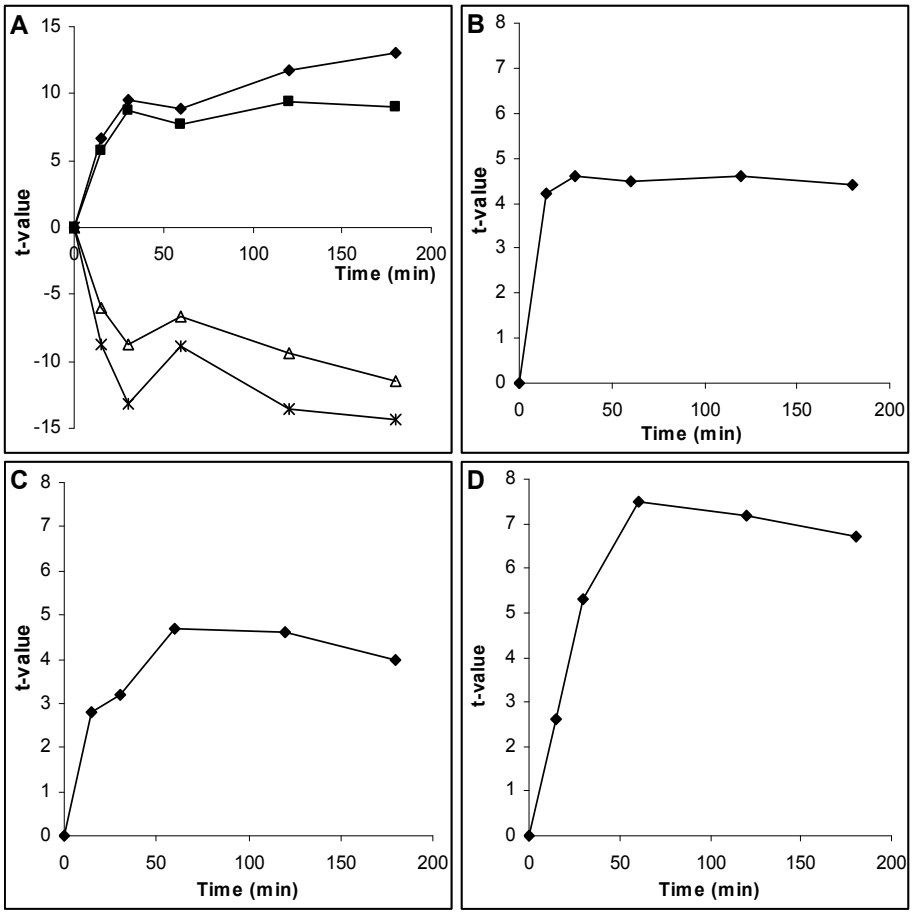


Figure 3. **Transcritpional response of yeast cells to chitosan in time as analyzed by T-profiler.** The presented t-values are calculated on the basis of three experiments. The groups of genes presented here contain the indicated binding sites in their upstream region.
A. Genes regulated by the environmental stress response. solid diamonds, Msn2-4-binding motif (AGGGG); solid squares, Msn2-4-binding motif (CCCCT); stars, PAC motif (CGATGAG); open triangles, rRPE motif (AAAATTT). B. Genes containing the Cin5p-binding motif (MTTAYRTAAK). C. Genes containing the Crz1p-binding motif (GAGGCT). D. Genes containing the Rlm1p-binding motif (TAWWWWTAGM). K, G or T; M, A or C; R, A or G; W, A or T; Y, C or T.

Transcriptional analysis of yeast cells treated with a mild concentration of chitosan revealed the induction of the ESR (Figure 3A). T-profiler analysis showed significant changes

in transcriptional upregulation of genes containing the STRE binding motif. We also found, in agreement with earlier published data, that the genes involved in RNA processing and ribosome biogenesis are down regulated in response to chitosan. The behavior of these genes, containing the PAC and rRPE motifs (Figure 3A), closely mimics the pattern of down-regulation of genes upon different kinds of stress including heat shock, sorbitol, and hydrogen peroxide treatment (Gasch *et al.*, 2000), thus strongly indicating the involvement of PAC and rRPE motifs in the environmental stress response program. The first significant response to chitosan was observed already at 15 min of treatment. The data from following time points showed stronger induction of ESR, both up- and downregulation patterns occurring in parallel.

Chitosan Induces Three Major Specific Transcriptional Responses

T-profiler analysis of microarray data revealed three additional *cis*-regulatory motifs apart from the ESR that were correlated positively with expression in chitosan-treated yeast. The three identified motifs were the binding sites for the transcription factors Cin5p, Crz1p, and Rlm1p (Figure 3B, 3C and 3D, respectively). The Cin5p/Yap4p motif – MTTAYRTAAK – has been identified by REDUCE analysis of the Lee–binding dataset (Lee *et al.*, 2002) and confirmed by REDUCE analysis of 700 published microarray data (Boorsma, unpublished data). Interestingly, in a recent study of 203 DNA-binding transcriptional regulators (Harbison *et al.*, 2004) the reported binding site for Cin5p, TTACRTAA, shows large overlap with the Cin5p - binding site identified in this work. Cin5p is involved in multiple stress responses (Furuchi *et al.*, 2001; Nevitt *et al.*, 2004a; Nevitt *et al.*, 2004b). When overexpressed, it increases tolerance to sodium and lithium (Mendizabal *et al.*, 1998).

Analysis based on FunSpec, which is a web-tool that calculates the significant overlap of two sets of genes based on hypergeometric distribution (Robinson *et al.*, 2002), showed that genes containing a Cin5p binding motif are enriched in the Gene Ontology category of the “Plasma membrane”, thus implicating a role for Cin5p in the regulation of plasma membrane functions (data not shown). The Cin5p-mediated response to chitosan was significantly increased after 15 minutes of treatment and remained at a similar level throughout the course of the experiment (Figure 3B). The group of strongly upregulated genes (at least 2-fold at 180 minutes of treatment) with a Cin5p-binding site contains five hypothetical ORFs, namely *YLR327C, YOR385W, YLR345W, YHR140W* and *YMR090W* (Table 4). Expression of *DDR48, YRO2, NTH1, SAM2* and *COX15* was also increased. *DDR48* and *NTH1* are known to be induced in response to various stress conditions (Boorsma *et al.*, 2004; Zahringer *et al.*, 1997). The gene *YRO2*, which codes for a putative plasma membrane protein, shows strong similarity to *HSP30/YRO1* encoding a plasma membrane protein involved in response to heat shock, ethanol stress and entry into stationary phase (Seymour and Piper, 1999). Hence it seems to be involved in environmental stress response. Overall, the Cin5p-dependent response was mild, but consistent throughout the course of the experiment.

Table 4. ***Saccharomyces cerevisiae*** **genes induced by chitosan treatment: genes sharing the Cin5p-binding site**.

The genes are grouped according to the regulatory elements present in their upstream regions. There are 119 ORFs that share the Cin5p-binding site, MTTAYRTAAK. The values presented are log_2 ratios of normalized signal intensities from pooled experiments. The genes presented here have log_2 ratios equal or above 1 at the 180-min time point.

ORF	Protein	Description	Log_2 ratio at time (min):				
			15	30	60	120	180
YLR327C		Hypothetical ORF	0.87	0.69	0.73	1.03	1.79
YMR173W	Ddr48	DNA damage-responsive protein	0.44	0.64	0.93	1.11	1.52
YOR385W		Hypothetical ORF	0.7	1.26	1.16	1.16	1.38
YLR345W		Hypothetical ORF	0.56	0.56	0.51	0.83	1.17
YBR054W	Yro2	Putative plasma membrane protein of unknown function, transcriptionally regulated by Haa1p	1.24	1.78	1.07	1.06	1.1
YDR001C	Nth1	Neutral trehalase, degrades trehalose; required for thermotolerance and may mediate resistance to other cellular stresses	0.34	0.51	0.42	0.71	1.07
YDR502C	Sam2	methionine biosynthesis regulation	0.14	0.51	0.36	0.79	1.06
YHR140W		Hypothetical ORF	0.46	0.48	0.42	0.55	1.03
YER141W	Cox15	Protein required for the hydroxylation of heme O to form heme A, which is an essential prosthetic group for cytochrome c oxidase	0.47	0.64	0.55	0.74	1.01
YMR090W		Hypothetical ORF	0.38	0.26	0.28	0.68	0.99

The Calcineurin-dependent Pathway is involved in the Response to Chitosan

The regulatory site (GAGGCT) binding Crz1p was identified by REDUCE analysis of transcriptome data from yeast exposed to Ca^{2+} and Na^{+} (Yoshimoto *et al.*, 2002). Crz1p is regulated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. In *S. cerevisiae*, calcineurin is activated under certain environmental conditions, such as exposure to high levels of calcium or sodium, elevated temperature or prolonged incubation with α-factor, and in cell wall mutants (Lagorce *et al.*, 2003). Calcineurin controls Crz1p activity by regulating its subcellular localization. The Crz1p-mediated response was significantly induced in chitosan-treated yeast cells at 60 minutes and 120 minutes, and started to decrease at 180 minutes (Figure 3C). We found sixteen genes to be induced by at least 2-fold at 180 minutes of chitosan treatment (Table 5).

Nine out of these were shown previously to be regulated by Calcineurin/Crz1p (Yoshimoto *et al.*, 2002). We detected 6 hypothetical ORFs, namely, *YJR008W, YOL159C, YIL023C, YOR220W, YOR385W,* and *YBR005W*. The latter three have been identified previously as calcineurin-dependent genes regulated by Ca^{2+} and Na^{+}.

Table 5. ***Saccharomyces cerevisiae*** **genes induced by chitosan treatment: genes sharing the Crz1p-binding site.**

The genes are grouped according to the regulatory elements present in their upstream regions. There are 252 ORFs that share the Crz1p-binding site, GAGGCT. The values presented are log_2 ratios of normalized signal intensities from pooled experiments. The genes presented here have log_2ratios equal or above 1 at the 180 minutes time point.

ORF	Protein	Description	Log_2 ratio at time (min):				
			15	30	60	120	180
YLR136C	Tis11	Zinc finger containing homolog of mammalian TIS11	0.05	0.74	1.58	1.51	2.12
YFR015C	Gsy1	Glycogen synthase with similarity to Gsy2p, expression induced by glucose limitation, nitrogen starvation, enviromental stress, and entry into stationary phase	1.09	0.68	0.50	1.33	1.96
YJR008W		Hypothetical ORF	0.31	0.53	0.60	1.41	1.89
YOL016C	Cmk2	Calmodulin-dependent protein kinase	0.95	2.10	2.09	1.71	1.82
YOR273C	Tpo4	Polyamine transport protein	0.20	0.43	0.49	1.09	1.65
YLL039C	Ubi4	UBI4 locus contains five-six tandem, in-frame copies of ubiquitin protein coding sequence, transcription is induced in response to several stress conditions	0.22	0.23	0.57	1.10	1.59
YOR220W		Hypothetical ORF	0.49	1.16	1.27	1.14	1.51
YOR385W		Hypothetical ORF	0.70	1.26	1.16	1.16	1.38
YNL160W	Ygp1	may be involved in cellular adaptations prior to stationary phase	0.89	1.18	0.93	1.04	1.19
YDL234C	Gyp7	GTPase-activating protein for yeast Rab family members, involved in vesicle mediated protein trafficking	0.37	0.58	0.70	0.80	1.17
YOL159C		Hypothetical ORF	0.34	0.39	0.64	1.06	1.13
YBR005W		Hypothetical ORF	0.17	0.72	0.82	0.76	1.10
YJR010W	Met3	ATP sulfurylase, catalyzes the primary step of intracellular sulfate activation, involved in methionine metabolism	0.24	0.76	0.82	0.89	1.08
YFL030W	Agx1	Alanine : glyoxylate aminotransferase, catalyzes the synthesis of glycine from glyoxylate	0.22	0.29	0.22	0.47	1.06
YIL023C		Hypothetical ORF	0.11	0.15	0.39	0.89	1.02
YGL006W	Pmc1	May be involved in depleting cytosol of Ca^{2+} ions	0.73	0.84	0.68	0.74	0.91

In the group of highly upregulated genes we found *CMK2*, coding for the calmodulin-dependent protein kinase involved in the calcineurin-mediated pathway, which is consistent with published data (Yoshimoto ***et al.***, 2002). The expression of three stress-related genes, *GSY1, UBI4* and *YGP1*, was also induced. Two genes that localize in the yeast membranes, *TPO4* (encoding polyamine transport protein) and *PMC1* (coding for vacuolar membrane Ca^{2+}/ATPase), also showed increased expression upon chitosan treatment of the cells. Both have been identified to be calcineurin-dependent and regulated by Ca^{2+} (Cyert, 2003;

Yoshimoto *et al.*, 2002). Among other genes in this group we found one gene with an unknown function, *TIS11*, and three genes involved in various cellular processes, *GYP7,* involved in vesicle mediated protein trafficking, *MET3,* implicated in methionine metabolism, and *AGX1,* required for glycine biosynthesis. The first three genes of this group have also been found in the genome-wide screen for calcineurin-regulated genes (Yoshimoto *et al.*, 2002). The strength of the Crz1p-dependent response was similar to the Cin5p-mediated induction of expression. However, we observe that the response developed more slowly and reached its maximum at a later time point, indicating that it is mounted later than Cin5p.

Calcineurin is a heterodimer comprised of a catalytic subunit A and a regulatory subunit B. The function of calcineurin can be specifically inhibited by the immunosuppressant FK506. FK506 first binds to immunophilin FKBP12, and then as a complex inhibits calcineurin by binding to the interface between the A and B subunits (Watanabe *et al.*, 1995b). Transcriptome analysis of cells treated with FK506 revealed downregulation of genes, whose transcription depends on the Crz1p regulatory protein (Yoshimoto *et al.*, 2002). We thus tested the effect of simultaneous treatment of yeast with chitosan and FK506 (Figure 4).

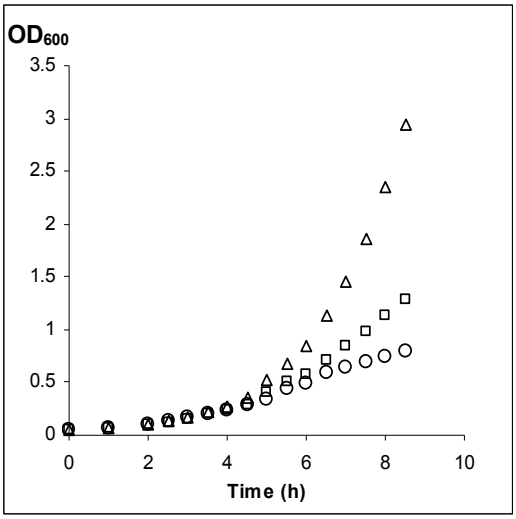


Figure 4. FK506 sensitizes *S. cerevisiae* cells to chitosan.

Strain X2180-1A was grown in SC medium, pH 5.5, to OD_{600} ~0.1. Next, FK506 and chitosan were added at concentrations of 2 μg/ ml and 25 μg/ ml, respectively. Growth was followed for 6.5 hours by OD_{600} measurement. The experiment was performed three times. Control (open triangles), chitosan (open squares), chitosan and FK506 (open circles). Addition of FK506 only did not affect growth.

The exponentially growing cells were exposed to 2 μg/ ml FK506 and 25 μg/ ml chitosan. We monitored growth by measuring OD_{600}. We detected no significant growth rate reduction when FK506 was added to the control (relative growth rates were 0.58±0.05 h^{-1} for

the control and 0.57±0.04 h^{-1} for the FK506-treated cells). Chitosan reduced the relative growth rate to 0.39±0.02 h^{-1}. Interestingly, when both drugs were combined, the relative growth rate was further reduced to 0.30±0.02 h^{-1}. This result suggests that the calcineurin-dependent response is necessary for adaptation of yeast cells exposed to chitosan.

The Cell Wall Integrity Signaling Pathway is Induced in Response to Chitosan

The *cis*-regulatory motif that binds to Rlm1p – TAWWWWTAGM – was identified by REDUCE analysis of transcriptome data from cells exposed to Calcofluor White and Zymolyase (Boorsma *et al.*, 2004). A highly similar sequence has been identified by Dodou and Treisman (Dodou and Treisman, 1997) and Jung and Levin (Jung and Levin, 1999). Rlm1p is a transcription factor involved in induction of the cell wall integrity signaling pathway. The Rlm1p-mediated response also developed slower than the Cin5p-mediated response (Figure 3D). It reached its maximum at 60 minutes and was slightly decreased over the next two hours. Comparison of the chitosan transcriptional profile to recently published microarray data of transient cell wall damage caused by Congo Red, Zymolyase and Calcofluor White (Boorsma *et al.*, 2004; Garcia *et al.*, 2004) and the recent transcriptional analysis of cell wall mutants (Lagorce *et al.*, 2003) shows a large overlap in induction of expression of genes involved in cell wall organization and biogenesis as well as in signal transduction.

In total, we detected 30 genes containing an Rlm1p binding site in the promoter region that increased expression by 2-fold or more at 180 minutes of chitosan treatment (Table 6). Of 16 genes identified as cell wall-related (Jung and Levin, 1999), we found seven to be strongly induced, five slightly induced, two not changed and two missing from the dataset. *SLT2*, encoding a MAP kinase of the cell wall integrity pathway, and *YKL161C/MLP1* coding for a putative MAP kinase that has been shown to interact with Rlm1p (Watanabe *et al.*, 1995a; Watanabe *et al.*, 1997) were positively regulated by chitosan stress. Three strongly upregulated genes code for GPI-CWPs, namely *CWP1*, *PST1* and a gene with unknown function, *YLR194C*. *YLR194C* was also upregulated in all five cell wall mutants investigated in a recent genome-wide study (Lagorce *et al.*, 2003). Taken together, these data strongly suggest a role for *YLR194C* in cell wall biogenesis and structure. Other members of the GPI-CWP family, *SED1* and *CRH1*, showed a slight induction. The gene most strongly upregulated was *PIR3*, which encodes a structural constituent of the cell wall. Interestingly, two other members of the PIR-CWP family (*PIR1* and *PIR2*) were not induced and *CIS3/PIR4* was not present in the dataset. The gene *GSC2* coding for an alternative catalytic subunit of 1,3-β-glucan synthase was highly upregulated in response to chitosan. The expression of *GSC2* is increased upon activation of the cell wall integrity pathway (Jung and Levin, 1999). In addition, it has been shown to be induced during activation of the calcineurin-dependent pathway (Yoshimoto *et al.*, 2002), and to be dually regulated by both pathways (Zhao *et al.*, 1998) indicating that it is subject to combinatorial control. The genes *BGL2, FKS1,* and *CHS3* showed a slight increase of expression in chitosan-treated cells (data not shown). The

expression of *KTR2*, implicated in glycosylation of cell wall mannoproteins, was also induced. In addition to genes known to be involved in cell wall biogenesis and structure, we discovered four members of the GPI-anchored aspartic protease family, namely, *YPS1*, *YPS3*, *YPS5*, and *YPS6*. Although this family of genes codes for proteins residing in the plasma membrane, they might also have a function in cell wall maintenance.

Table 6. ***S. cerevisiae* genes induced by chitosan: genes sharing the Rlm1p-binding site.**
There are 351 ORFs that share the Rlm1p-binding site, TAWWWWTAGM. The values presented are $\log_2$ ratios of normalized signal intensities from pooled experiments. The genes presented here have $\log_2$ratios equal or above 1 at the 180 minutes time point.

ORF	Protein	Description	$\log_2$ ratio at time (min):				
			15	30	60	120	180
		Cell wall maintenance [1)]					
YKL163W	Pir3	Cell wall protein containing tandem internal repeats	0.13	0.59	1.59	2.52	3.05
YGR032W	Gsc2	Catalytic subunit of 1,3-beta-glucan synthase, has similarity to an alternate catalytic subunit, Fks1p (Gsc1p);	0.17	0.64	1.27	1.26	1.73
YLR194C		Cell wall protein	0.13	0.94	1.32	1.47	1.71
YIR039C	Yps6	Gpi-anchored aspartic protease (Yapsin 6)	0.60	0.74	0.69	1.16	1.57
YKL096W	Cwp1	Cell wall protein, involved in O and N glycosylation, acceptor of β−1,6-glucan	0.28	0.46	0.79	1.23	1.35
YDR055W	Pst1	Protoplasts-secreted protein	0.30	0.55	0.76	1.05	1.32
YLR120C	Yps1	Gpi-anchored aspartic protease (Yapsin 1)	0.42	1.01	1.1	1.15	1.31
YLR121C	Yps3	Gpi-anchored aspartic protease (Yapsin 3)	-0.22	0.27	0.74	0.89	1.20
YGL259W	Yps5	Gpi-anchored aspartic protease (Yapsin 5)	0.33	0.39	0.36	0.66	1.01
YKR061W	Ktr2	Involved in N-linked glycosylation of cell wall mannoproteins	-0.29	0.02	0.5	0.81	1.06
		Signal transduction					
YKL161C	Mlp1	Mpk1-like protein kinase; associates with Rlm1p	-0.09	0.64	1.38	1.83	1.98
YOL016C	Cmk2[2)]	Calmodulin-dependent protein kinase	0.95	2.1	2.09	1.71	1.82
YHR030C	Slt2	MAP kinase of the Cell Wall Integrity Pathway	0.38	0.89	1.08	1.22	1.43

ORF	Protein	Description	Log2 ratio at time (min):				
			15	30	60	120	180
		General stress response					
YLL039C	Ubi4	Protein involved in ubiquitination of proteins	0.22	0.23	0.57	1.1	1.59
YDR533C	Hsp31	Possible chaperone and cysteine protease with similarity to *E. coli* Hsp31 and *S. cerevisiae* Hsp32p, Hsp33p, and Sno4p;	0.10	0.24	0.42	0.86	1.37
YER062C	Hor2	Involved in glycerol biosynthesis; induced in response to hyperosmotic stress and oxidative stress, and during the diauxic transition	0.62	0.75	0.60	0.82	1.22
YDR001C	Nth1	Neutral trehalase, degrades trehalose; required for thermotolerance and may mediate resistance to other cellular stresses	0.34	0.51	0.42	0.71	1.07
		Other/ unknown function					
YHR209W		Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family	0.48	0.82	1.64	2.25	2.84
YGL255W	Zrt1	High-affinity zinc transporter of the plasma membrane	0.18	0.20	0.53	0.69	2.65
YDR085C	Afr1	Protein coordinates regulation of alpha-factor receptor signaling and induction of morphogenesis during conjugation	0.5	0.62	0.91	1.35	1.77
YPL088W		Putative aryl alcohol dehydrogenase	0.01	0.39	1.07	1.4	1.66
YJL082W	Iml2	Protein of unknown function	0.36	0.73	0.83	1.21	1.32
YOL084W	Phm7	Protein of unknown function, expression is regulated by phosphate levels	0.30	0.68	0.38	0.72	1.29
YIL107C	Pfk26	6-Phosphofructose-2-kinase	0.42	0.47	0.46	0.84	1.25
YOL159C		Hypothetical ORF	0.34	0.39	0.64	1.06	1.13
YJL132W		Hypothetical ORF	0.22	0.55	0.60	0.87	1.12
YBR071W		Hypothetical ORF	0.31	0.59	0.78	0.92	1.12
YLR133W	Cki1	Choline kinase	0.04	0.42	0.37	0.92	1.11
YMR315W		Hypothetical ORF	0.27	0.44	0.66	0.94	1.10
YIL108W		Hypothetical ORF	0.18	0.32	0.40	0.73	1.00

1. *SED1* and *CRH1*, which both encode GPI-modified CWPs, are also upregulated (log_2 ratios of 0.4 and 0.9 at 180 minutes, respectively)
2. Probably has an ineffective Rlm1p binding site (see text)

Interestingly, we also found *CMK2* in the group of upregulated genes containing an Rlm1p-binding site. However, the Rlm1p-binding site in the promoter region of this gene

localizes only 9 bp from the start codon, thus indicating this gene is not controlled by Rlm1p. This is also consistent with the different time-course of the expression levels of this gene compared to the other genes in Table 3C. In the Rlm1p-dependent set of responsive genes we further identified three genes that are induced under different stress conditions, *UBI4*, *NTH1,* and *HOR2* (Boorsma *et al.*, 2004). The latter is known to be involved in response to hyperosmotic and oxidative stress. Among other genes in this category *YHR209W* (coding for a putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family (Niewmierzycka and Clarke, 1999), and the gene coding for the zinc transporter of the plasma membrane (*ZRT1*) were both highly upregulated (6-7 fold) in response to chitosan. In summary, the Rlm1p-mediated response was the strongest out of the three specific positive responses identified in transcriptional profiles of chitosan-treated yeast. However, it was delayed as compared to Cin5p-response, hence suggesting that it is an indirect response.

β 1,3 - Glucanase Resistance Increases upon Chitosan Treatment

One of the known outcomes of the cell wall integrity pathway is cell wall remodeling, which can be measured with glucan-degrading enzymes. Induction of the cell wall integrity pathway in response to Calcofluor white and Zymolyase treatment leads to a significant increase of resistance to β-1,3-glucanase (Boorsma *et al.*, 2004; de Nobel *et al.*, 2000). To confirm the role of the Rlm1p-regulated response in the chitosan response, we tested whether chitosan induces resistance to glucanase activity.

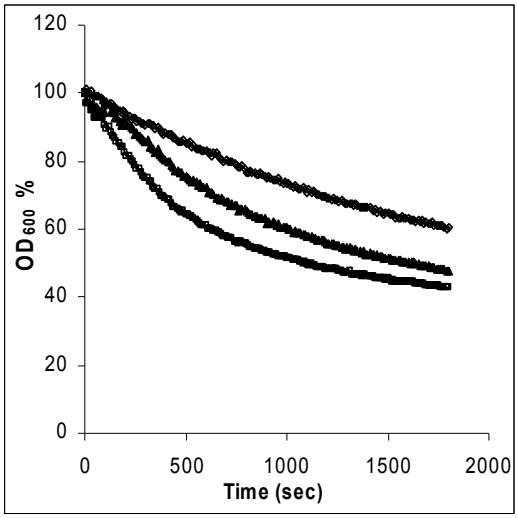


Figure 5. Chitosan increases *S. cerevisiae* resistance to β-1,3-glucanase.

Strain X2180-1A was grown for 3 h in SC medium, pH 5.5. Then, chitosan was added at a concentration of 50 or 100 μg/ml. After 3h of chitosan treatment cells were harvested and treated with Quantazyme (see Experimental procedures). Directly after Quantazyme addition cell lysis was measured every 10 seconds for 30 minutes at OD_{600}, and was expressed as percentage of the starting OD_{600}. The values are means

of two independent experiments. Lower curve (control), middle curve (50 μg/ml chitosan), and upper curve (100 μg/ml chitosan).

Quantazyme resistance of WT cells exposed to chitosan in a time range for up to 4 hours gradually increased, consistent with the notion that chitosan causes Rlm1-mediated cell wall remodeling (results not shown). We further showed that pre-treatment for 3 hours with 50 and 100 μg/ml chitosan caused significant increase in Quantazyme resistance, which depended on chitosan concentration (Figure 5).

The Sensitivity of Transcription Factor Deletion Mutants is altered upon Chitosan Treatment

Analysis of the transcriptional response of yeast to chitosan identified in addition to the general stress response three regulatory pathways, which are mediated by the transcription factors Cin5p, Crz1p, and Rlm1p. In order to investigate whether loss of these regulators leads to increased sensitivity to chitosan, we tested deletion mutants of these genes. We observed a substantial increase in sensitivity of *cin5Δ* and *crz1Δ* cells as compared to WT.(Figure 6).

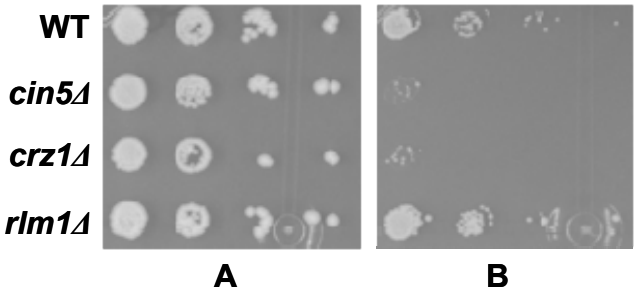


Figure 6. Chitosan susceptibility of *S. cerevisiae* deletion mutants.
Wild-type (WT) and mutant strains were grown overnight in SC at 30°C, the OD_{600} was normalized, and 10-fold dilutions were spotted onto SC plates with or without chitosan. Plates were photographed after 3 days of growth at 30°C. Panel A - growth on SC medium without chitosan. Panel B – growth on SC medium with 200 μg/ ml chitosan.

Interestingly, *rlm1Δ* cells showed no increased sensitivity, but seemed to be slightly more resistant to chitosan, than the WT. Similar results have been obtained before with *rlm1Δ* cells subjected to cell wall stress (Garcia *et al.*, 2004; Reinoso-Martin *et al.*, 2003).

Discussion

Chitosan is the naturally occurring, deacetylated derivative of chitin and has been shown to exert antimicrobial action against a broad range of microorganisms without having a significant effect on mammalian cells (Rhoades and Roller, 2000; Roller and Covill, 1999). For this reason, it is attractive as a potential antimicrobial compound. We have shown here that chitosan affects growth of the model fungus *S. cerevisiae*, the food spoilage fungus *Z. bailii*, and two medically important fungi (*C. albicans* and *C. glabrata*).

To characterize the fungal response to chitosan on a genome-wide scale, we used *S. cerevisiae* as a model organism. Transcriptome data were analyzed with a newly-developed tool, namely T-profiler (Boorsma *et al.*, 2005). T-profiler is a highly sensitive and relatively unbiased method that can be applied to single data sets and does not require introduction of arbitrary cut-offs, in contrast to methods such as for example hierarchical or k-means clustering. This analysis led to identification of four groups of co-expressed genes participating in the environmental stress response, the Cin5p-, the Crz1p-, and the Rlm1p-mediated responses, respectively. The environmental stress response (ESR) was induced rapidly together with the Cin5p-regulated response. The induction of ESR upon chitosan treatment resembled the response patterns observed for other stress conditions (Gasch and Werner-Washburne, 2002). The upregulated genes controlled by Msn2p and Msn4p were acting in parallel with the downregulated genes containing PAC and rRPE motifs. Already after 15 minutes of treatment, the Cin5p-mediated response was almost fully induced and it remained consistently upregulated throughout the duration of the experiment. The Cin5p-controlled genes are known to be involved in multiple stress responses (Furuchi *et al.*, 2001; Nevitt *et al.*, 2004a; Nevitt *et al.*, 2004b). Induction of the Cin5p-mediated response in cells treated with the plasma membrane perturbant, chitosan, suggests a possible role for this transcription factor in dealing with stress targeted at the plasma membrane. This was also supported by the observation that *cin5*Δ cells were hypersensitive to chitosan.

The Crz1p-mediated response was of similar strength as the Cin5p-mediated response, yet it increased more gradually and reached its maximal value only after 60 minutes of treatment, suggesting that it may represent a late response. The Crz1p-dependent response is activated by many forms of stress, including cell wall stress (Lagorce *et al.*, 2003). Crz1p also induces a number of genes involved in ion homeostasis (Yoshimoto *et al.*, 2002). Induction of cell leakage by chitosan stress (Jaspers *et al.*, 1975), could lead to activation of the calcineurin–dependent pathway in order to deal with ion fluctuations. In addition, the calcineurin-dependent pathway has also been shown to play an essential role in the survival of *C. albicans* subjected to fluconazole-induced membrane-stress (Cruz *et al.*, 2002). We observed that blocking the Calcineurin/Crz1p-pathway with the specific calcineurin inhibitor FK506 led to increased sensitivity to chitosan, thus supporting the involvement of the calcineurin-dependent pathway in the response to chitosan-induced membrane stress. In addition, a *crz1*Δ strain was found to be hypersensitive to chitosan.

The Rlm1p-mediated response was considerably stronger than the Cin5p and Crz1p- regulated responses. Since Rlm1 is under the sole control of the cell integrity signaling pathway (Watanabe *et al.*, 1997), it suggests that this pathway is relatively strongly induced by chitosan. Of 16 genes identified as cell wall–related, and under control of Rlm1p (Jung and Levin, 1999), we found 12 to be induced. When we compared our data with transcriptional studies of transient cell wall damage caused by Congo Red, Zymolyase, and Calcofluor White (Boorsma *et al.*, 2004; Garcia *et al.*, 2004) we found strong similarity to the chitosan stress response with respect to the group of upregulated genes involved in cell wall biogenesis and structure. In a recent study, global transcription analysis of five *S. cerevisiae* cell wall deletion mutants, *fks1Δ*, *kre6Δ*, *mnn9Δ*, *gas1Δ* and *knr4Δ*, was used to define a consensus group of genes that are significantly altered in expression by cell wall stress (Lagorce *et al.*, 2003). The genes in this "cell wall compensatory cluster" suggest the involvement of three major signal transduction pathways: the environmental stress response mediated by Msn2 and 4, the Ca^{2+}/calcineurin-mediated pathway and the Cell Integrity Pathway. We found that the same three pathways were induced in response to chitosan stress. Interestingly, on the level of single genes we also observed many similarities, especially in the group of genes containing an Rlm1p-binding site, supporting the hypothesis that chitosan leads to induction of the cell wall integrity pathway.

The involvement of the cell wall integrity pathway in the chitosan-response was validated by another experiment. It has been shown that various forms of cell wall stress result in cell wall modifications, which can be measured as increased resistance of intact cells to digestion with β-1,3-glucanase (Boorsma *et al.*, 2004; de Nobel *et al.*, 2000; Kapteyn *et al.*, 2001). In exponentially growing cells, this increase in resistance is dependent on *SLT2* (de Nobel *et al.*, 2000). We found that treatment of wild-type cells with 50 or 100 μg/ml chitosan caused a significant increase in β-1,3-glucanase resistance, in a time- and concentration-dependent manner. The changes in the cell wall that cause the increased resistance to Quantazyme observed upon cell wall stress remain to be discovered. Possibly, it is due to an increase in the chitin content of the cell wall, and the cross-linking of this polymer to cell wall glucans, which are known responses to cell wall stress (Klis *et al.*, 2002). This idea is consistent with our observation that genes involved in chitin biosynthesis, such as *CHS1* and *CHS3*, are induced by chitosan. Increased incorporation of proteins into the outer layer of the cell wall, which make the β-1,3-glucan layer less accessible, might also contribute to increased resistance to β-1,3-glucanase. This possibility is supported by the strong upregulation of the genes *CWP1* and *PIR3*, which encode cell wall proteins. We also tested the sensitivity of cells deleted for *RLM1*. In contrast to *cin5*Δ and *crz1*Δ cells, which as expected became more sensitive to chitosan, *rlm1*Δ cells were slightly more resistant to chitosan. This is consistent with earlier observations. For example, *rlm1*Δ cells do not become more sensitive to caspofungin and Congo Red, although both compounds are known to induce the cell wall integrity pathway (Garcia *et al.*, 2004; Reinoso-Martin *et al.*, 2003). It has

further been shown that deletion of *RLM1* does not cause a loss in cell integrity (Watanabe *et al.*, 1997). The behavior of the *rlm1*Δ strain under cell wall stress conditions suggests that the Cell Wall Integrity Pathway can be activated via an Rlm1p-independent circuit. It also implies that Rlm1p is not the only target of Slt2p. A possible explanation is that Rlm1p is a member of a protein complex and that its deletion allows another protein to occupy its place in the protein complex and take over its function.

There are four tyrosine phosphatases known to negatively regulate Slt2 activity. These are Ptp2, Ptp3, Sdp1, and Msg5. Of the corresponding genes, only *PTP2* was significantly upregulated in this study, in parallel with *SLT2*. This is in agreement with the finding that *PTP2* is the only one of the four protein tyrosine phosphatase-encoding genes whose expression is regulated by Slt2 itself (Hahn and Thiele, 2002).

The Rlm1p-mediated response was delayed as compared to the Cin5p-mediated response. Conceivably, chitosan-induced loss of plasma membrane integrity may affect the activity of plasma membrane-associated cell wall polysaccharide synthases such as β-1,3-glucan or chitin synthase, and other membrane-bound enzymes involved in cell wall formation. This would result in defective cell wall formation, which the cells may try to counteract by mounting the Rlm1p-mediated response. Interestingly, it has been shown that the cationic drug chlorpromazine, which induces membrane stretch by inserting itself into the lipid bilayer, also activates the Pkc1-Slt2 signaling pathway (Kamada *et al.*, 1995). Furthermore, T-profiler analysis of previously published expression profiles of other plasma membrane-perturbing compounds such as itraconazole and lovastatin (Hughes *et al.*, 2000) also shows activation of the Crz1p- and Rlm1p-mediated responses, suggesting an induction of a similar compensatory response on the cell wall level (data not shown).

In numerous studies the binding of chitosan to anionic compounds has been demonstrated, both on the surface of yeasts and that of Gram-negative bacteria (Helander *et al.*, 2001; Horisberger and Clerc, 1988). It is also known that the presence of divalent cations impairs the effect of chitosan, probably by binding to chitosan targets (Helander *et al.*, 2001). This makes it plausible that the cationic nature of chitosan is part of its mode of action. In a recent study of chitosan effect on bacterial membranes, it has been shown to interact with negatively charged phospholipids, with the NH_3^+ groups being responsible for this interaction (Liu *et al.*, 2004). Yeast sphingolipids, which comprise a major part of plasma membrane lipids and reside in the outer layer, are negatively charged. The most abundant yeast sphingolipid, $M(IP)_2C$, contains even two negative charges. Thus sphingolipids may represent possible binding sites for chitosan. Importantly, mammalian cells do not possess negatively charged sphingolipids, and that may explain why they are much less sensitive to chitosan. Another indication that chitosan may target sphingolipids is provided by the observation that a conserved group of cationic antimicrobial peptides, namely plant defensins, exert their antifungal effect by inducing membrane permeabilization through specific interaction with high affinity binding sites on fungal cell surface (Thevissen *et al.*, 2004). The involvement of

sphingolipids in sensitivity towards plant defensins has been shown for the filamentous fungus *N. crassa* (Ferket *et al.*, 2003). Among other plant defensins, the radish plant defensin RsAFP2 has been demonstrated to cause sphingolipid-dependent antifungal activity against the yeast species *P. pastoris* and *C. albicans* (Thevissen et al., 2004). DmAMP1, a plant defensin from *Dahlia merckii*, induces yeast membrane permeabilization that correlates with its antifungal activity (Im *et al.*, 2003; Thevissen *et al.*, 1999). In the ELISA-based binding assay, DmAMP1 was found to interact with sphingolipids purified from *S. cerevisiae*, with optimal results obtained when an equimolar mixture of ergosterol and yeast sphingolipids was used to reflect the *in vivo* plasma membrane composition in specific domains, namely lipid rafts (Thevissen *et al.*, 2003b) . Yeast lipid rafts are known to be enriched both in sphingolipids and ergosterol, raising the question if chitosan may affect plasma membrane integrity by perturbing lipid rafts. The polycationic nature of chitosan, supported by our transcriptome analysis revealing involvement of plasma membrane in the chitosan stress response, suggests a mode of antifungal activity similar to that of defensins. Finally, chitosan has been shown to greatly improve the fungistatic effects of the classical weak acid preservative benzoic acid (Sagoo *et al.*, 2002). The question whether other synergistically active compounds exist will be addressed in future research together with further studies of exact mechanism of yeast response to chitosan on the molecular and cellular level.

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Chapter 3

Saccharomyces cerevisiae responds to plasma membrane stress by concerted upregulation of Crz1p- and Rlm1p-controlled genes

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Abstract

Integrative analysis of yeast microarray data improves our understanding of how yeast responds to changes in environmental conditions. We used the average expression levels of predefined gene groups with a common regulatory motif to transform genomic expression profiles into t-profiles. Clustering analysis of 46 motif-based t-profiles revealed that two anionic detergents, LAS and SDS, elicit similar transcriptional responses as chitosan, involving upregulation of genes controlled by the transcription factors Crz1p, Rlm1p, Msn2p, and Msn4p. In addition, treatment with LAS, SDS, or chitosan resulted in comparable phenotypes: (i) increased *FKS2-LacZ* expression, which is under the dual control of Crz1p and Rlm1p; (ii) a synergistic growth defect when combined with FK506, a specific inhibitor of calcineurin, and (iii) increased resistance of cells to β-1,3-glucanase, a known result of activation of the cell wall integrity pathway. The t-profiles of these compounds clustered with the t-profiles of cell wall biosynthetic mutants, and the tested cell wall mutants (*fks1*Δ, *kre6*Δ, and *knr4*Δ) were hypersensitive to these agents. We propose that plasma membrane stress causes an orchestrated transcriptional response mediated by Crz1p and Rlm1p, in addition to the environmental stress response, and that this is a transcriptional signature for loss of membrane integrity.

Keywords: *SDS, LAS, chitosan, T-profiler, cell wall integrity pathway*

Introduction

In *S. cerevisiae* plasma membrane stress can be caused by chemical or genetic perturbation. There are two main categories of chemicals that cause membrane stress: compounds that act indirectly by inhibiting membrane lipid biosynthesis, and those that directly interact with plasma membrane lipids. The first category is represented by ergosterol biosynthesis inhibitors, such as azoles, allylamines, and morpholines, and inhibitors of sphingolipid biosynthesis, such as myriocin, and aureobasidin A. The second group comprises such agents as the anionic detergents (LAS - sodium n-dodecyl benzene sulfonate, SDS-sodium dodecyl sulfate), ergosterol – binding antimicrobials (amphotericin B, nystatin, natamycin), chlorpromazine, which causes plasma membrane stretch, antimicrobial peptides, which are often cationic, and chitosan, which is a polycation causing considerable leakage of cellular components and leads to cell wall remodeling (Kamada *et al.*, 1995; Sirisattha *et al.*, 2004; Thevissen *et al.*, 1999; Thevissen *et al.*, 2004; Zakrzewska *et al.*, 2005).

The abrupt changes in external environment instigate rapid adjustments of the genomic expression program for adaptation to the new conditions. Integration of expression data originating from multiple microarray experiments is essential for understanding the transcriptional response of yeast to various environmental stimuli (Causton *et al.*, 2001; Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). For example, yeast expression analysis of over 140 different conditions revealed the induction of the general Environmental Stress Response program (ESR). The ESR is characterized by upregulation of genes mediated by the transcription factors Msn2p and Msn4p, and downregulation of genes involved in various aspects of RNA metabolism and ribosomal biogenesis (Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). The induction of the ESR program is generally accompanied in yeast by activation of condition-specific expression programs.

The expression analysis of yeast cell wall perturbation caused by CFW (Calcofluor White), and Zymolyase revealed induction of the ESR and the gene group regulated by the transcription factor Rlm1p, a downstream activator of the cell wall integrity pathway (Boorsma *et al.*, 2004; Levin, 2005). A genome-wide screen of expression changes in response to activation of the cell wall integrity pathway, identified 20 genes positively regulated by Rlm1p, out of which 16 are involved in cell wall biogenesis (Jung and Levin, 1999; Jung *et al.*, 2002). The cell wall integrity pathway is also activated upon treatment of yeast with other cell wall perturbing compounds such as Congo Red and caspofungin (Garcia *et al.*, 2004; Reinoso-Martin *et al.*, 2003). The activation of the cell wall integrity pathway leads to cell wall remodeling (Smits *et al.*, 2001). Interestingly, chlorpromazine and chitosan, compounds leading to plasma membrane perturbation, also activate the cell wall integrity pathway (Kamada *et al.*, 1995; Zakrzewska *et al.*, 2005). The transcriptional signature of five deletion mutants of genes involved in cell wall construction revealed the induction of the “cell wall compensatory-dependent gene cluster”, which involved the aforementioned cell wall integrity

pathway as well as two others: the ESR, and the Ca^{2+}/calcineurin-dependent pathway (Lagorce *et al.*, 2003). The Ca^{2+}/calcineurin-dependent pathway in *S. cerevisiae* is required for survival of endoplasmic reticulum stress, exposure to ions, incubation with mating pheromone, and alkaline stress (Bonilla *et al.*, 2002; Bonilla and Cunningham, 2003; Cyert, 2003; Viladevall *et al.*, 2004). In a study of the yeast transcriptional response to activation of the Ca^{2+}/calcineurin pathway, expression of three distinct classes of genes was shown to depend on the major transcription factor of the pathway, Crz1p (Yoshimoto *et al.*, 2002). In *S. cerevisiae*, *crz1*Δ was found to be hypersensitive to drugs inhibiting ergosterol biosynthesis (Edlind *et al.*, 2002). In *C. albicans* the Ca^{2+}/calcineurin-dependent pathway is implicated in the response to plasma membrane stress, as the deletion strain of *C. albicans CRZ1* is hypersensitive to plasma membrane stress. In addition, the inhibition of the pathway with FK506 renders the cells hypersensitive to drugs inhibiting ergosterol biosynthesis. Finally, the deletion mutant of the regulatory subunit of calcineurin in *C. albicans*, *CNB1*, has increased sensitivity to plasma membrane stress (Cruz *et al.*, 2002; Karababa *et al.*, 2006; Marchetti *et al.*, 2000; Onyewu *et al.*, 2003; Sanglard *et al.*, 2003; Santos and de Larrinoa, 2005).

Interestingly, the cell wall integrity pathway and the Ca^{2+}/ calcineurin pathway seem to perform independent but related functions in maintenance of cell integrity in *S.cerevisiae* and functioning of one of these pathways is essential for cell viability when the other pathway is defective. The *S.cerevisiae* mutants of genes involved in cell wall integrity signaling *SLT2*, *PKC1*, and the mutant of β-1,3 glucan synthase, *FKS1*, require calcineurin for normal growth, as they are hypersensitive to the calcineurin inhibitor FK506 (Garrett-Engele *et al.*, 1995). The *cnb1*Δ mutant in *S. cerevisiae* is synthetically lethal with *SLT2*, *BCK1*, *GAS1*, *FKS1,* and *KNR4*, and a double mutant in *CRZ1* and *FKS1* is inviable due to deficiency in *FKS2* expression (Lesage *et al.*, 2004; Parsons *et al.*, 2004; Tong *et al.*, 2004).

In this study we have integrated approximately 600 public expression profiles of various stress treatments and mutants, including our data on the transcriptional response to chitosan, in order to gain insight in the transcriptional response of *S.cerevisiae* to plasma membrane stress. Combination of t-profiler and clustering analysis showed that chitosan clusters closely with two other known plasma perturbants, LAS and SDS. All three compounds induce expression of *FKS2-LacZ* mediated by Rlm1p and Crz1p. We present evidence that LAS and SDS, like chitosan, lead to cell wall remodeling and that their growth inhibitory activity is enhanced when combined with FK506, a potent inhibitor of the Ca^{2+}/ calcineurin pathway. Furthermore, we show that cell wall biogenesis mutants are hypersensitive to these three compounds. Finally, we report that transcriptional responses to deletion of plasma membrane and cell wall biosynthetic genes, and of cells treated with membrane perturbants involve induction of both the Crz1p-, and Rlm1p- mediated responses in addition to the Msn2/4p-mediated response.

Experimental procedures

Microarray data

We used publicly available microarray datasets for our analysis. The microarray data of mutants used in this study were obtained from a compendium of approximately 300 yeast deletion mutant expression profiles (Hughes *et al.*, 2000), a genome-wide analysis of the response to cell wall mutations (Lagorce *et al.*, 2003), and the collection of expression profiles of strains with essential genes under tet-regulatable promoters (Mnaimneh *et al.*, 2004). The transcriptome data of chemical and physical stress conditions were collected from three major publicly available datasets; the yeast environmental stress data (Gasch *et al.*, 2000), a yeast compendium (Hughes *et al.*, 2000), Egenomix (http://kasumi.nibh.jp/~egenomix/) and two other publications (Boorsma *et al.*, 2004; Bro *et al.*, 2003). All data were obtained in the form of $\log_2$ ratios, and subsequently analyzed with T-profiler.

T-profiler analysis of DNA microarray data

To assess the contribution of the expression of genes from specific gene classes to the total gene expression, we used T-profiler (Boorsma *et al.*, 2005). This algorithm uses an unpaired t-test to obtain a measure of significance for the difference between the mean of a specific class of genes and the mean of the remaining genes of the total gene expression profile. Gene expression profiles were analyzed using two types of gene groups. (1) Gene groups with a common regulatory motif in their 600-base pair upstream region. All regulatory motifs (in total: 153) used for analysis and their characteristics can be found at www.T-profiler.org. Note that T-profiler does not take into account how often a particular motif occurs in a promoter region. (2) The TFO (Transcription Factor Occupancy) – based categories, which define groups of genes by actual binding of a transcription factor to their promoter regions (Harbison *et al.*, 2004; Lee *et al.*, 2002). We set constraints on our analysis such that all predefined gene groups used had to have more than seven gene members, and had to be active in at least 4 experiments out of ~1000 tested. For each microarray experiment we obtained two t-profiles based on the motif-derived and TFO-derived gene groups. These t-profiles were used for clustering.

Hierarchical clustering

Microarray data clustering was performed in J-express (Dysvik and Jonassen, 2001). We used the hierarchical clustering method (Eisen *et al.*, 1998). Agglomerative hierarchical clustering is an unsupervised procedure where objects are subjected to pair wise similarity measurement, in order to obtain a hierarchy of objects reflecting their relationship to one another. Instead of the commonly used clustering input, that is the $\log_2$ ratios of all genes of one experiment, we used the t-profile of each experiment, which contains the t-values of all predefined gene groups from one category (*i.e.* regulatory motif, or TFO-based).

Strains, plasmids and growth conditions

Saccharomyces cerevisiae strain BY4741 (*MAT**a** his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and individual deletion strains in this genetic background were used in this study. Synthetic Complete medium (SC: 0.67% Yeast Nitrogen Base without amino acids, 0.5% casamino acids, 2% glucose), with 25 mM phthalate, pH 5.5 set with 10 M NaOH, was used for growth. Cells were pre-cultured overnight in SC before inoculating in fresh medium to an OD_{600} of 0.05 (~0.5 x 10^6 cells/ ml). The cultures were grown in SC medium at 30°C. Test compounds were added when the OD_{600} was 0.1 (~1 x 10^6 cells/ ml). In exponentially growing culture growth was measured by following OD_{600}, unless indicated otherwise. The sensitivity of deletion mutants, *fks1*Δ, *kre6*Δ, and *knr4*Δ, to chitosan, LAS, or SDS, was measured as an endpoint OD_{600} after 18 hours of growth (stationary phase), and expressed as the relative OD_{600} of the treated culture compared to the control.

Plasmid *FKS2–lacZ* [*FKS2*(*-928* to *-1*)*–lacZ*] contains 928 bp of the *FKS2* upstream non-coding region in front of the bacterial *lacZ* gene (Zhao *et al.*, 1998) and was kindly provided by D. Levin. This reporter plasmid is based on the yeast episomal plasmid containing a 2μ origin of replication and *URA3* as selectable marker (Marguet & Lauquin, 1986; Zhao *et al.*, 1998). *Escherichia coli* strain DH5α was used for plasmid analysis and amplification.

Preparation of chitosan

Chitosan was obtained from SIGMA (crab shells, minimum 85% deacetylated). It was fragmented using nitrous deamination. Chitosan was dissolved in 10% acetic acid and incubated with sodium nitrite at a concentration of 20 mg per gram of chitosan at room temperature for 17 h. To stop the reaction, the pH of the sample was adjusted to 5.5 with NaOH. The final concentration of chitosan was measured as glucosamine equivalents after hydrolysis in 6 M HCl for 17 h at 100°C (Popolo *et al*.). The average fragment size was about 50 residues (Zakrzewska *et al.*, 2007).

Yeast susceptibility assays to drug combinations

Saccharomyces cerevisiae strain BY4741 was grown overnight in SC medium and diluted to OD_{600} equal to 0.1 in SC medium the next day. Growth, in the absence or presence of LAS, SDS, or FK506, was followed in 96-well plates in a Spectramax spectrophotometer (Molecular Devices). The relative growth rates were calculated by linear regression from logarithmic plots of the OD_{600} data versus time.

β-Galactosidase assay

Cells containing the *FKS2–lacZ* reporter plasmid were precultured in selective medium for ~24 h at 28 °C. Fresh precultures were used to inoculate liquid cultures (SC, pH 5.5). The transformants were grown for an additional 3 h at 28 °C in the presence or absence of the compounds to be tested. Growth was determined as OD_{600} and the cell number was

measured in a Coulter Counter. The cell number was adjusted to approximately 15 x 10^6 per sample to obtain similar permeabilization efficiency and the cells were permeabilized immediately using chloroform/SDS as described previously (Guarente, 1983). ß-Galactosidase activity was determined at 30°C (Guarente, 1983) and was expressed in Miller units/10^4 cells.

***β-1,3*-Glucanase sensitivity**

Strain BY4741 was inoculated in SC medium, pH 5.5. Cells were cultured at 30°C in Erlenmyer flasks (200 rpm) at a culture/air ratio of 1/5. Compounds were added at an OD_{600} = 0.1 (~1 x 10^6 cells/ml) at various concentrations.OD_{600} was measured every hour. After 3 hours of treatment a small volume of the culture was centrifuged (2 minutes at 3,500 rpm) and the cells were resuspended in 65 mM Tris-HCl, pH 7.4, to an OD_{600} of 1 (de Nobel *et al.*, 2000). β-Mercaptoethanol was added to a concentration of 40 mM. After 1 h incubation at ambient temperature without agitation, 60 units of Quantazyme/ml (Quantum Biotechnologies) were added. After vigorous shaking, the OD_{600} was measured every 30 seconds for 30 minutes in a microtiterplate-reader (Spectramax Plus^{384}, Molecular Devices).

Results

Correlation analysis of transcription profiles based on t-values of predefined gene groups

Cluster analysis is commonly used to classify genes that show correlation of expression levels over many experiments (Eisen *et al.*, 1998; Hughes *et al.*, 2000; Mnaimneh *et al.*, 2004). However, microarray data viewed on a single gene level are generally noisy, due to biological and experimental variability (Hatfield *et al.*, 2003). To avoid the influence of intrinsic variability of single gene expression levels we clustered the data using t-values based on gene groups sharing a common feature (Materials and Methods). First, to test whether correlation on the basis of t-values improves as compared to $\log_2$ ratios, we analyzed the transcriptional responses to compounds with a known mode of action. We calculated correlation coefficients based either on $\log_2$ ratios or on the t-values of motif- and TFO-based gene groups (Materials and Methods). For most data tested we observed a substantial increase in correlation when t-values were used (Table 1).

Table 1. Correlation analysis of transcriptional profiles using t-values of predefined gene groups. Correlation coefficients between indicated pairs of experiments were calculated based on t-values for motif-, and TFO-based gene groups, and based on $\log_2$ ratios of individual genes. The experiments in the pair-wise comparisons are known to share a similar mode of action or cellular target.

Microarray experiments	Correlation coefficient		
	t-values		$\log_2$ ratios
	Motif	TFO	
Itraconazole / Lovastatin	0.83	0.92	0.44
Itraconazole / Terbinafine	0.68	0.93	0.60
Lovastatin / Terbinafine	0.72	0.94	0.45
CFW / Zymolyase	0.90	0.93	0.69
LAS / SDS	0.93	0.64	0.66
SDS / Chitosan	0.85	0.33	0.38
LAS / Chitosan	0.85	0.27	0.33

Next, we compared the correlation coefficients calculated for motif- and TFO-based gene groups, and found that in some cases, especially when the cells were treated with direct plasma membrane perturbants, TFO-based gene groups scored lower. A possible explanation is that the TFO-binding data do not include any tests on plasma membrane stress-causing compounds and thus are not representative for this type of stress (Harbison *et al.*, 2004; Lee

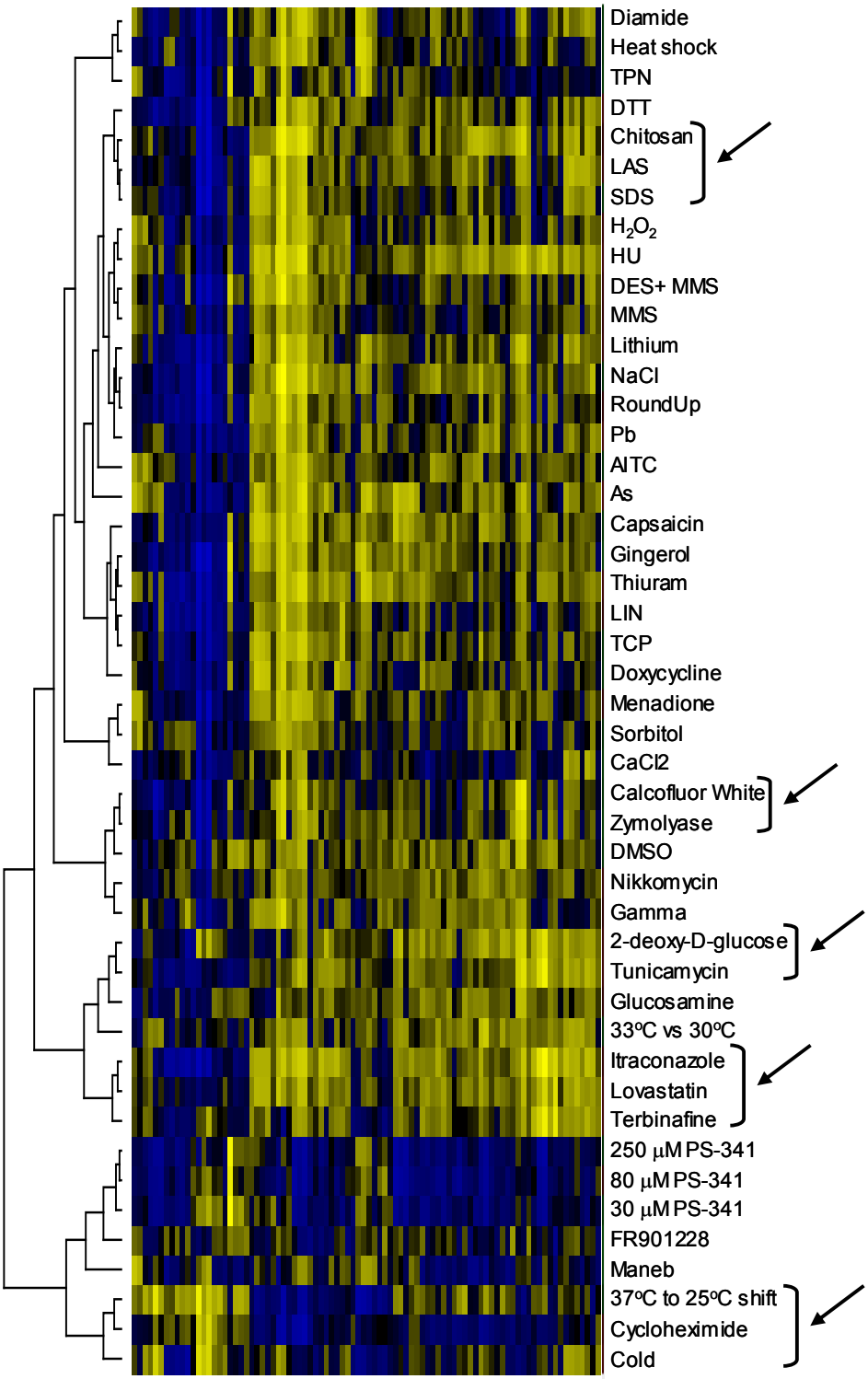
et al., 2002). Since we were particularly interested in unraveling the transcriptional responses to plasma membrane stress, we chose to use the t-profiles based on motif-based gene groups for further comparison of expression data.

Yeast cells treated with chitosan, LAS, or SDS show a similar transcriptional response

We clustered the motif-based t-profiles of 46 microarrays resulting from various chemical perturbations including the chitosan dataset from our previous study (Zakrzewska *et al.*, 2005). Yeast transcriptional responses to changes in environmental conditions are transient and differ in strength (Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). To obtain a complete picture of a transcription response program, initiated upon a stress condition, it would be best to analyze the whole time-spectrum of such a treatment. Yet, most of the available microarray data represent a single time-point only. In order to be able to compare the yeast t-profiles obtained with various chemical perturbations we focused on datasets obtained at 120 min, because the majority of the transcription microarray experiments used that time-point.

Figure 1 presents the results of our global clustering analysis (Figure 1). As expected, we found a tight cluster formed by transcription profiles of yeast treated with three known ergosterol biosynthesis inhibitors (itraconazole, lovastatin, and terbinafine), which confirmed our approach based on clustering of t-profiles. In addition, a cluster containing two well-known perturbants of the yeast cell wall, CFW and Zymolyase, was identified. Further, we found that the t-profiles of three conditions expected to slow down protein biosynthesis, cold shock, temperature downshift from 37^{o}C to 25^{o}C, and addition of cycloheximide, which inhibits protein synthesis, clustered together, indicating induction of a similar transcription response. We also identified a cluster formed by 2-deoxy-D-glucose and tunicamycin. As 2-deoxy-D-glucose is identical to 2-deoxy-D-mannose, one may expect that a treatment with this compound could strongly affect protein mannosylation, a process which is known to be inhibited by tunicamycin. This could explain why the t-profiles of these two compounds clustered together. Finally, we found a cluster formed by t-profiles of cells treated with chitosan, LAS and SDS (Figure 1). These three compounds are known to perturb the plasma membrane, leading to leakage of cellular components.

Figure 1. Clustering analysis of transcriptional profiles using t-values of motif-based gene groups. T-values of motif-based gene groups were calculated for 46 expression datasets of various stress treatments. The obtained t-profiles were clustered using software package J-express. Blue indicates downregulated, and yellow indicates upregulated motif-based gene groups. Arrows point to clusters discussed in the text. A detailed list of experimental conditions can be found in Supplemental Data at the end of the chapter.


Diamide
Heat shock
TPN
DTT
Chitosan
LAS
SDS
H_2O_2
HU
DES+ MMS
MMS
Lithium
NaCl
RoundUp
Pb
AITC
As
Capsaicin
Gingerol
Thiuram
LIN
TCP
Doxycycline
Menadione
Sorbitol
CaCl2
Calcofluor White
Zymolyase
DMSO
Nikkomycin
Gamma
2-deoxy-D-glucose
Tunicamycin
Glucosamine
33ºC vs 30ºC
Itraconazole
Lovastatin
Terbinafine
250 µM PS-341
80 µM PS-341
30 µM PS-341
FR901228
Maneb
37ºC to 25ºC shift
Cycloheximide
Cold

Genes regulated by Crz1p and Rlm1p are induced in yeast cells treated with anionic detergents

To further examine the common characteristics of the yeast transcription programs activated by LAS, SDS, and chitosan, we investigated the significantly induced motif-based gene groups in all three experiments. Interestingly, we found that treatment with LAS and SDS leads to the induction of three responses, namely, the ESR, and those mediated by Rlm1p (LAS), and Crz1p (LAS, and SDS), that were identified previously in the yeast transcriptional response to chitosan (Table 2) (Zakrzewska *et al.*, 2005).

Table 2. Yeast transcriptional response to perturbations of the plasma membrane caused by chitosan, LAS, or SDS, involves induction of the Crz1p- and Rlm1p-mediated responses.
T-values of upregulated motif-based gene groups are presented. Significant t-values are shown in bold. Experiments are ordered according to t-value scores for the Crz1 motif, AGCCWC. M=A/C, W=A/T.

Plasma membrane perturbation	Crz1 AGCCWC	Rlm1 $TA(W)_4TAGM$	Msn2-4 AGGGG/ CCCCT	Publication
LAS	**4.16**	**3.79**	**9.05**	Sirisattha *et al.*
SDS	**4.08**	2.38	**10.31**	Sirisattha *et al.*
Chitosan	**4.01**	**8.08**	**11.68**	Zakrzewska *et al.*

Next, we examined the expression levels of single genes known to be induced by the Rlm1p and Crz1p transcription factors in LAS and SDS treatment (Jung and Levin, 1999; Sirisattha *et al.*, 2004; Yoshimoto *et al.*, 2002). In the group of 18 Rlm1p-regulated genes we found 8 genes to be induced, the two most highly induced genes being *SLT2*, and *GSC2/FKS2*, that code for the MAP kinase of the cell wall integrity pathway and the catalytic subunit of β-1,3-glucan synthase, respectively (Table 3A). Finally, the *RLM1* gene itself was upregulated after treatment with LAS, but not with SDS. The Crz1p – responsive gene group showed strong induction in both treatments (Table 3B). Seventeen out of 19 genes shown previously to be upregulated upon induction of Crz1p response were substantially induced in both, LAS and SDS, experiments (Sirisattha *et al.*, 2004; Yoshimoto *et al.*, 2002). The induction of many genes known to be regulated by the cell wall integrity and Ca^{2+}/ calcineurin pathways indicate that both transcription factors, Crz1p and Rlm1p, contribute to transcriptional response in cells treated with LAS and SDS, similar to chitosan treatment (Zakrzewska *et al.*, 2005).

Table 3. Expression levels of genes activated by transcription factors Rlm1p and Crz1p upon exposure to LAS, SDS, and chitosan.

A. Genes activated by Rlm1p according to Jung and Levin, 1999.

B. Genes activated by Crz1p according to Yoshimoto *et al*., 2002.

A

Gene	Log_2 ratio		
	LAS	SDS	chitosan
SLT2	1.02	0.86	1.22
GSC2	1.7	1.49	1.26
PST1	1.01	0.96	1.05
SED1	0.55	0.07	0.33
PIR1	0.03	-0.36	0.2
PIR3	0.03	0.01	2.52
MLP1	0.47	0.95	1.83
KRE6	0.43	0.18	0.49
CRH1	1.24	0.77	0.65
RLM1	0.86	0.11	0.51
CWP1	-0.89	-0.54	1.23
YLR194C	0.04	-0.07	1.47
CIS3	0.81	0.73	0.22
BGL2	0.5	0.62	0.21
CHS3	-0.58	0.11	0.46
SPS100	0.58	0.36	0.13
YGP1	-0.04	0.23	1.04
PGK1	0.07	0.37	-0.04

B

Gene	Log_2 ratio		
	LAS	SDS	chitosan
CMK2	2.11	2.02	1.71
DIA1	2.8	2.7	1.59
GYP7	1.21	1.85	0.8
YPT53	1.59	1.64	NA
MNT4	1.55	1.23	0.39
CPS1	0.98	0.88	0.91
CRH1	1.24	0.77	0.65
YOR385W	2.19	2.57	1.16
YLR414C	1.98	1.85	1.11
YBR005W	1.61	2.21	0.76
YOR220W	1.81	1.83	1.14
PMC1	1.26	2.1	0.74
ENB1	0.45	0.82	0.42
PHO89	1.91	2.03	-0.22
BAG7	1.25	2.21	0.37
SMP1	1.78	0.65	-0.19
PRM8	1.15	1.81	0.3
UIP3	0.97	2.23	0.89
RCN1	0.64	0.63	-0.14

The cell wall integrity and calcineurin pathways collaborate to induce FKS2 expression upon treatment with plasma membrane stress-inducing compounds.

Both the Ca^{2+}/ calcineurin and the cell wall integrity pathway have been shown to participate in controlling *GSC2/FKS2* expression (de Nobel *et al*., 2000; Zhao *et al*., 1998). A *FKS2-lacZ* reporter plasmid, which is activated by both pathways, was previously shown to induce β – galactosidase activity upon temperature shift (Zhao *et al*., 1998), as well as in cells treated with cell wall stress agents *i.e.* CFW, and in cell wall mutants (de Nobel *et al*., 2000). Here we examined the induction of β – galactosidase activity in cells treated with LAS, SDS, or chitosan. The WT strain bearing the reporter plasmid showed considerable induction of β –

galactosidase activity upon all three stresses tested (Table 4). The strongest (~40-fold) induction was observed upon treatment with SDS.

Table 4. Activation of the *FKS2-lacZ* reporter in cells exposed to cell wall or plasma membrane stress.

β-Galactosidase activity was measured after a 3-h exposure of exponentially growing yeast cells to the indicated concentrations of the test compounds. The presented values are means of two independent biological experiments with standard deviations.

Treatment	Concentration μg/ ml	β-galactosidase activity (Miller units/ 10^4 cells)
Control	-	3.3 ± 0.1
Calcofluor white	50	18.0 ± 1.9
Chitosan	50	18.2 ± 3.6
LAS	50	14.7 ± 3.7
SDS	100	125.1 ± 4.7

The differences in strength of the induction of β – galactosidase activity could be related to the observed growth inhibition, with SDS causing the largest decrease in growth. Thus, plasma membrane perturbation with LAS, SDS, or chitosan leads to induction of *GSC2/FKS2* expression, mediated by the transcription factors Rlm1p and Crz1p.

Inhibition of the Ca^{2+}/ calcineurin pathway with FK506 sensitizes the cells to LAS and SDS

We found that yeast cells treated with the anionic detergents LAS and SDS, induced a group of genes regulated by the transcription factor Crz1p. Crz1p activity is regulated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. The immunosuppressant FK506 specifically inhibits the function of calcineurin. FK506 first binds to immunophilin FKBP12, and the resulting complex inhibits calcineurin by binding to the interface between its A and B subunits (Griffith *et al.*, 1995). Transcriptome analysis of cells treated with FK506 revealed downregulation of genes, whose transcription depends on the Crz1p regulatory protein (Yoshimoto *et al.*, 2002). We thus expected that inhibiting the Crz1p-calcineurin pathway with FK506 should sensitize yeast cells to LAS and SDS. Addition of FK506 to a control culture did not cause any growth rate reduction (Table 5). Addition of LAS or SDS led to a substantial reduction of the relative growth rate (to 54 and 46% of the wild-type growth rate, respectively). However, when either of the two drugs was combined with FK506, the relative growth rates were reduced further, to ~ 44% for LAS, and to ~ 30% for SDS. These data demonstrate that the Ca^{2+}/ calcineurin pathway is necessary for optimal growth of yeast cells exposed to these two plasma membrane perturbants.

Table 5. The calcineurin inhibitor FK506 enhances the growth inhibitory effect of LAS and SDS on *S. cerevisiae*.

Cells were grown in SC medium. The following compound concentrations were used: 50 μg/ ml LAS, 100 μg/ ml SDS, 2 μg/ ml FK506. OD_{600} was measured every 20 minutes, and the relative growth rate was calculated from time-points between 2 and 8 hours. The data presented are the average of three independent experiments with standard errors.

Treatment	RGR (h^{-1})	
	-FK506	+FK506
Control	0.52±0.02	0.53±0.02
LAS	0.28±0.02	0.23±0.01
SDS	0.24±0.01	0.16±0.02

β 1, 3 - Glucanase Resistance Increases upon Treatment with LAS and SDS

The induction of the cell wall integrity pathway leads to cell wall remodeling, which can be measured as increased resistance of the cells to glucan-degrading enzymes. Both, treatment with Calcofluor White and Zymolyase led to induction of the cell wall integrity pathway and increased resistance to β-1,3-glucanase (de Nobel *et al.*, 2000).

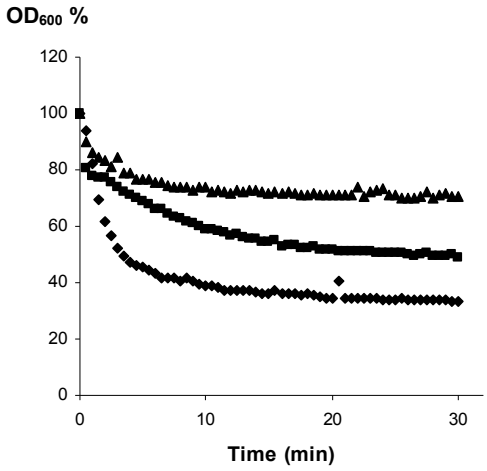


Figure 2. Treatment with LAS or SDS increases the resistance of *S. cerevisiae* to β-1,3-glucanase.

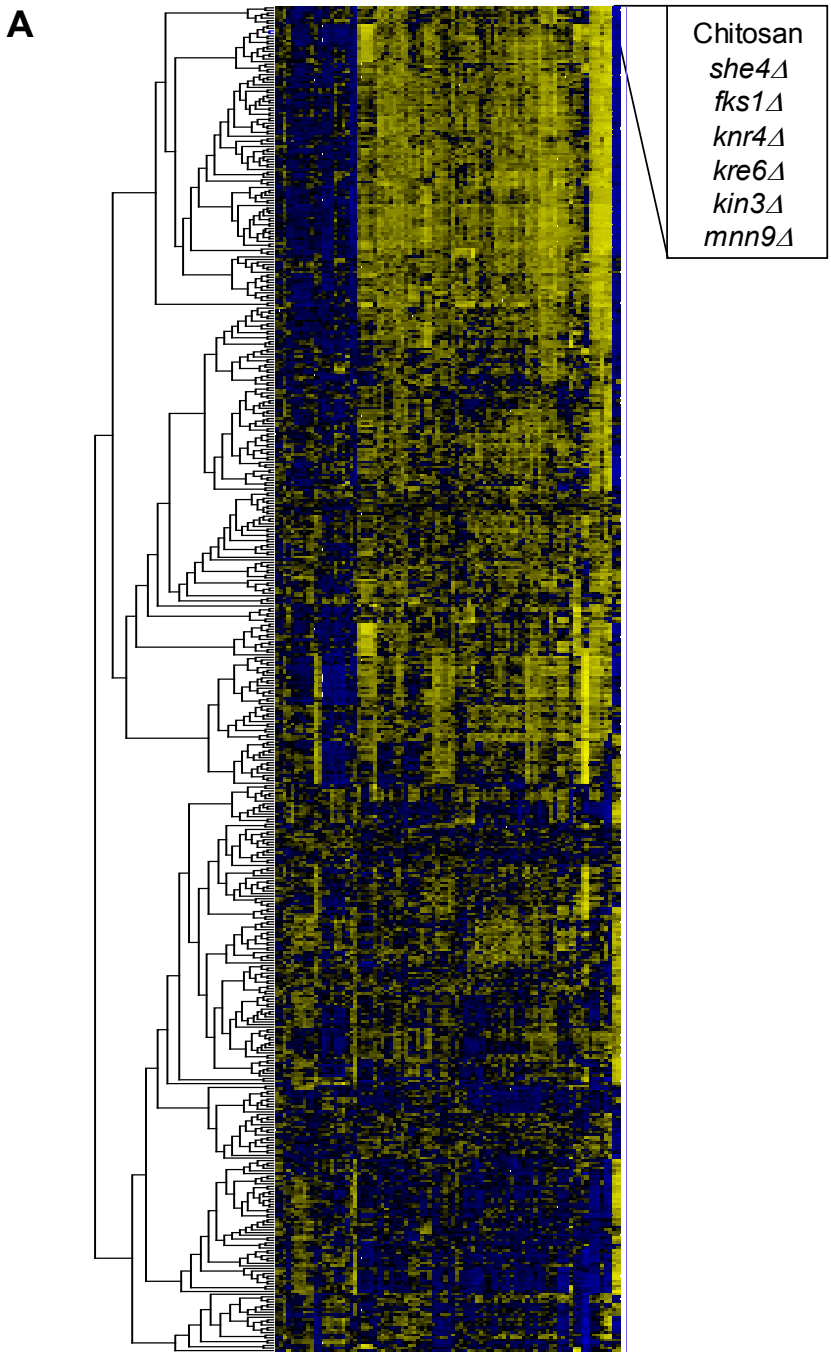
Strain BY4741 was grown until early exponential phase. Then, LAS and SDS were added. After 3h of treatment the cells were harvested and treated with Quantazyme (see Materials and Methods). Directly after Quantazyme addition cell lysis was measured every 30 seconds for 30 minutes at OD_{600}, and was expressed as percentage of the starting OD_{600}. Lower curve (control), middle curve (LAS), and upper curve (SDS).

We have shown previously that treatment with the plasma membrane perturbant chitosan leads to increased resistance to β-1,3-glucanase, in a time- and concentration-dependent fashion (Zakrzewska *et al.*, 2005). To verify the role of the cell wall integrity pathway in cells treated with LAS or SDS we exposed *S.cerevisiae* cells to sublethal concentrations of these two plasma membrane stress compounds, and tested their sensitivity to β-1,3-glucanase. We observed that treatment with both compounds for 3 hours led to increased resistance to cell wall digestion with Quantazyme as compared to the non-treated cells (Figure 2). Interestingly, treatment of yeast cells with SDS led to stronger Quantazyme resistance than treatment with LAS, possibly due to higher concentration of SDS used. These data supports the hypothesis that LAS and SDS cause similar cellular effects as chitosan and lead to cell wall remodeling.

T-profiles of yeast treated with LAS, SDS, or chitosan cluster with deletion mutants of genes involved in cell wall biogenesis.

The transcriptional responses to gene mutations have been widely studied in *S. cerevisiae*. Recent studies revealed that different mutants participating in the same cellular process display related expression profiles. In addition, clustering of expression profiles of deletion mutants with an unknown compound provides insights into its mode of action (Hughes *et al.*, 2000). We re-investigated the expression profiles derived from yeast mutants (Hughes *et al.*, 2000; Lagorce *et al.*, 2003; Mnaimneh *et al.*, 2004) and their similarity to expression profiles derived from chemical perturbation of plasma membrane with chitosan, LAS and SDS, by clustering analysis using t-profiles. We found that the t-profiles of chitosan- and SDS-treated cells cluster closely with t-profiles of six deletion mutants, namely *she4Δ*, *kre6Δ*, *fks1Δ*, *knr4Δ*, *kin3Δ*, and *mnn9Δ* (Figure 3), while the t-profile of LAS-treated cells clusters with the t-profiles of four of those mutants, namely *she4Δ*, *kre6*Δ, *fks1Δ*, and *knr4Δ*. Interestingly, four of these mutants represent loss of genes which are involved in cell wall biosynthesis and protein glycosylation (*kre6Δ*, *fks1Δ*, *knr4Δ*, *mnn9Δ*). *FKS1* encodes a catalytic subunit of a β-1,3-glucan synthase, *KNR4* encodes a protein involved in control of cell wall synthesis, Kre6p is required for β-1,6-glucan synthesis, and Mnn9p is a component of a mannoprotein glycosylation complex. *SHE4* plays a role in endocytosis, polarization of the actin cytoskeleton, and asymmetric mRNA localization, whereas *KIN3* codes for a protein of unknown function that exhibits kinase activity. These results show that the transcriptional program of deletion mutants in genes involved in cell wall biosynthesis is similar to that activated by plasma membrane perturbation with chitosan, LAS, or SDS.

Figure 3. Clustering of t-profiles of compounds with t-profiles of deletion mutants.
The motif-based t-profiles of chitosan, LAS, or SDS were clustered with t-profiles of 523 mutants using J-Express (Materials and Methods). Yellow indicates induction of expression and blue indicates repression.


A
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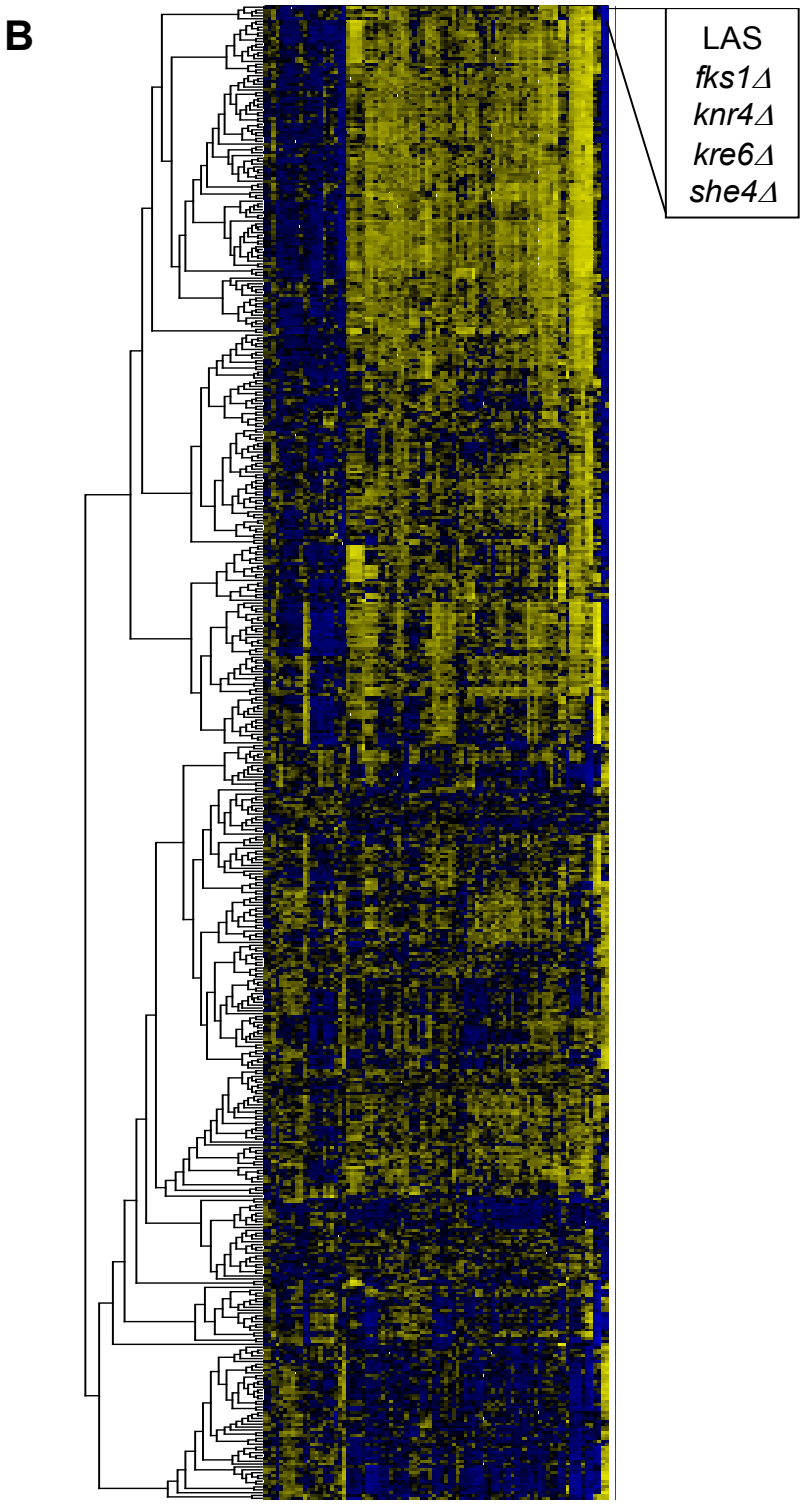
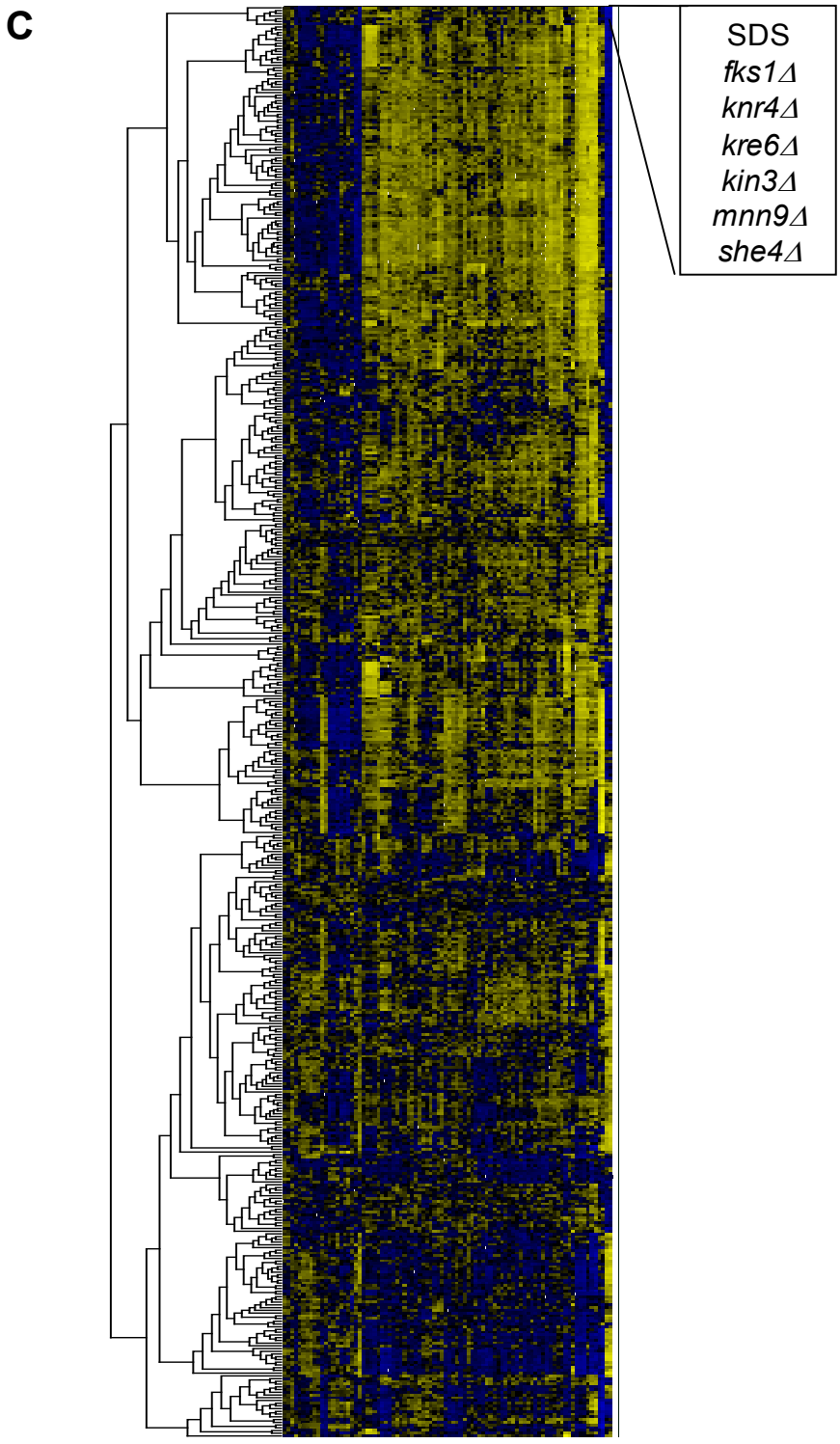
fks1Δ
knr4Δ
kre6Δ
kin3Δ
mnn9Δ


B
LAS
fks1Δ
knr4Δ
kre6Δ
she4Δ


C
SDS
fks1Δ
knr4Δ
kre6Δ
kin3Δ
mnn9Δ
she4Δ

Deletion mutants of genes involved in cell wall biogenesis are hypersensitive to treatment with plasma membrane perturbants

Next, the three deletion mutants in genes involved in cell wall biogenesis, *kre6*Δ, *fks1*Δ, *knr4*Δ, whose t-profiles cluster with t-profiles obtained from cells treated with LAS, SDS, and chitosan, were examined for their sensitivity to these plasma membrane stress compounds (Table 6).

Table 6. Mutants with a defective cell wall are hypersensitive to plasma membrane perturbation by chitosan, LAS, or SDS.

Cells were grown in SC medium with or without drug for 18h. Next, the final OD_{600} was measured. The values (relative growth) represent the final OD_{600} as percentage of the control condition for each strain. The data presented are the average of two independent experiments with standard errors. All three mutants have spherical, enlarged cells.

Strain	Relative growth			
	Control	+ Chitosan	+ LAS	+ SDS
WT	100	83 ± 8	77 ± 6	41 ± 8
*fks1*Δ	100	4 ± 3	57 ± 8	18 ± 4
*kre6*Δ	100	21± 6	24 ± 17	6 ± 2
*knr4*Δ	100	16 ± 7	54 ± 9	13 ± 1

The deletant strain *kre6*Δ exhibited the highest increase in sensitivity to SDS, while the *Δfks1* strain was most sensitive to chitosan. Our data clearly show that the defects in cell wall maintenance experienced by these deletion mutants render them more sensitive to direct plasma membrane perturbation with chitosan, LAS, and SDS.

The Crz1p- and Rlm1p-controlled gene groups are involved in the transcriptional response to loss of plasma membrane integrity

As shown above, we found that three membrane perturbants, LAS, SDS, and chitosan elicit similar transcriptional responses. This raised the question whether common transcriptional features can be found in cells treated with plasma membrane stress-inducing compounds or in mutants lacking a plasma membrane biosynthetic gene. We investigated all significantly upregulated motif-based gene groups in t-profiles of all available microarray experiments that have an effect on the yeast plasma membrane, including the three mentioned above and an additional nine that are caused by genetic perturbations (Table 2 and Table 7). First, we found considerable activation of the ESR program (Msn2-4 motif) in all 12 transcription profiles. Second, the Crz1p-mediated response was induced in 11 experiments. The gene group positively regulated by Crz1p comprises genes involved in ion homeostasis, plasma membrane and cell wall maintenance. Interestingly, we found that the Rlm1p-mediated response was also induced in 11 experiments. Rlm1p is a transcriptional activator of genes involved mainly in maintenance of the cell wall integrity. Altogether, in all 16

datasets resulting from chemical or genetic perturbation of the plasma membrane the gene groups regulated by Crz1p, Rlm1p, or Msn2/4p were generally upregulated.

Table 7. Mutations of genes involved in plasma membrane or cell wall biosynthesis induce the Crz1p- and Rlm1p-mediated transcriptional responses.

T-values of upregulated motif-based gene groups are presented. The significant t-values are shown in bold. Experiments are ordered alphabetically. M=A/C, W=A/T.

Motifs are: Crz1p – AGCCWC, Rlm1p – $TA(W)_4TAGM$, Msn2-4p – AGGGG or CCCCT.

Genetic perturbation	Description of gene function	Crz1p	Rlm1	Msn2-4	Publication
Plasma membrane					
CDS1 (tet)	CDP-diglyceride synthetase, catalyzes critical step in yeast phospholipid synthesis	**6.44**	**9.27**	**7.18**	Mnaimneh *et al.*
ERG1 (tet)	Squalene epoxidase, plays an essential role in the ergosterol-biosynthesis pathway	**3.63**	**11.76**	**5.52**	Mnaimneh *et al.*
ERG11 (tet)	Lanosterol 14-alpha-demethylase, involved in ergosterol biosynthesis	**4.90**	**6.28**	**6.26**	Hughes *et al.*
ERG25 (tet)	C-4 methyl sterol oxidase, involved in ergosterol biosynthesis	2.56	**7.00**	**5.43**	Mnaimneh *et al.*
FAS2 (tet)	Protein catalyzes the synthesis of long-chain saturated fatty acids	**4.67**	**5.78**	**8.05**	Hughes *et al.*
HEM12 (tet)	Involved in heme biosynthesis, heme is required for ergosterol biosynthesis	**4.09**	**11.85**	**7.86**	Mnaimneh *et al.*
HMG2 (tet)	One of two isozymes of HMG-CoA reductase, catalyzes a rate-limiting step in sterol biosynthesis	**3.75**	**4.42**	**4.50**	Hughes *et al.*
MVD1 (tet)	Mevalonate pyrophosphate decarboxylase, involved in isoprenoid and sterol synthesis	**5.02**	**10.92**	**5.67**	Mnaimneh *et al.*
SEC14 (tet)	protein involved in regulation of PtdIns and PtdCho metabolism	**5.56**	**11.18**	**7.41**	Mnaimneh *et al.*
Cell wall					
*fks1*Δ	Catalytic subunit of β-1,3-glucan synthase, crucial for cell wall biogenesis	**5.43**	**10.64**	**3.89**	Hughes *et al.*
FKS1 (tet)	"	**4.07**	**11.53**	**6.90**	Hughes *et al.*
*fks1*Δ	"	1.92	**7.30**	**6.61**	Lagorce *et al.*
*gas1*Δ	transglucosylase, required for cell wall assembly	**5.41**	**10.89**	3.17	Hughes *et al.*
*gas1*Δ	"	**5.01**	**8.99**	**6.19**	Lagorce *et al.*
*knr4*Δ	Protein involved in the regulation of cell wall synthesis	**4.53**	**6.55**	**4.85**	Lagorce *et al.*
*kre6*Δ	Protein required for β-1,6 glucan biosynthesis	**4.06**	**7.25**	**8.16**	Lagorce *et al.*
*mnn9*Δ	Subunit of Golgi mannosyltransferase complex	**5.21**	**6.62**	**3.98**	Lagorce *et al.*

Interestingly, significant induction of gene groups regulated by Crz1p, Rlm1p, or Msn2/4p was found for t-profiles of yeast cells treated with ergosterol biosynthesis inhibitors, namely itraconazole and lovastatin, and lithium, a compound that influences the phospholipid composition of the yeast plasma membrane (data not shown).

A study of global expression changes in five cell wall mutants uncovered a "cell wall compensatory-dependent gene cluster" where both Rlm1p-, and Crz1p-dependent signaling were shown to play an important role (Lagorce *et al.*, 2003). We have re-analyzed the available 8 expression profiles of the cell wall mutants and indeed found significant t-values for gene groups sharing the motifs; AGCCWC, TA(W)$_4$TAGM, and AGGGG or CCCCT, indicating a consistent induction of the Crz1p-, Rlm1p-, and Msn2/4p-mediated responses (Table 7).

To investigate whether Rlm1p- and Crz1p- mediated responses are generally activated in parallel, we have calculated the correlation coefficients between the t-values of both motif-groups in all microarray experiments, and compared them to correlations obtained with t-values of other motif-based groups. Interestingly, we found that the Crz1p and Rlm1p motif-based groups show the highest correlation, with r = 0.77, and show a similar pattern of activation in all expression datasets (Figure 3). This suggests that in most conditions both groups behave similarly, or in other words, genes regulated by these two transcription factors share a comparable pattern of expression.

To conclude, our integrative analysis of expression t-profiles shows that the Ca^{2+}/ calcineurin pathway mediated by Crz1p and the cell wall integrity pathway mediated by Rlm1p are both induced in the transcriptional response of *S.cerevisiae* to plasma membrane stress caused by either chemical or genetic perturbations.

A

Motif	TF	Correlation coefficient	
		TA(W)$_4$TAGM	AGCCWC
TA(W)$_4$TAGM	RLM1	1.00	0.77
AGCCWC	CRZ1	0.77	1.00
TATAWAW	TBP	0.74	0.66
TCTCC	ADR1	0.67	0.65
CCCCT	MSN2-4	0.67	0.59
CCNNNWWRGG	MCM1	0.65	0.59
RG(T)$_5$CCG	RGT1	0.65	0.55
CGG(N)$_{10}$CCG	PUT3	0.57	0.55
CWTCC	GCR1	0.52	0.59
CCG(N)$_5$CCG	CAT8	0.52	0.52
AGGCACA	NRG1	0.52	0.39
CACGTK	PHO4	0.48	0.59

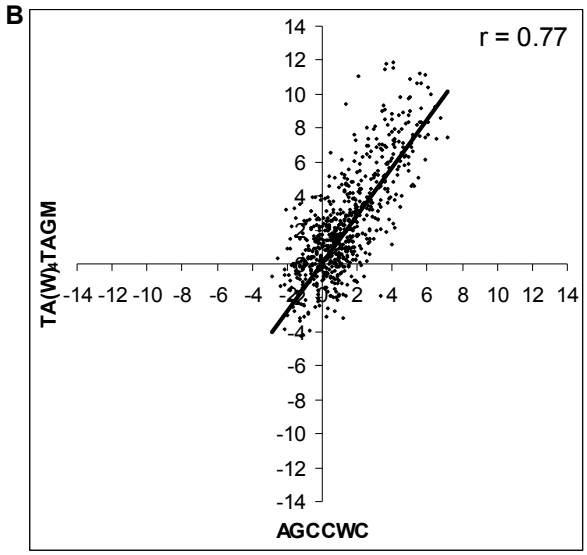


Figure 3. The transcriptional responses of the Crz1p and Rlm1p motif-based gene groups are strongly correlated.

The t-values of motif-based gene groups were calculated for all available expression experiments. Next, the correlation coefficients were calculated based on t-values of gene groups containing the AGCCWC (Crz1p-binding) or TA(W)$_4$TAGM (Rlm1p-binding) motif with all other motif-based groups. (A) The top 10 motif-based gene groups with the highest correlation coefficients with the AGCCWC or TA(W)$_4$TAGM motifs. (B) The scatter plot of t-values of gene groups containing the AGCCWC or the TA(W)$_4$TAGM motif over all experiments. M=A/C, W=A/T.

Discussion

Integrative analysis of yeast transcriptional profiles derived from different chemical or genetic perturbations provides insights into the cellular effects elicited by various compounds (Agarwal *et al.*, 2003; Bammert and Fostel, 2000; Hughes *et al.*, 2000). We have used T-profiler, which transforms gene expression datasets into a limited set of t-values corresponding to predefined gene groups, which can be directly linked to cellular functions, and which has the added advantage that it lowers the noise inherent to microarray data (Boorsma *et al.*, 2005). In this study, we investigated the t-profiles of various chemical treatments while focusing on compounds that show a similar transcriptional response as chitosan, which we studied previously (Zakrzewska *et al.*, 2005). Our analysis shows that LAS and SDS, two anionic detergents, form a tight cluster with chitosan, a polycationic plasma membrane perturbant that activates the Crz1p- and Rlm1p-controlled gene groups (Zakrzewska *et al.*, 2005). The *S. cerevisiae* transcriptional response to LAS and SDS is similar to that to chitosan in that in all three experiments the Crz1p and Rlm1p-regulated genes are induced together with the activation of the ESR.

Expression of *FKS2*, which codes for an enzyme synthesizing the main yeast cell wall polysaccharide, β-1,3-glucan) under stress conditions is controlled by the transcription factors Crz1p and Rlm1p. We showed that treatment of *S.cerevisiae* with LAS, SDS or chitosan led to induction of *FKS2-lacZ*. Treatment of *S.cerevisiae* with terbinafine and miconazole, two drugs that inhibit the biosynthesis of the plasma membrane lipid ergosterol also results in increased expression of *FKS2* (Edlind *et al.*, 2002). Additionally, exposure of yeast cells to the ergosterol-binding drug amphotericin B, induces expression of the *FKS2* gene (Agarwal *et al.*, 2003). Induction of *FKS2-lacZ* was also observed in several cell wall mutants (*fks1Δ*, *gas1Δ*, *kre1Δ*, and *kre9Δ*), in WT cells stressed with the cell wall perturbants CFW and Zymolyase, and upon temperature stress (de Nobel *et al.*, 2000; Zhao *et al.*, 1998). Interestingly, upregulation of *FKS2-lacZ* in response to CFW and Zymolyase fully depends on the presence of Slt2p, while during temperature stress it is under dual control of the transcription factors Crz1p and Rlm1p (de Nobel *et al.*, 2000; Zhao *et al.*, 1998). The relative contribution of each transcription factor to induction of *FKS2-lacZ* upon plasma membrane perturbation remains to be resolved.

The synergistic growth inhibitory effect of LAS and SDS with the calcineurin inhibitor FK506 suggests that the Ca^{2+}/ calcineurin pathway is important for adaptation to plasma membrane perturbation. This is in agreement with the synergistic growth defect observed for the combination of chitosan and FK506 (Zakrzewska *et al.*, 2005). We found a similar synergism when FK506 was combined with chlorpromazine, a compound causing membrane stretch (our unpublished data). Further, blocking of calcineurin with FK506 leads to increased sensitivity to azoles and an allylamine, which both inhibit sterol synthesis (Edlind *et al.*, 2002). Interestingly, Viladevall *et al.* (Viladevall *et al.*, 2004), have shown that exposure of yeast cells to alkaline stress resulted in a strong influx of extracellular calcium and subsequent triggering

of the Ca^{2+}/ calcineurin pathway. Thus, a possible explanation for our observations is that plasma membrane perturbation results in Ca^{2+} influx as well, thus inducing the Ca^{2+}/ calcineurin pathway.

Cell wall remodeling, measured as increased resistance of yeast cells to cell wall digestion with β-1,3-glucanase, is a result of activation of the cell wall integrity pathway in exponentially growing cells (de Nobel *et al.*, 2000). An increased resistance to β-1,3-glucanase was also observed in mutants lacking genes involved in cell wall biogenesis, and cells treated with cell wall stress agents. Interestingly, we found that LAS and SDS also induced cell wall remodeling. Additionally, treatment of *S.cerevisiae* with chlorpromazine caused a significant increase in β-1,3-glucanase resistance (our unpublished data). A similar effect was found previously in cells treated with the plasma membrane perturbant chitosan (Zakrzewska *et al.*, 2005). On the basis of these findings, we propose that cell wall remodeling is a process induced not only upon exposure to cell wall stress but also when yeast cells are treated with plasma membrane perturbants, suggesting that perturbation of the plasma membrane induces a compensatory response that may involve cell wall strengthening.

The t-profiles of yeast treatments with LAS, SDS and chitosan cluster with the t-profiles of mutants deleted for cell wall biosynthetic genes, indicating that the transcriptional response to plasma membrane perturbation and to genetically induced cell wall weakening is similar. It was shown previously that addition of 1M sorbitol to the medium leads to a reduction in the strength of the phenotypes shown by cell wall mutants, indicating that providing osmotic support for the plasma membrane rescues cells from the effects of cell wall weakening (de Nobel *et al.*, 2000). Importantly, all four cell wall biosynthesis mutants are more spherical, and swollen, suggesting that their cell wall is weakened and that as a consequence the plasma membrane is subject to increased stretching, which could explain why the three tested cell wall mutants: *fks1Δ*, *kre6Δ*, and *knr4Δ*, show increased sensitivity to plasma membrane perturbation by LAS, SDS, and chitosan.

The integrative analysis of t-profiles derived from treatment with various plasma membrane perturbants, mutants of membrane lipid and cell wall biosynthetic genes, revealed an expression program that involves gene groups regulated by Rlm1p, and Crz1p. Previously, the Ca^{2+}/ calcineurin pathway and its main transcription factor Crz1p have been shown to play a crucial role in the survival of *C. albicans* exposed to plasma membrane stress (Cruz *et al.*, 2002; Karababa *et al.*, 2006; Onyewu *et al.*, 2003; Santos and de Larrinoa, 2005). Our current findings indicate that similar to *C. albicans*, *S. cerevisiae* requires the Ca^{2+}/calcineurin pathway for dealing with plasma membrane stress. Interestingly, the induction of the cell wall integrity pathway and its transcriptional activator Rlm1p in *S.cerevisiae* has been mostly associated with the response to cell wall stress (Boorsma *et al.*, 2004; Garcia *et al.*, 2004; Jung and Levin, 1999; Lagorce *et al.*, 2003; Reinoso-Martin *et al.*, 2003). Our data extend the range of conditions that lead to activation of the cell wall integrity pathway, by including

several chemical and genetic perturbations of the plasma membrane. Finally, we propose that the transcriptional induction of gene groups controlled by Rlm1p and Crz1p can be a common signature for the response of *S.cerevisiae* to stresses affecting plasma membrane integrity.

Acknowledgments

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Chapter 4

Cellular processes and pathways that protect yeast cells against the plasma membrane-perturbing compound chitosan

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Abstract

Global fitness analysis makes use of a genomic library of tagged deletion strains. We used this approach to study the effect of chitosan, which causes plasma membrane stress. The data were analyzed using T-profiler, which was based on determining the sensitivity to chitosan of groups of deletion strains, as defined by Gene Ontology (GO) and by genomic synthetic lethality screens, in combination with t-statistics. The chitosan-hypersensitive groups included a group of deletion strains characterized by a defective HOG signaling pathway, indicating that the HOG pathway is required for counteracting chitosan-induced stress. Consistent with this, activation of this pathway in wild-type cells by hypertonic conditions offered partial protection against chitosan, whereas hypotonic conditions sensitized the cells to chitosan. Other chitosan-hypersensitive groups were defective in RNA synthesis and processing, actin cytoskeleton organization, protein N-glycosylation, ergosterol synthesis, endocytosis, or in cell wall formation, predicting that these cellular functions buffer the cell against the deleterious effect of chitosan. These predictions were supported by showing that tunicamycin, miconazole, and staurosporine (which target protein N-glycosylation, ergosterol synthesis, and the cell wall integrity pathway, respectively) sensitized yeast cells to chitosan. Intriguingly, the GO-defined group of deletion strains ´cytosolic large ribosomal subunit´ was more resistant to chitosan. We propose that global fitness analysis of yeast in combination with T-profiler is a powerful tool to identify specific cellular processes and pathways that are required for survival under stress conditions.

Keywords: fitness profiling, T-profiler, plasma membrane stress, cell wall stress, HOG pathway, N-glycosylation, ergosterol biosynthesis.

Introduction

Chitosan, a linear β-1,4-D-glucosamine polymer, is a de-acetylated derivative of chitin. When added exogenously, it is known to cause cell leakage and to inhibit the growth of fungi and bacteria; importantly, it seems to be less toxic to mammalian cells. These properties make it a valuable antimicrobial compound with potential applications in medicine and in the food industry (Liu *et al.*, 2004; Rabea *et al.*, 2003; Rhoades and Roller, 2000; Roller and Covill, 1999; van der Lubben *et al.*, 2001; Zakrzewska *et al.*, 2005). Chitosan has a pK_a value of around 6.5. Thus, at neutral and acidic pHs, chitosan molecules become positively charged as a result of the protonation of the amino groups in the glucosamine residues, enabling them to interact with negatively charged components of the plasma membrane such as phospholipids (Liu *et al.*, 2004). Chitosan also induces the formation of mass-transfer channels in artificially created lipid bilayers, thus providing additional evidence for the disturbing effect of chitosan on the plasma membrane (Yang *et al.*, 2002). When Saccharomyces cerevisiae is challenged with sub-lethal concentrations of chitosan, it induces a specific transcriptional expression program comprising the environmental stress response and three more major transcriptional responses mediated by the transcription factors Cin5p, Crz1p, and Rlm1p, respectively (Zakrzewska *et al.*, 2005). This is accompanied by structural changes in the cell wall, as reflected by the increased resistance of living cells to β-1,3-glucanase.

Here, we report a genome-wide screen of heterozygous essential and homozygous non-essential deletion strains for altered sensitivity to chitosan. This type of approach is complementary to transcriptional profiling (Birrell *et al.*, 2002; Brown *et al.*, 2006; Giaever *et al.*, 1999; Giaever *et al.*, 2002; Lum *et al.*, 2004; Parsons *et al.*, 2004; Parsons *et al.*, 2006; Wu *et al.*, 2004; Zewail *et al.*, 2003). Homozygous, non-essential deletion strains have been extensively used to examine the response of yeast deletion strains to various environmental conditions. Strains that are heterozygous for a deletion mutation of an essential gene are assumed to produce less of the essential gene's product and so tend to be hypersensitive to drugs that target that protein. For this reason, they may be used to identify novel drug targets.

We have used T-profiler (Boorsma *et al.*, 2005) to identify pre-defined groups of gene deletion strains that are hypersensitive to chitosan stress. Chitosan-hypersensitive categories of deletion strains included groups with defects in: RNA synthesis and processing; actin cytoskeleton organization; protein N-glycosylation; ergosterol synthesis; endocytosis; and cell wall formation. The results of this analysis were supported by using drugs that target specific cellular processes. For example, tunicamycin, which blocks the N-glycosylation of secretory proteins and thus causes hypoglycosylation of yeast mannoproteins, strongly sensitized yeast cells to chitosan. Chitosan sensitivity was also elevated when it was combined with miconazole (an inhibitor of ergosterol biosynthesis) and with staurosporine, which inhibits the Pkc1p complex - a protein kinase complex that controls the cell wall

integrity pathway. In addition, we demonstrate that various forms of plasma membrane stress, such as that caused by hypotonic conditions, treatment with chlorpromazine (a drug that causes membrane stretch) or a high growth temperature, also dramatically increase the sensitivity of the cells to chitosan. We conclude that analyzing global fitness experiments with T-profiler is a powerful approach to identify cellular processes and pathways that are essential for survival under drug-induced or other stress conditions.

Materials and Methods

Strains and growth conditions

Saccharomyces cerevisiae strain BY4743 (MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ura3Δ0/ura3Δ0) and individual deletion strains in this genetic background were used in this study. Synthetic Complete medium (SC: 0.67% Yeast Nitrogen Base without amino acids, 0.5% casamino acids, 2% glucose), with 25 mM phthalate-NaOH buffer, pH 5.5, was used to culture the wild-type (WT) strain, the homozygous deletion strains (in case of non-essential genes) and the heterozygous deletion strains (in case of essential genes). Cells were pre-cultured overnight in SC (in the case of the WT), for 12 hours in YPD (homozygous deletion pool), or 5 hours in SC (heterozygous essential pool). Batch fermentors were inoculated with cells to an OD_{600} of 0.07 (~0.7 x 10^6 cells/ml). The cultures were grown in 1L SC medium at 30°C, with an aeration rate of 1.0 L/min, fast stirring (200 rpm), and monitoring of pH. Chitosan (final concentration 25 μg/ ml) was added at an OD_{600} = 0.1 (~1 x 10^6 cells/ml). Growth was measured by monitoring OD_{600}. Samples for genomic DNA isolation were taken after 5 and 9 hours of growth. Reference samples were taken at the same time points from a culture grown without chitosan. An appropriate volume (20-25 ml) of the culture was centrifuged at 4,000 rpm for 5 minutes and the cell pellets were stored at – 20°C.

Preparation and analysis of chitosan

Chitosan was obtained from SIGMA (crab shells, minimum 85% deacetylated; average molecular weight ≥ 600 kDa (Ngimhuang J., 2004)). It was fragmented using nitrous deamination. Chitosan was dissolved in 10% acetic acid and incubated with sodium nitrite at a concentration of 20 mg per gram of chitosan at room temperature for 17 h. To stop the reaction, the pH of the preparation was adjusted to 5.5 with NaOH. The final concentration of chitosan was measured as glucosamine equivalents after hydrolysis in 6 M HCl at 100°C for 17 h (Kapteyn *et al.*, 1997). The average size of the chitosan fragments was defined as the ratio of the number of glucosamine residues measured after acid hydrolysis of chitosan and the number of reducing ends measured after fragmentation of chitosan using the method of Lever et al (Lever *et al.*, 1973), and was ~50 glucosamine residues. This was supported by gel filtration, which indicated a molecular weight of about 10 kDa (~60 glucosamine residues). We used chitosan fragments, because we reasoned that native chitosan (average MV ≥ 600 kDa) might be too large to easily pass the cell wall (De Nobel *et al.*, 1990) and because an earlier study had shown that fragmented chitosan had higher antifungal activity than native chitosan (Rhoades and Roller, 2000).

SYTOX Green uptake

Cell samples from exponential-phase cultures of the Saccharomyces cerevisiae wild-type strain BY4741, grown in either the absence or presence of 25 μg/ml chitosan, were spun down at 3,500 rpm, and the cell pellet was washed twice with 50 mM MES-NaOH buffer, pH 5.5. Ninety μl of cell suspension in buffer (OD_{600}=1) were transferred to a 96-well plate with flat bottom wells, and SYTOX Green was added to a final concentration of 1 μM. The SYTOX Green fluorescence was read at 1-min intervals for 30 minutes in a GeminiXS microtiter plate reader, using 488 nm as the excitation wavelength, 544 nm as the emission wavelength, and 530 nm as the cut-off. To calculate relative fluorescence units, all measurements were expressed as a percentage of the fluorescence of a sample of cells treated with 70% v/v ethanol for 5 minutes for complete cell permeabilization, spun down at 3,500 rpm, re-suspended in buffer, and measured as above.

PCR amplification, hybridization, and data acquisition

Yeast cells were lysed at 37^{o}C with 0.5 mg/ml Zymolyase 100T for 60 minutes in sorbitol buffer (1M sorbitol, 100mM sodium EDTA, 14mM β-mercaptoethanol). Genomic DNA was isolated with the Qiagen Dneasy kit. The PCR amplification of the TAGs was performed on 200 ng of genomic DNA template. Both, UPTAGs and DOWNTAGs were amplified in separate reactions using biotinylated PCR primers complementary to common regions in the replacement cassette. PCR conditions and primers used were as described previously (Giaever *et al.*, 2002). The final, labeled UPTAGs and DOWNTAGs were purified on a YM10 Microcon column, combined, and hybridized to custom-made oligonucleotide microarrays (DNA TAG3, Affymetrix, Santa Clara, CA) as described previously (Giaever *et al.*, 2002) with the exception that four blocking primers were used in the hybridization mixture. After 16 hours of hybridization at 42^{o} C, the arrays were stained with streptavidin-phycoerythrin (Molecular Probes) and scanned with an Affymetrix GeneChip Scanner. The hybridization intensities for each probe were determined using Affymetrix GeneChip® Operating Software (GCOS).

Data processing

The majority of knock-out strains are represented by four values of signal intensity (sense and antisense array elements for each UPTAG and DOWNTAG). We have calculated the mean of the signal intensities of tags representing each ORF on the basis of two biological replicates of the experiment. The signal intensities of the UPTAGs and DOWNTAGs were calculated separately. Next, the ORFs whose averaged signal intensity was below the background level in the control experiment were discarded from further analysis. We defined the background as the average of all signal intensities of non-hybridizing spots and found that, for all arrays, it was below 20. To quantify the strain susceptibility to chitosan we calculated the $\log_2$ ratio ($\log_2$ R) of normalized signal intensities

for each strain in the control condition over the chitosan treatment. This results in positive values for sensitive strains. To define significantly sensitive or resistant mutants in strains homozygous for the deletion of non-essential genes or heterozygous for essential genes, we used a cut-off ≥ 1 for the $\log_2$ R at 5 h (corresponding to a ratio of two), and a cut-off ≥ 1.585 for the $\log_2$ R at 9 h (corresponding to a ratio of three).

Data analysis using T-profiler

We used an unpaired t-test, which gives a measure of significance to the difference between the mean of a specific group of deletion mutants and the mean of the remaining deletion mutants of the total dataset (Boorsma *et al.*, 2005). To increase the robustness of the t-test, we discarded the highest and lowest mutant $\log_2$ ratio of all deletion mutant groups. This method is comparable to the 'jack knife' procedure and reduces the effect of outliers, which might cause false-positive or false-negative results. Only groups consisting of ≥ 5 members were used for analysis. The P-values obtained in this way were Bonferroni-corrected for multiple testing by multiplying them by the number of mutant groups tested in parallel. Resulting E-values that were ≤ 0.05 were considered to be significant.

The following two types of pre-defined groups of deletion strains were used in our analysis: (1) Gene Ontology (GO) categories. These are defined according to function, biological process, or cellular localization (Dwight *et al.*, 2002). Deletion mutant groups containing more than 100 members were left out. In total, 961 categories were tested. (2) Synthetic Lethality- (SL-) based deletion mutant groups. These groups have been obtained by crossing a query deletion strain into a set of ~4900 viable deletion mutants and by screening the resulting double mutants for synthetic lethality or slow growth (Tong *et al.*, 2001; Tong *et al.*, 2004). There have been ~500 deletion strains used as queries in such experiments, resulting in the 462 deletion mutant groups used in our analysis. The complete gene composition of each SL- and GO-based deletion mutant group can be found on SGD website (Hong *et al.*).

Growth experiments

Exponential-phase cultures of the Saccharomyces cerevisiae wild-type strain BY4741 were diluted to OD_{600} = 0.1 and cultures of 200 μl were grown further in 96-well plates at 30°C. Either no drug, 25 or 50 μg/ml chitosan, 1.25 μg/ml tunicamycin, 250 μM chlorpromazine, 1.6 μM staurosporine, 2 μg/ml miconazole, or a combination of chitosan with one of the drugs was added. To combine chitosan with hypo-osmotic stress, the cells were suspended in SC medium (control culture) or 75% SC medium, and chitosan was added at 50 μg/ml. To test growth at 37°C, cells were suspended in SC medium, with or without 50 μg/ml chitosan, and placed in a 96-well microtiter plate, which was positioned in the reader that was set at 37°C. Growth was followed for 16 hours by OD_{600} measurements at 20-min

intervals. The relative growth rates were calculated by linear regression from logarithmic plots of the OD_{600} data versus time.

Chitosan susceptibility assays were carried out as described by Zakrzewska et al (Zakrzewska *et al.*, 2005). In short, 10-fold serial dilutions of yeast cells were spotted on solid, synthetic complete medium prepared by using 2% agarose instead of agar, and the plates were incubated at 30^{o}C for three days.

Results

Overview of the chitosan susceptibility of all deletion mutants

Treatment of the *S. cerevisiae* wild-type strain with 25 μg/ml or 50 μg/ml chitosan resulted in a mild reduction in its relative growth rate (Table 1). SYTOX Green is a dye that fluoresces when bound to nucleic acids but that can only pass the plasma membrane when that membrane's integrity is compromised. When exponentially growing cells were treated with 25 μg/ml chitosan and stained with this dye, the fluorescence increased from about 0.5 % of the fluorescence of ethanol-treated cells to ~6% at 15 min, and increased further with time (7% at 30 min, 11% at 60 min, and 13% at 120 min), whereas the fluorescence of untreated cells remained unchanged (Figure 1). These results suggest that chitosan at 25 μg/ml slightly affected the integrity of the plasma membrane. However, after a 4-h exposure to chitosan the fluorescence decreased to 8%, and to 2% at 6 h, suggesting that the cells may partially recover from treatment with this mild chitosan concentration.

Table 1. Conditions and drugs that sensitize yeast cells to chitosan.

WT cells were cultured in SC medium, pH 5.5 in the absence or presence of the indicated drugs. Growth was monitored as OD_{600} in a 96-well microtiter plate for 16 h. The data are from two independent experiments; the differences in values between the two experiments were generally 0.01 – 0.02 and never exceeded 0.04. RGR, Relative Growth Rate. ND, not determined.

Treatment	RGR (h^{-1})		
	- chitosan	+ 25 μg/ml	+ 50 μg/ml
Control	0.52	0.46	0.32
Decreased osmolarity (0.75 SC)	0.52	ND	0
Chlorpromazine (250 mM)	0.25	0	ND
Miconazole (2 mg/ml)	0.33	0.13	ND
Growth at 37ºC	0.59	ND	0
Tunicamycin (1.25 mg/ml)	0.35	0.28	0.24
Staurosporine (1.6 mM)	0.48	0	ND

To identify the genes and the cellular processes involved in chitosan sensitivity, a global fitness experiment was carried out in the presence of 25 μg/ml of chitosan. The abundance of the deletion strains in the control culture and in the chitosan-treated culture was determined after 5 and 9 h of growth, which corresponds to 3-4 and 6-7 generation times of the wild-type strain, respectively.

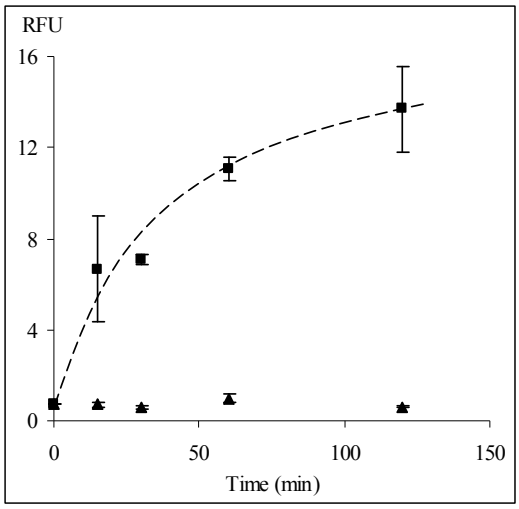

Figure 1. SYTOX Green uptake of yeast cells grown in the presence of chitosan.
Yeast cells were grown exponentially in the absence or presence of 25 μg/ml chitosan. Samples for fluorescence measurements were taken at the indicated time points. RFU – relative fluorescence units, expressed as a percentage of the fluorescence obtained by treating the same number of cells with 70% ethanol. After four and six hours of incubation the RFU values were 8% and 2%, respectively. Closed triangles (control), closed squares (25 μg/ml chitosan); means and standard deviations (n = 3).

These late time points were chosen to be able to detect even relatively small differences in growth. Among the ~4900 homozygous deletion mutants of non-essential genes, we found 184 strains with a $\log_2$ R ≥ 1 (this corresponds to a two-fold reduction in strain abundance in the presence of chitosan) at 5 h and 153 strains with a $\log_2$ R ≥ 1.585 (this corresponds to a three-fold reduction) at 9 h (see Figure 2 for Venn diagrams). One hundred and one mutants were chitosan-hypersensitive at both time points. To establish how reliable the results obtained by the genomic approach were, we retested a number of individual strains that varied widely in their sensitivity to chitosan. Both approaches generally gave the same results, both for the sensitive and the resistant strains (Table 2). As shown before (Zakrzewska *et al.*, 2005), rlm1Δ cells were less sensitive to chitosan than wild-type cells. Interestingly, mnn4Δ and mnn6Δ cells, which are responsible for the introduction of (negatively charged) phosphodiester groups in N- and O-linked protein side-chains, were also less sensitive to chitosan, indicating that binding of chitosan to phosphodiester linkages enhances its effect. Conceivably, binding of chitosan to such groups in plasma membrane proteins, and the resulting local increase in the concentration of chitosan, is largely responsible for this stimulatory effect.

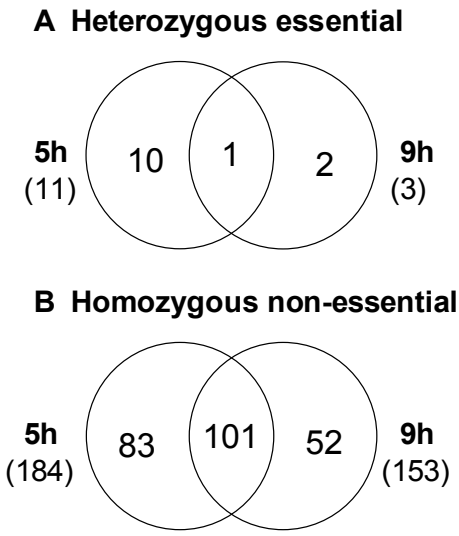

Figure 2. Overview of chitosan-hypersensitive deletion mutants at 5 and 9 hours.
A. Venn diagram summarizing the results for strains heterozygous for the deletion of an essential gene. B. Venn diagram summarizing the results for strains homozygous for the deletion of a non-essential gene. The cut-offs for the $\log_2$ values of R (signal in the absence of chitosan)/(signal in the presence of chitosan) were 1 at 5 h (R = 2), and 1.585 at 9 h (R = 3). The numbers in brackets refer to the total number of sensitive strains identified at the indicated time points.

Among the ~1100 strains heterozygous for the deletion of an essential gene, we detected only 11 and 3 chitosan-hypersensitive mutants at 5 and 9 h, respectively. In addition, the highest observed sensitivity of the mutants, expressed as $\log_2$ R, was much lower than in the homozygous deletion strains, both at 5 h (1.46 and 9.57, respectively) and at 9 h (1.75 and 9.39, respectively). These observations indicate that, in most heterozygotes, the activity of the remaining copy of the gene is sufficient to withstand chitosan-induced stress to a large extent. As previous studies have shown that heterozygous deletants are often highly sensitive to drugs that specifically target the product of the deleted gene (Giaever *et al.*, 1999; Giaever, 2003; Giaever *et al.*, 2004; Lum *et al.*, 2004), our data also indicate that chitosan does not have a unique protein target.

Table 2. Chitosan susceptibility of individual strains.
Growth of individual mutants was determined by serial plate dilution assays, unless indicated otherwise. The individual mutants represent a wide range of $\log_2$ R values. R = (signal control condition)/(signal chitosan treatment). Note that the distribution of $\log_2$ R values tends to be skewed in favor of the positive values.

ORF	Strain	Log_2 ratio at 9h	Sensitivity of individual mutants
YDR200C	*vps64Δ*	6.64	++*
YJL062W	*las21Δ*	4.16	++*
YNR031C	*ssk2Δ*	4.13	++*
YBR171W	*sec66Δ*	3.22	++*
YLR006C	*ssk1Δ*	2.33	++*
YJL183W	*mnn11Δ*	2.29	++
YOR028C	*cin5Δ*	0.53	++
YKL126W	*ypk1Δ*	0.5	++
YPL050C	*mnn9Δ*	0.23	++
YNL237W	*ytp1Δ*	2.4	+
YNL098C	*ras2Δ*	1.39	+
YOL016C	*cmk2Δ*	0.58	+
YER001W	*mnn1Δ*	0.46	+
YBR182C	*smp1Δ*	0.38	+
YMR251W-A	*hor7Δ*	0.26	+
YGR248W	*sol4Δ*	0.08	=
YGR138C	*tpo2Δ*	0.07	=
YKL161C	*mlp1Δ*	0.06	=
YPR008W	*haa1Δ*	0.04	=
YPR005C	*hal1Δ*	-0.07	=
YBR054W	*yro2Δ*	-0.09	=
YOR101W	*ras1Δ*	-0.18	=
YLR258W	*gsy2Δ*	-0.08	-
YGL006W	*pmc1Δ*	-0.35	-
YKL201C	*mnn4Δ*	-0.05	--
YPL053C	*mnn6Δ*	-0.17	--
YPL089C	*rlm1Δ*	-0.46	--
YOL108C	*ino4Δ*	-0.8	--

= similar sensitivity to WT
+ slightly more sensitive than WT
++ hypersensitive
- slightly more resistant than WT
-- more resistant
++* growth of these strains was determined in liquid cultures as opposed to plate dilution assays

Identification of GO-based groups of deletion strains with altered fitness in the presence of chitosan

To acquire easily interpretable biological information about the response of deletion strains to chitosan treatment, we used T-profiler (which is based on t-statistics) to identify changes in susceptibility of predefined groups of mutants (Materials and Methods). We first tested groups of strains deleted for a gene with a common regulatory motif in their upstream promoter region, and groups deleted for a gene bound by a common transcription factor as determined by transcription factor occupancy analysis (Harbison *et al.*, 2004; Lee *et al.*, 2002). None of those groups of deletants had significant t-values, indicating that there is no simple relationship between the transcriptional response of co-regulated genes in response to stress and the fitness of the corresponding deletion mutants under the same condition.

Next, we investigated the response of Gene Ontology- and Synthetic Lethality-based deletion mutant groups to chitosan. Gene Ontology categories were included in our study in order to investigate whether the genes deleted in chitosan-hypersensitive GO-based groups of mutants share any functional features, or participate in the same biological process. The advantage of this approach is that all yeast genes are annotated to these categories, thus giving a comprehensive overview of the whole yeast genome. On the other hand, due to the hierarchical structure of the categories, there is considerable overlap between them. We further used 462 Synthetic Lethality-based gene groups in our analysis, representing a wide spectrum of biological processes and cellular functions (for a detailed discussion of these results, see below). The major advantage of this approach is that it represents experimentally validated relationships between genes, rather than the researchers' view of those relationships. However, it is incomplete, as only around 10% of the genes have been tested as query gene (Tong *et al.*, 2001; Tong *et al.*, 2004).

We detected 13 significant GO groups, 12 of which were hypersensitive to chitosan, whereas one of them contained resistant mutants (Table 3). These 13 groups can be further ordered into several biologically relevant categories.

Four groups of strains that were deleted for genes involved in RNA polymerase II transcription or in RNA splicing were found to be hypersensitive to chitosan. The five most sensitive mutants are paf1Δ, snf5Δ, rtf1Δ, srb5Δ, and med1Δ. Interestingly, Paf1p and Rtf1p belong to the Paf1p complex that is required for full transcription of several cell wall biosynthetic genes (Chang *et al.*, 1999). Ten mutants deleted for genes involved in processes occurring at the endosome membrane (snf8Δ, snf7Δ, vps20Δ, vps28Δ, vps22Δ, vps36Δ, vps25Δ, vps24Δ, did4Δ, srn2Δ) are highly sensitive to chitosan. Deletion mutants of five genes that encode proteins participating in retrograde transport from the Golgi to the ER (get1Δ, get2Δ, get3Δ, glo3Δ, sec22Δ), also show increased sensitivity to chitosan. In addition, the mutants in the group "nuclear envelope-endoplasmic reticulum network" are hypersensitive to chitosan. These observations indicate that proper functioning of the intracellular transport machinery is crucial for counteracting chitosan stress. We also found

that mutants from the group “glycoprotein metabolism”, which includes N-glycosylation, are highly sensitive to chitosan. In addition, we identified the category “response to osmotic stress” to be hypersensitive to chitosan. These findings will be the subject of a more extended discussion later in this paper.

Table 3. Identification of GO-based gene groups with altered fitness in the presence of chitosan.
Significant t-values ($t \geq 4$) are in bold.

Gene groups	ORFs	t-value	
		5h	9h
General RNA polymerase II transcription factor activity	14	3.4	**5.0**
DNA-directed RNA polymerase II, holoenzyme	14	2.8	**4.7**
RNA polymerase II transcription mediator activity	5	2.8	**4.4**
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	22	1.6	**4.8**
Endosome membrane	10	2.5	**4.7**
Retrograde transport, Golgi to ER	8	1.6	**4.5**
Nuclear envelope-endoplasmic reticulum network	64	**5.2**	**4.4**
Glycoprotein metabolism	44	3.9	**4.6**
Response to osmotic stress	40	1.7	**4.7**
Osmosensory signaling pathway via two-component system	8	1.7	**4.2**
Signal transducer activity	44	2.2	**4.5**
Receptor signaling protein activity	10	2.0	**4.3**
Cytosolic large ribosomal subunit (sensu Eukaryota)	59	-3.0	**-4.2**

We also detected one GO functional category that contained genes whose deletants exhibited increased resistance to chitosan. This category, designated as “cytosolic large

ribosomal subunit", comprises genes coding for structural proteins of the large ribosomal subunit. Interestingly, a similar behavior of deletion mutants in ribosomal proteins was observed in other genome-wide fitness studies in yeast (Warringer *et al.*, 2003; Wu *et al.*, 2004).

Deletion mutants of genes that synthesize a truncated lipid-linked oligosaccharide for protein N-glycosylation are highly susceptible to chitosan

The pathway of protein N-glycosylation in yeast begins with the assembly of a lipid-linked oligosaccharide, which is later transferred in the endoplasmic reticulum to suitable protein N-glycosylation sites. The first part of the lipid-linked oligosaccharide assembly in yeast takes place on the cytoplasmic side of the ER membrane. The second part, which involves the addition of the final four mannose residues and three glucose residues, occurs in the ER lumen (Burda and Aebi, 1999). None of the heterozygote deletion strains of essential genes showed increased sensitivity to chitosan ($\log_2$ R ~ 0), consistent with our previously noted observation that most heterozygous deletants were not strongly affected by chitosan. We found that 3 out of 6 homozygous deletion mutants of genes involved in the second part of lipid-linked oligosaccharide biosynthesis (alg9Δ, alg6Δ, alg8Δ) are hypersensitive to chitosan (Table 4). All three mutants exhibit hypoglycosylation of secretory proteins. We found that the alg5Δ deletion mutant, which lacks the gene coding for Dol-P-Glc synthase, is also highly sensitive to chitosan. Dol-P-Glc is the sugar donor of the three glucose residues in the N-chain precursor.

The sensitizing effect of incomplete N-glycosylation was also observed when yeast cells were treated with a combination of chitosan and tunicamycin, which blocks N-glycosylation (Table 1).

Table 4. Chitosan sensitivity of deletion strains of genes that synthesize a truncated lipid-linked oligosaccharide for protein *N*-glycosylation.

R= (signal in the absence of chitosan)/ (signal in the presence of chitosan). Significant values are in bold. The values are organized according to $\log_2$ R at 9h.

ORF	Gene name	$\log_2$ R	
		5h	9h
YOR002W	*ALG6*	**1.17**	**1.93**
YPL227C	*ALG5*	**0.91**	**1.66**
YNL219C	*ALG9*	**1.55**	**1.50**
YOR067C	*ALG8*	**0.92**	**1.44**
YGR227W	*ALG10*	0.40	0.89
YNR030W	*ALG12*	0.11	0.21
YBL082C	*ALG3*	0.25	0.15

Hypoglycosylation probably leads to a lower efficiency of folding of secretory proteins in the endoplasmic reticulum, and thus to lower levels of the mature proteins (Riederer and Hinnen, 1991). As many known cell-wall polysaccharide synthases and cell wall construction enzymes are N-glycosylated, tunicamycin-induced hypoglycosylation may result in a weakened cell wall and, indirectly, in increased membrane stress - potentially rendering the cells more prone to the membrane-perturbing effect of chitosan.

HOG-pathway mutants are highly susceptible to chitosan

The HOG pathway is crucial for regulation of the intracellular osmotic balance of the yeast cell in response to osmotic changes in its surrounding environment and also for cell wall maintenance (Alonso-Monge *et al.*, 2001; Garcia-Rodriguez *et al.*, 2000; Kapteyn *et al.*, 2001). The HOG pathway comprises two upstream signaling branches, mediated by the osmosensing, transmembrane proteins Sln1p and Sho1p, and a downstream MAP kinase cascade. In the Sln1p branch, the signal is transferred from Ssk1p to two redundant MAPKKKs, Ssk2p and Ssk22p, which activate the MAPKK Pbs2p, which in turn activates Hog1p. We found that the homozygous deletion mutants of genes in this branch (*ssk1*Δ) and in the MAP kinase cascade (*ssk2*Δ, *pbs2*Δ, and *hog1*Δ, but not the redundant gene *ssk22*Δ), are extremely sensitive to chitosan (Table 5). Consistent with these observations, the gene PBS2 was initially identified by the sensitivity of the deletion mutant to polymyxin B, a decapeptide that (like chitosan) is a cationic molecule and causes plasma membrane disintegration and leakage of cellular components (Storm *et al.*, 1977). The hypersensitivity of mutants of the HOG pathway to chitosan indicates that the proper functioning of this pathway is required for counteracting chitosan stress.

Table 5. Chitosan sensitivity of strains homozygous for the deletion of a non-essential gene from the HOG pathway.

R= (signal in the absence of chitosan)/(signal in the presence of chitosan). Significant values are in bold. The values are organized according to $\log_2$ R at 9h.

ORF	Gene name	$\log_2$ R	
		5h	9h
YJL128C	PBS2	**1.85**	**4.46**
YNR031C	SSK2	**1.72**	**4.13**
YLR113W	HOG1	**1.55**	**3.99**
YLR006C	SSK1	**1.15**	**2.33**
YDL006W	PTC1	0.33	**1.99**
YCR073C	SSK22	0.09	0.09

This was experimentally validated by incubating yeast cells before and during chitosan treatment in the presence of 1 M sorbitol, which partially protected the cells against the deleterious effect of chitosan on growth (Table 6). Conversely, hypotonic growth conditions made the cells more vulnerable to chitosan (Table 1).

Table 6. Pretreatment with 1 M sorbitol protects yeast against chitosan-induced stress.
Yeast cells were exposed to chitosan after pre-treatment with 1 M sorbitol for the indicated times. Relative growth rates (RGR) were expressed as a percentage of the RGR in the absence of chitosan. The results are the means of three experiments. The standard errors were ≤ 1%. The RGR of the control culture grown without sorbitol was ~0.52±0.02 h^{-1}.

Treatment		RGR (%) Chitosan +25 μg/ml	RGR (%) Chitosan +50 μg/ml
No sorbitol added		77	66
Sorbitol added for [a)]	1h	93	80
	2h	92	82
	3h	89	80

[a] Preincubation time

Identification of chitosan-hypersensitive, SL-based groups of deletants.

In synthetic genetic analysis, a strain bearing a mutation in a query gene is crossed with a set of ~4700 deletion mutants and the resulting double mutants are screened for synthetic lethal (SL) or slow-growth ('sick', SS) phenotypes (Tong *et al.*, 2001). Thus, a synthetic lethality group is composed of the strain deleted for the query gene and all mutants that have an SL or SS interaction with this deletion mutant. Twenty-five SL-based deletion mutant groups were hypersensitive to chitosan (Table 7), comprising 61 chitosan-hypersensitive mutants. The membership of these SL-groups only partially overlaps with that of the GO-based mutant groups. For example, two SL-groups are involved in protein N-glycosylation (the HOC1- and the CWH41-group), in agreement with the results obtained with the GO-group analysis (Table 3). In addition, both GO- and SL-groups identify inter-compartmental transport in the secretory pathway as important for counteracting chitosan-induced stress. On the other hand, both approaches point to several unique cellular functions that are needed to counteract chitosan-induced stress, indicating that the two approaches complement each other.

Table 6. Identification of chitosan-hypersensitive SL-deletion mutant groups.
Significant values ($t \geq 4$) are in bold. [a)] our own unpublished data

Query Gene of the SL-group	ORFs	t-value	
		5h	9h
Cell wall integrity			
BNI4	16	1.53	**4.12**
CHS3	41	**5.51**	**4.74**
SKT5/CHS4	34	**7.31**	**5.61**
SMI1	44	3.62	**4.5**
Glycoprotein biosynthesis			
CWH41	9	3.27	**7.46**
HOC1	32	**4.18**	**4.16**
Plasma membrane integrity			
ERG11	23	3.83	**6.19**
CNB1[a]	26	2.57	**4.09**
Intercompartmental transport in the secretory pathway			
CHS5	59	**5.37**	**8.45**
CHS6	19	**8.37**	**7.74**
GYP1	24	3.1	**4.41**
RIC1	118	**4.3**	**6.66**
YPT6	117	3.96	**6.56**
Cytoskeleton-related			
ARC40	33	**5.3**	**6**
ARP2	40	**4.18**	**5.64**
GIM3	73	3.42	**5.07**
GIM5	102	**5.27**	**6.46**
PAC10/GIM2	107	3.72	**4.76**
RVS161	45	2.88	**4.64**
RVS167	37	3.43	**5.42**
YKE2/GIM1	106	3.72	**4.99**
Nuclear proteins			
ARP6	37	3.36	**5.64**
CDC73	94	3.69	**5.57**
CTF4	84	2.52	**4.37**
SET2	31	3.49	**4.9**

The first four chitosan-hypersensitive groups are based on query genes that are involved in the formation of the cell wall, and its localization and regulation (Table 7). CHS3 and CHS4 are directly involved in chitin synthesis, whereas BNI4 is involved in the polymer's localization (DeMarini *et al.*, 1997). The gene SMI1 encodes a signaling protein that is involved in regulating cell wall synthesis (Basmaji *et al.*, 2006). These observations indicate that cell wall integrity is required to withstand chitosan-induced stress. Consistent with this, staurosporine (a potent inhibitor of the protein kinase C complex, which controls the cell wall integrity signaling pathway (Levin, 2005; Watanabe *et al.*, 1994; Yoshida *et al.*, 1992)) showed a dramatic growth-inhibitory effect when combined with chitosan (Table 1).

Plasma membrane integrity also seems to be a major contributing factor to the yeast cell's ability to cope with chitosan-induced stress. Members of both the ERG11-based SL-group and the CNB1-based SL-group are chitosan-hypersensitive. Ergosterol is the main component responsible for the rigidity of the plasma membrane, and ERG11 encodes an essential enzyme in ergosterol biosynthesis, the cytochrome P-450-dependent C14 lanosterol demethylase. The hypersensitivity of the ERG11-based SL-group to chitosan was experimentally confirmed by incubating yeast cells in the presence of a combination of chitosan and miconazole (a specific inhibitor of Erg11p), resulting in a synergistic effect on growth (Table 1). CNB1 encodes the regulatory subunit of calcineurin, which is involved in coping with plasma membrane stress (Cruz *et al.*, 2002). The identification of the CNB1-based SL-group is in agreement with our previous study, where cells exposed to the calcineurin inhibitor FK506 showed increased chitosan sensitivity (Zakrzewska *et al.*, 2005). Additional experimental validation for a key role of plasma membrane integrity in coping with chitosan-induced stress was obtained by subjecting the cells to hypotonic stress and to chlorpromazine, a cationic amphipathic drug that inserts itself into the plasma membrane lipid bilayer, thus leading to plasma membrane stretch (Kamada *et al.*, 1995). Table 1 shows that both treatments acted synergistically in combination with chitosan. Consistent with this, cells grown at 37°C, a temperature that is believed to increase membrane fluidity, were also more sensitive to chitosan.

Finally, eight groups consist of mutants that show synthetic lethality with mutants of genes encoding various cytoskeleton-related proteins. These include the four members of cytoplasmic prefoldin protein complex (GIM1, GIM2, GIM3, and GIM5), which participates in the folding of tubulin and actin; RVS161 and RVS167, both subunits of the same complex that regulates cell polarity, actin cytoskeleton polarization and endocytosis; and, finally, ARC40 and ARP2, the components of the actin nucleation center, involved in endocytosis, membrane growth, and polarity. In addition, we detected four groups of chitosan-hypersensitive mutants that have synthetic lethal interactions with CFT4, SET2, ARP6 and CDC7, genes participating in DNA binding and histone methylation.

Discussion

Like many natural antimicrobial compounds found in vertebrates, invertebrates, and plants, chitosan is positively charged (at least, at neutral and acidic pHs) and perturbs the plasma membrane of bacteria and fungi (Thevissen *et al.*, 1999; Thevissen *et al.*, 2003b; Thevissen *et al.*, 2004). Cationic peptides and other cationic compounds, including chitosan, have been shown to facilitate the entry of molecules into mammalian cells (Fuchs and Raines, 2006). Chitosan may thus function as an antimicrobial compound itself, and as a model compound to understand the deleterious effects of other cationic antimicrobial compounds on microorganisms. In an earlier study, we analyzed the transcriptional response of yeast cells to chitosan (Zakrzewska *et al.*, 2005). Here, we have complemented the transcriptional analysis of chitosan-treated cells with a global fitness study. Global fitness studies make use of a genomic library of deletion strains, allowing the parallel measurement of their growth response to a chosen stress condition (Parsons *et al.*, 2004; Parsons *et al.*, 2006; Tong *et al.*, 2001). For the analysis and interpretation of our data we have applied and extended the approach developed for global transcriptional analysis, called T-profiler, which is based on the study of the transcriptional behavior of related groups of genes using t-statistics (Boorsma *et al.*, 2005; Zakrzewska *et al.*, 2005). In this study, we have determined the response to chitosan of pre-defined groups of mutant strains deleted for related genes. Two types of deletion mutant groups were successfully tested: (i) groups based on GO categories (Ashburner *et al.*, 2000), and (ii) groups based on global synthetic lethality screens (Tong *et al.*, 2001; Tong *et al.*, 2004). GO-groups have the advantage that they cover all deletions, in contrast to the incomplete coverage by SL-groups. However, the SL-groups have the intrinsic advantage that they are defined by the yeast cell, rather than the investigator.

Both approaches led to testable predictions. For example, the analysis based on GO-groups identified a role for the Sln1 signaling branch and the Hog1 MAP kinase module of the HOG pathway in protecting the cell against chitosan-induced stress. In contrast, the Sho1 signaling branch is not involved. We hypothesize that chitosan may make the plasma membrane leaky, for example, by inducing the formation of mass-transfer channels as has been observed in artificial lipid bilayers (Yang *et al.*, 2002). This would result in uptake of (acidic) medium and, consequently, cytosolic acidification. The HOG pathway genes SSK1, SSK2, PBS2, and HOG1, which function in the Sln1 branch and the Hog MAP kinase module, respectively, might then be expected to offer protection not only against chitosan (this paper), but also against cytosolic acidification resulting from other stresses. Evidence for this comes from the work of Mollapour and Piper (Mollapour and Piper, 2006), who found that mutants deleted for either SSK1, PBS2, or HOG1 are highly sensitive to acetic acid/ acetate at pH 4.5. At this pH, which is below the pKA of acetic acid, undissociated, and thus uncharged, acetic acid molecules form the majority. The uncharged acetic acid molecules can easily pass the plasma membrane. They will immediately dissociate on arrival in the

cytosol, resulting in acid stress. Mollapour and Piper (Mollapour and Piper, 2006) also found that the *sho1*Δ strain does not show increased sensitivity to acetic acid/acetate, demonstrating that the Sho1 signalling branch is not involved in coping with acid stress. Similar results have been described by Lawrence et al. (Lawrence *et al.*, 2004), who found that *ssk1*Δ, *pbs*2Δ, and *hog*1Δ cells, but not *sho1*Δ cells, were more sensitive to citric acid/citrate at pH 3.5, and by Kapteyn et al. (Kapteyn *et al.*, 2001), who showed that Hog1p is required for a cell wall strengthening response induced by culturing cells at pH 3.5. Using global transcript analysis, we have previously shown that in the presence of chitosan yeast cells strongly and continuously activate the cell wall integrity pathway (Zakrzewska *et al.*, 2005). This raises the question how cells challenged with chitosan can have the cell wall integrity pathway turned on, whereas simultaneously the HOG pathway is functional in protecting the cells against chitosan. Interestingly, Mollapour and Piper (Mollapour and Piper, 2006) have shown that in yeast cells challenged with acetic acid/acetate at pH 4.5 the doubly phosphorylated forms of Slt2p and Hog1p can be simultaneously present in the cell, indicating that at least under some stress conditions simultaneous activation of both MAP kinases is possible. This further suggests that the HOG pathway might have functions other than organizing a response to hypertonic stress, as also indicated by the observations that the HOG pathway is induced by cold stress and dimethyl sulfoxide (Hayashi and Maeda, 2006; Panadero *et al.*, 2006).

Analysis of both types of deletant groups identified defective protein N-glycosylation as an important contributing factor to chitosan hypersensitivity, and this was supported experimentally by the observation that tunicamycin sensitized yeast cells to chitosan (Table 1). As defective protein N-glycosylation affects protein folding in the endoplasmic reticulum, it seems likely that many secretory proteins involved in cell wall construction will be produced in insufficient amounts, affecting cell wall integrity and indirectly resulting in increased membrane stress.

Several SL-groups are related to actin cytoskeleton organization. Previous studies have shown that actin cytoskeleton depolarization occurs upon heat and cell wall stress (Delley and Hall, 1999), as well as in hypo- and hyper-osmotic conditions (Chowdhury *et al.*, 1992; Gualtieri *et al.*, 2004). Interestingly, actin cytoskeleton depolarization was also observed upon treatment with two plasma membrane perturbants, SDS and chlorpromazine (Delley and Hall, 1999). Actin cytoskeleton is also required for stress-dependent cellular redistribution of the cell wall biosynthetic protein, Fks1p (Delley and Hall, 1999). The transient depolarization of actin cytoskeleton may therefore act as a homeostatic mechanism to repair cell wall and membrane damage, which can explain why mutants defective in actin cytoskeleton organization are hypersensitive to membrane stress caused by chitosan. Interestingly, actin patches co-localize with sites of endocytosis, associate with endosomes, and their movement is mediated by polarized actin cables (Huckaba *et al.*, 2004). Endocytosis in *S. cerevisiae* is essential for plasma membrane-associated functions,

because it is required for recycling of plasma membrane components, uptake of nutrients, and regulation of cell-surface signaling receptors (Engqvist-Goldstein and Drubin, 2003; Walther *et al.*, 2006). The observation that mutants that are defective in endocytosis are hypersensitive to chitosan-induced plasma membrane perturbation supports the idea that endocytosis is crucial for maintenance of plasma membrane integrity and functioning.

Recent studies in S. cerevisiae have demonstrated that on the level of individual genes there is often no correlation between the transcriptional activation of a gene upon stress and the sensitivity of the corresponding deletion strain to the same stress (Birrell *et al.*, 2002; Giaever *et al.*, 2002). Similarly, we have found that there is no clear relationship between the transcriptional response of co-regulated genes in case of stress and the stress sensitivity of the corresponding groups of deletion strains. The transcriptional response to stress is unavoidably confounded by a slow-growth response (Castrillo *et al.*, 2006). We have separated these two responses by focusing our analyses at the level of cellular functions and pathways, thus gaining results in which transcriptional and fitness profiling show a larger degree of similarity.

Zakrzewska *et al*. (2005) have shown that the main transcriptional responses of yeast cells during the first 180 min of incubation in the presence of chitosan are mediated by the transcription factors Msn2p/Msn4p, which mediate a general stress response (Gasch and Werner-Washburne, 2002); Crz1p, which probably mediates the transcriptional response to plasma membrane stress (Santos and de Larrinoa, 2005); Rlm1p, which activates a group of mostly cell-wall-related genes in case of cell-wall stress (Jung and Levin, 1999); and Cin5p, which mediates salt tolerance (Mendizabal *et al.*, 1998).

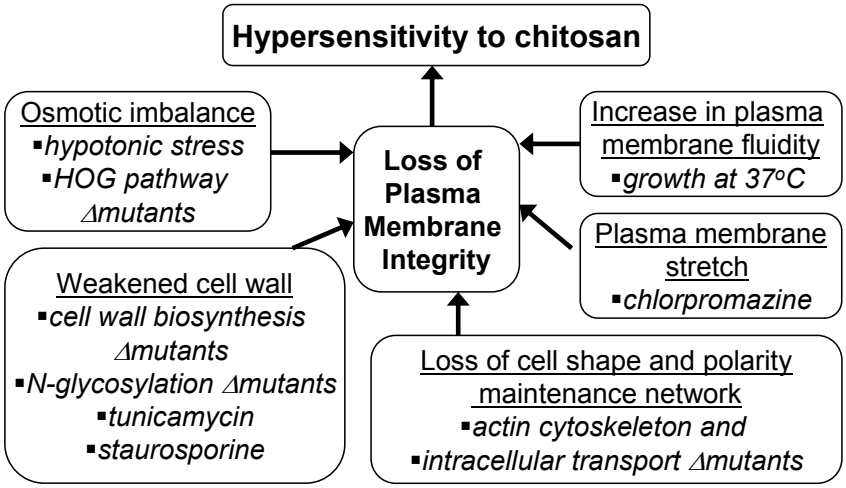

Figure 3. Factors contributing to chitosan hypersensitivity in *S. cerevisiae*.
Δmutants refers to deletion mutants.

Although the global fitness experiment described in this paper covers a period of 9 h and the data are thus not directly comparable to the transcriptional profiling data obtained during a 180-min chitosan treatment, our current results clearly point to a major role for cell wall and plasma membrane integrity in counteracting and recovering from chitosan-induced stress. They also show the benefit of analyzing the data on the level of functional categories of genes using T-profiler.

In summary, our genome-wide analysis of fitness of all yeast deletion mutants in response to chitosan treatment indicates that the loss of genes that are involved (directly or indirectly) in maintaining plasma membrane integrity is the primary cause of chitosan hypersensitivity (summarized in Figure 3). In addition, we have shown that T-profiler analysis of yeast fitness data, based on GO- and SL-based gene groups, is highly effective in identifying cellular processes and pathways that are crucial for defending the cell against chitosan-induced stress. This approach is equally effective for the analysis of other fitness profiles (our own unpublished data) and may eventually provide a useful platform for comparison and integration of other large-scale genomic studies. Finally, it will also allow the systematic identification of combinations of drugs to prevent or minimize fungal infections in food and hosts.

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Chapter 5

Global responses of *Saccharomyces cerevisiae* to changing environment; a meta-analysis of transcription and fitness profiles.

Manuscript in preparation

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Abstract

A major question in biology is how an organism responds and adapts to changes in its environment. To tackle that question we can utilize large-scale genomic tools. Taking advantage of the existing yeast fitness datasets we created and analyzed 159 fitness profiles and integrated them with ~ 950 yeast transcription profiles. We show that correlation of group fitness and transcriptional regulation is very weak, with the exception of mutants in ribosomal protein genes. Furthermore, we find that the functional groups required for growth in a certain condition do not overlap with those that are transcriptionally upregulated in response to the same condition. We reveal general features of yeast fitness in response to various stresses; in particular, we find the overall resistance of deletion mutants in ribosomal protein encoding genes. Importantly, we also identify groups of mutants involved in vesicle-mediated transport, actin cytoskeleton organization and cellular communication that exhibit broad sensitivity to many stress conditions. Therefore, these functions seem to play a crucial role in yeast adaptation to stress. In conclusion, we present a comprehensive study of global fitness data and its integration with expression data, which provide new insights into multilevel responses to changing environmental conditions. We propose that both fitness and expression studies provide complementary information about the complexity of cellular responses and as such should be analyzed in an integrative way.

Key words: global fitness, fitness t-profiles, expression t-profiles, t-statistics, general stress response

Introduction

Understanding the complexity of cellular responses to changes in the external environment has been and still is one of the major challenges in biology. Genomic tools that help to face the above challenge, such as global expression or fitness measurements, were first developed for the model organism *S. cerevisiae*. Global transcription experiments measure in a single assay the changes in expression levels of all genes in response to any environmental condition and as such provide snapshots of the changes occurring in genome-wide expression programs in response to various stresses. For example, the extensive studies of stress-induced global expression levels in *S. cerevisiae* led to discovery of the environmental stress response program (ESR). This program is activated in response to majority of the stress conditions, and comprises upregulated genes mediated by the Msn2, and Msn4 transcription factors and downregulated genes involved in ribosome biogenesis (Causton *et al.*, 2001; Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). Finally, expression studies coupled with statistical methods allow for linking global transcriptional mechanisms to physiology. One such example is a recent study, which reported a large-scale modulation of transcriptional networks of mitochondrial and cytoplasmic ribosomal proteins that is connected to the capacity for anaerobic growth of *S. cerevisiae* (Ihmels *et al.*, 2005). Another study reveals the interplay of metabolic and transcriptional regulation in response to sudden relief from glucose limitation (Kresnowati *et al.*, 2006). Finally, a comprehensive study has been just recently published that explores in detail the multi-level control of growth rate (Castrillo *et al.*, 2007).

Recently, a genomic library of genetically tagged gene deletion mutants was constructed, covering both the essential and non-essential genes of the *S. cerevisiae* genome. This deletant collection can be used to determine fitness of all mutants in response to a given condition in a single experiment (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). Such a genome-wide approach has been extensively utilized to detect pathways, functions and novel genes necessary for optimal growth in a wide range of stress conditions (Birrell *et al.*, 2002; Brown *et al.*, 2006; Giaever *et al.*, 2002; Wu *et al.*, 2004). Another collection of fitness data, obtained by fitness analysis of a haploid yeast deletant strain set in response to 82 bioactive compounds greatly facilitated studies on cellular effects of novel drugs (Parsons *et al.*, 2006). Finally, global analysis of heterozygous mutants, where lowering the gene dosage from two to one in a heterozygote supposedly results in hypersensitivity to a compound specifically targeting that gene, was used to confirm known targets of drugs and to identify target genes of novel compounds (Giaever *et al.*, 1999; Lum *et al.*, 2004).

Several studies of global fitness and transcript levels reported that individual genes required for optimal growth are not activated transcriptionally under the same condition, (Birrell *et al.*, 2002; Giaever *et al.*, 2002; Smith *et al.*, 2006). Discrepancies were also observed between mRNA levels and corresponding protein levels upon growth of yeast in the same conditions (Griffin *et al.*, 2002; Kolkman *et al.*, 2006). These studies indicate that there

is no clear understanding as to which cellular functions are required to cope with imposed stresses or how these functions are regulated, and warrant the need for integrative analysis of genome-wide data in yeast systems biology.

To address this issue we set out to integrate data from two types of high-throughput experiments, fitness and expression, in order to better understand the global changes occurring on different levels of biological control. In this study, we use T-profiler, a method based on t-statistics, to integrate and analyze ~950 expression and 159 fitness experiments. We find that mutant-groups with a significantly altered fitness are in majority not transcriptionally co-regulated; with the exception of the ribosomal protein coding genes, which form a fixed functional group that is also tightly co-regulated transcriptionally. This indicates that transcriptional regulators mediate expression of genes involved in different functions. Next, we identify similar numbers of significantly altered functional categories in both transcription and fitness data, yet they represent very distinct functional classes. In general, we observe that functions required for optimal growth are not subject to transcriptional regulation indicating a different level of biological control. Clustering of t-values of GO-based groups for all fitness profiles reveals the resistance of mutants of RP genes and mutants involved in rRNA processing to be a general feature of genomic fitness data. In addition, there is a general sensitivity of mutants involved in the intracellular transport machinery and the actin cytoskeleton. These functions are not regulated at the transcriptional level, but they seem to constitute the general adaptive mechanisms of *S. cerevisiae*. In conclusion, we are the first to report global properties in yeast fitness data and provide a quantitative comparison of global fitness and transcription differences on the level of cellular functions and biological processes.

Materials and Methods

Fitness Library of genome-wide fitness profiles.

Our library of fitness profiles contains data of 159 fitness experiments carried out with all non-essential deletion mutants of *S. cerevisiae* (Table 1). The yeast deletion collection can be tested for susceptibility to any type of stress in at least three various ways. The growth of individual mutants can be detected, the colony size of each mutant can be determined, and finally by amplification of unique tags for each mutant, the abundance of all mutants can be scored simultaneously on a microarray (Ericson *et al.*, 2006; Fernandez-Ricaud *et al.*, 2005; Warringer *et al.*, 2003). This fitness library contains data from all three different experimental platforms and includes experiments where cells are subjected to various stress conditions (see supplemental data).

T-profiler.

We use T-profiler to calculate the t-values corresponding to the average fitness levels of predefined groups of deletion mutants (mutant groups). Previously, the same method was used to quantify the average expression levels of predefined gene groups in genomic expression experiments (Boorsma *et al.*, 2005; Zakrzewska *et al.*, 2005). A mutant group represents a group of deletion mutants defined by the genes annotated to that group on the basis of various parameters (see below for a description of each category of mutant groups). Both gene and mutant groups contain the same members, yet to avoid confusion we generally use the term “gene group” for the description of expression data, and the term “mutant group” for the description of fitness data.

For a given mutant group M, the t-value is given by the following formula:

$$t_M = \frac{\mu_M - \mu_{M'}}{s\sqrt{\frac{1}{N_M} + \frac{1}{N_{M'}}}}$$

where

.

$$s = \sqrt{\frac{(N_M - 1) \times s_M^2 + (N_{M'} - 1) \times s_{M'}^2}{N_M + N_{M'} - 2}}$$

Here μ_M is the mean fitness $\log_2$ ratio of the N_M mutants in a mutant group M; $\mu_{M'}$ is the mean fitness $\log_2$ ratio of the remaining $N_{M'}$ mutants; and s is the pooled standard deviation, as obtained from the estimated variances for groups M and M’. To reduce the influence of outliers, which may result in false positives or false negatives, we discard the

highest and lowest fitness value in each mutant group. This method is similar to the jack-knife procedure (Heyer *et al.*, 1999). The associated two-tailed p-value can be calculated from t using the t-distribution with N-2 degrees of freedom. We accounted for multiple testing by computing an E-value equal to the p-value multiplied by the number of mutant groups, which is known as the Bonferroni correction for multiple comparisons. All groups with an E-value of 0.05 or smaller are considered to be significantly altered in fitness. The fitness datasets in our library have been analyzed using T-profiler and the resulting fitness t-profiles have been uploaded to a database, which can be found at http://www.science.uva.nl/~boorsma/t-base-fitness/.

Motif-based mutant groups.

Motif-based groups are defined as groups of mutants where each mutant is deleted for a gene that contains a particular consensus motif within 600 base pairs upstream of the ORF (van Helden *et al.*, 2000), allowing no overlap between neighboring ORFs. The consensus motifs used in T-profiler (Boorsma *et al.*, 2005) are derived from three different sources. First, motifs were extracted from the SCPD database (http://cgsigma.cshl.org/jian /). Additionally, motifs were found by comparing the genome sequence of highly related yeast species (Gasch *et al.*, 2000; Kellis *et al.*, 2003). Finally, motifs were discovered in various microarray experiments by the REDUCE algorithm (Bussemaker *et al.*, 2001; Roven and Bussemaker, 2003). Most of these motifs are similar or identical to motifs described in the literature. In total, 153 motif groups have been included in T-profiler calculations.

TFO (Transcription factor occupancy) - based mutant groups

We used the transcription factor occupancy data from ChIP-chip studies as input in T-profiler (Harbison *et al.*, 2004; Lee *et al.*, 2002). These datasets contain results of 203 ChIP-chip experiments performed in rich medium (YPD). For 84 of these transcription factors, their binding to promoter regions was also measured in at least 1 of 12 other environmental conditions. A mutant was considered to be part of a TFO group if the P-value reported for its gene by the authors was smaller than 0.001. In addition, TFO groups were required to have at least 7 gene members. This resulted in 252 TFO groups that were used for T-profiler analysis.

Gene Ontology (GO)-based mutant groups

GO-based mutant groups contain the genes associated with a specific GO category as well as all of its child categories. Only Gene Ontology groups with at least 7 members were used for calculation. This approach resulted in a reduction of the original SGD 3836 GO-based groups to 1346 GO-based groups, which were used for T-profiler analysis. Significantly scoring GO-based mutant groups directly indicate which functions or cellular processes are required for optimal growth under a specified condition.

MIPS –based mutant groups

MIPS-based mutant groups contain genes annotated by the MIPS organization. These groups can be divided into three major categories based on function, protein complex and cellular localization. We only used the MIPS mutant groups with at least 7 members for our analysis. Significantly scoring MIPS-based mutant groups directly indicate which functions are required to maintain optimal fitness in a specific stress condition. In addition, the mutant groups from the three types of categories listed below: morphology, phenotype, and synthetic lethality, were also analyzed together with MIPS derived groups.

Morphology-based mutant groups

In the morphology database 4400 non-essential mutants were scored for their cellular shape and subsequently annotated to 11 categories (Giaever *et al.*, 2002). In our analysis we used 6 of the morphology groups, based on the number of their members and excluding the morphology group named WT (wild type).

Phenotype-based mutant groups

We tested 233 phenotype categories, derived from MIPS database, which describe various features of mutant strains ranging from mutants' drug susceptibilities, defects in cell shape or organelle morphology, cell cycle, mating, sporulation, to auxotrophies, and defects in utilization of carbon and nitrogen. The weakness of this database is such that it is not complete, due to the fact that it is only a collection of available facts from literature. However, since it is entirely based on analysis of mutants, it is very suitable for characterization of fitness data.

Synthetic Lethality-based mutant groups

The Synthetic Lethality Interactions database comprises 462 groups of mutants, representing a full spectrum of biological processes and cellular functions. The data contained there comes from genetic screens where a deletion mutant of a query gene is crossed into an array of viable gene deletions, which generates an array of double mutants that can be scored for synthetic lethal or sick (reduced fitness) phenotypes. The result of such screen provides information on genes that are involved in the same function or genes, which are able to buffer each other's functions (Tong *et al.*, 2001; Tong *et al.*, 2004). This database is not yet complete, as only around 10% of genes were tested as a query in the synthetic genetic interaction network. However, the enormous advantage of the Synthetic Lethality categories is that they are defined by the yeast cell itself, and not annotated by a researcher.

Cluster analysis of fitness t-profiles

Fitness data clustering was performed in J-Express (Dysvik and Jonassen, 2001). We used the hierarchical clustering method (Eisen *et al.*, 1998). Agglomerative hierarchical

clustering is an unsupervised procedure where objects are subjected to pair-wise similarity measurement, in order to obtain a hierarchy of objects reflecting their relationship to one another. The fitness t-profiles of all experiment were obtained from the database. Any given fitness t-profile of a single experiment contains the t-values calculated in T-profiler for all mutant groups of each type e.g. GO-based mutant groups. The fitness t-profiles were used for clustering analysis, with Pearson correlation as a distance measure, and complete linkage.

Results

Generation and analysis of fitness t-profiles

We collected 159 global fitness datasets of homozygous diploid or haploid deletions of all nonessential genes of *S. cerevisiae* exposed to various environmental conditions, including two datasets of our own (Table 1).

Table 1. List of publicly available experiments used in this study.
The list contains publication from which experimental data was obtained, the type of yeast deletion pool used, the platform that was used to perform the genome-wide experiment and the number of experiments per study.

Literature	Deletion pool used	Type of platform	#
Birell *et al.*, 2001	Homozygous non-essential diploid	Chip	2
Birrell *et al.*, 2002	Homozygous non-essential diploid	Chip	4
Giaever *et al.*, 2002	Homozygous non-essential and heterozygous essential diploid	Chip	6
Warringer *et al.*, 2003	Haploid non-essential	Individual growth curves	4
Wu *et al.*, 2004	Homozygous non-essential diploid	Chip	8
Dudley *et al.*, 2005	Homozygous non-essential diploid	Colony	21
Brown et al., 2006	Homozygous non-essential diploid	Chip	28
Parsons *et al.*, 2006	Haploid-non-essential	Chip	84
Zakrzewska *et al.*, 2007	Homozygous non-essential and heterozygous essential diploid	Chip	2

Each fitness experiment provides a quantitative description of a whole spectrum of susceptibilities, ranging from highly sensitive to highly resistant, of all individual mutants (Figure 1A). Each individual mutant is assigned a fitness value, which is the $\log_2$ ratio of abundance of a single mutant in a control versus treatment. We analyzed the fitness of mutant groups compared to the whole population using T-profiler (Boorsma *et al.*, 2005), which converts fitness values from all individual mutants into a set of t-values for all mutant groups (Figure 1B and 1C). A set of t-values for all mutant groups in one fitness experiment is termed a fitness t-profile. Similarly, an expression t-profile is the collection of all t-values of predefined gene-groups for each individual transcription experiment, and as such can be directly compared to a fitness t-profile, when using the same predefined groups. T-profiler was previously used to analyze the transcriptional behavior of predefined gene groups derived from TFO, motifs, GO (Gene Ontology), and MIPS functional categories (which contain also groups of genes defined by protein localization, and complex formation) in expression

microarray experiments (Boorsma *et al.*, 2005; Zakrzewska *et al.*, 2005). Here, we extend the analysis by 3 types of categories derived from the characterization of mutant morphology, phenotypes, and synthetic lethal interactions, which we combine with MIPS-based groups (Materials and Methods), to broaden the analysis of mutant fitness.

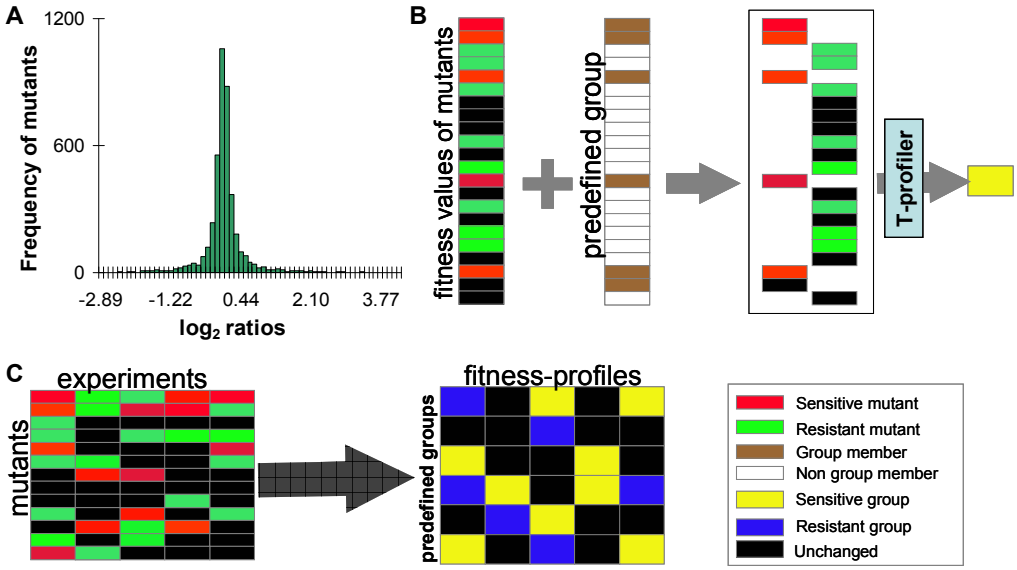

Figure1. Transformation of fitness data into fitness t-profiles.

(A) Frequency distribution of all $\log_2$ fitness ratios corresponding to treatment with oxaliplatin. (B) Calculation of an individual t-value. The difference in mean fitness of mutants (red corresponds to sensitive, and green to resistant mutants) belonging to a particular mutant group (brown) to the mean of the remaining mutants is compared in a t-test. For each mutant group a t-value is obtained. (C) Conversion of genomic fitness data into fitness t-profiles. Fitness data from multiple experiments are transformed into a matrix of t-values (yellow- positive, blue-negative t-values). The columns of the matrix contain t-values of individual mutant groups in each separate experiment, whereas the rows contain t-values of an individual mutant group over a set of experiments.

An important advantage of T-profiler over other methods used to analyze large-scale datasets is that by simplifying the data into a number of t-values for all groups and thus performing intrinsic normalization (Figure 1C), it allows for direct comparison of datasets from various types of experiments, such as colony size, arrays for assessing abundance of all deletion mutants by unique DNA tags, or individual growth curves, or finally between fitness and transcription experiments. In addition, this method is relatively insensitive to normalization and scaling procedures applied to the individual datasets. Furthermore, T-profiler is capable of detecting minor changes in average fitness, which means that individual mutants do not have to show significant changes in fitness in order for the group to show a significant

change. For example, treatment with lovastatin, a drug that inhibits ergosterol biosynthesis, leads to significant sensitivity of the "steroid biosynthesis" mutant group with a t-value of 5.30 (and E-value of 0.0002), yet only one of the individual mutants present in this group shows a $\log_2$ ratio over 1, which corresponds to 2–fold increase in sensitivity (Table 2).

Table 2. Example of t-profiler sensitivity; fitness values of individual mutants in Gene Ontology group "steroid biosynthesis" upon treatment with lovastatin.

The mutant group "steroid biosynthesis" scored significantly (t-value= 5.30, E-value=0.0002) upon treatment with lovastatin. The fitness values ($\log_2$ ratios) of all individual mutants members (13 ORFs) in this group are shown.

ORF	Mutant	Function of the corresponding gene	$\log_2$ ratio
YML075C	*hmg1*Δ	hydroxymethylglutaryl-CoA reductase (NADPH) activity	1.73
YDR213W	*upc2*Δ	RNA polymerase II transcription factor activity	0.82
YLR228C	*ecm22*Δ	RNA polymerase II transcription factor activity	0.53
YAR042W	*swh1*Δ	phosphatidylinositol binding	0.5
YHR073W	*osh3*Δ	oxysterol binding	0.48
YMR015C	*erg5*Δ	C-22 sterol desaturase activity	0.45
YPL145C	*kes1*Δ	oxysterol binding	0.42
YKL150W	*mcr1*Δ	cytochrome-b5 reductase activity	0.27
YLR450W	*hmg2*Δ	hydroxymethylglutaryl-CoA reductase (NADPH) activity	0.26
YGL012W	*erg4*Δ	delta24(24-1) sterol reductase activity	0.18
YNL231C	*pdr16*Δ	phosphatidylinositol transporter activity	0.05
YKR003W	*osh6*Δ	oxysterol binding	-0.11
YNL111C	*cyb5*Δ	electron transporter activity	-0.23

This property of T-profiler analysis provides a more realistic insight into cellular responses than any stringent cut-off, as biological processes are likely to be regulated in subtle and gradual ways.

Expression of transcriptionally co-regulated gene groups shows very weak correlation with the fitness of the corresponding mutant groups

A well-documented characteristic of yeast transcriptional responses to changes in environmental conditions is the regulation of expression of a specified group of genes by a common transcription factor (TF). For example, the transcription factor Gcn4p is the main positive regulator of amino acid biosynthetic genes, which is activated during amino acid starvation or nitrogen limitation (Hinnebusch and Fink, 1983; Hinnebusch, 1988). However, it has not been systematically analyzed if the members of a gene group regulated by a common

transcription factor are functionally involved in similar or the same cellular responses. Here, we specify a TF-regulated group in two ways; as those that contain a common regulatory motif in the 600bp of their promoter region (motif-based group), or as those that belong to the same transcription factor occupancy group (TFO-based group).

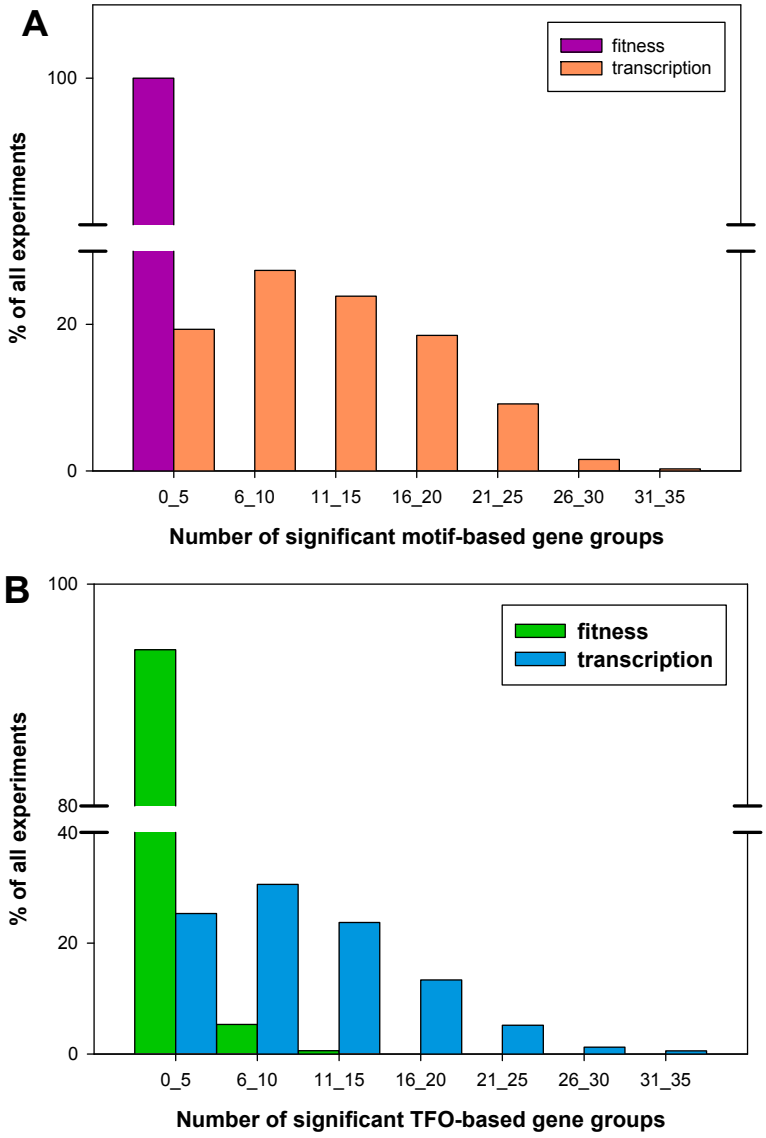

Figure 2. Distribution of significantly altered motif- and TFO- based gene groups in fitness and transcription experiments.

For each fitness and expression experiment the t-values of the motif- and TFO-based gene groups were calculated (see Materials and Methods for details) and the number of significant t-values per experiment

was determined. The percentages of experiments with the indicated numbers of significantly changed gene groups (in indicated bins) are shown; A- motif-based, B-TFO-based.

We were interested to test whether TF-regulated groups share similar fitness characteristics (sensitivity or resistance to a specified condition). The relevance of TF-regulation for fitness was assessed by scoring the number of significantly affected motif- and TFO-based mutant groups over all ~ 950 expression t-profiles (Harbison *et al.*, 2004; Lee *et al.*, 2002), (Boorsma *et al.*, 2005), and by comparing to the same analysis performed on 159 fitness t-profiles. As expected, the transcriptional co-regulation was a prominent feature of all tested expression t-profiles, as in approximately 80% of these we found more than six significantly regulated motif-based gene groups. In contrast, we found that in 93% of the fitness t-profiles there were no significantly affected motif-based mutant groups, and the rest showed only one or two significant t-values for motif-based groups (Figure 2A). Similarly, although 53% of all tested fitness t-profiles showed significant changes in TFO - based mutant groups (Figure 2B, and see below for further analysis), only 8 out of those fitness t-profiles scored more than five TFO-based groups. Conversely, in genomic transcription data 75% of all expression t-profiles had more than 5 significantly regulated TFO-based groups (Figure 2B). The data indicate that with some exceptions (see below) there is generally only very weak correlation between the average expression levels of co-regulated genes and the average fitness levels of the corresponding mutant groups under the same stress conditions. This implies that mutants of genes regulated by a common/ single transcription factor do not share similar or growth defects in a specific condition.

Transcriptional regulation and fitness properties of the ribosomal protein groups are highly correlated

We tested if any specific TFO-based groups were enriched among the 53% (90) fitness profiles that scored at least 1 significantly affected TFO-group. We found that 20 diverse specific transcription factors regulate the identified TFO-based groups (Table 3). Interestingly, only three groups were significant in more than 10% of all 159 fitness experiments. These groups are co-regulated by transcription factors Fhl1p (47% of all fitness experiments), Rap1p (28%), and Sfp1 (16%), (Table 3), where Rap1 and Sfp1 groups were always found together with the Fhl1-group in a fitness t-profile. The transcription factor Fhl1p is found almost exclusively at RP genes. Rap1p is the main transcriptional regulator of RP genes expression, which together with Fhl1p, and Ifh1p regulates expression of RP genes (Zhao *et al.*, 2006). Transcription factor Sfp1 directly regulates expression of RP genes under stress and nutrient-limited conditions (Marion *et al.*, 2004). To sum up, we found that the vast majority of all TFO-based groups that scored significantly in the fitness data are under regulation of transcription factors that mediate RP gene expression (Rap1, Fhl1, and Sfp1) (Table 3), which means that the deletion mutants of transcriptionally co-regulated RP genes share the same fitness properties in various environmental conditions.

Among other TFO-groups that were reasonably enriched in fitness profiles we identified two groups regulated by TFs involved in central carbon metabolism, *i.e.* Hap4 and Hap1, which were affected significantly in 5% and 3% respectively.

Table 3. Frequency of significant TFO-based gene groups in fitness experiments.
The number of all TFO-based gene groups with significant t-values that were found in fitness datasets was calculated using t-statistics. The enrichment of particular TFO-based gene groups over the 90 experiments that showed significant TFO-gene groups is shown. The values in bold represent the groups that were significant in at least 10% of all the fitness experiments tested.

TFO-group [a]	# of fitness experiments [b]
FHL1	**80**
RAP1	**47**
SFP1	**27**
HAP4	9
HAP1	5
GAL4	3
GCN4	3
BAS1	3
RTG3	2
ARG81	2
ABF1	2
ARG80	1
CBF1	1
MET32	1
STB2	1
PUT3	1
MSN2	1
PDR3	1
PHD1	1
DAL81	1

[a] TFO-group refers to a group of genes, whose intergenic regions bind the indicated transcriptional regulator

[b] the number of fitness experiments in which the TFO-group is significantly altered

These findings indicate that the expression of genes under control of transcription factors regulating carbon metabolism and the fitness of their mutants are sometimes well correlated. However, the strongest aspect of transcriptional regulation that was propagated into fitness was in the functional group of ribosomal protein genes.

Functionally related genes are required for optimal growth in stress environments

To respond and adapt to specific changes in growth conditions yeast requires specialized functions and cellular processes. Therefore, mutation of genes participating together in such functions or processes should result in similar phenotypes, or fitness properties. We found that fitness of yeast deletion strains does not correlate with transcriptional co-regulation of their respective genes. To examine whether fitness properties of mutants are linked by other than transcriptional characteristics, we studied the distribution of significantly altered mutant groups derived from Gene Ontology and MIPS, in both fitness and expression datasets (Figure 3).

The majority of fitness profiles (81%) scored between 11 and 90 significantly changed GO-based mutant-groups. Similar observations were made for expression profiles, where 61% of all experiments scored between 11 and 90 significantly changed GO-based groups. Above 80% of all fitness profiles scored between 11 and 90 significantly changed MIPS-based mutant-groups. Similarly, 83% of all expression profiles showed between 11 and 90 significantly changed MIPS-based groups. Our analysis shows that genes required for optimal growth under specific conditions are generally involved in similar functions or biological processes, their gene products localize to the same cellular compartments and form complexes together, or their mutants share morphological characteristics. In brief, we demonstrate that fitness of mutants under stress is determined by the specific cellular functions of their genes. Interestingly, similar results were obtained for expression profiles that also show a high number of significantly regulated GO- and MIPS-based gene groups. These data raise a question whether functions that are regulated transcriptionally are the same as those required for fitness under the same stress condition.

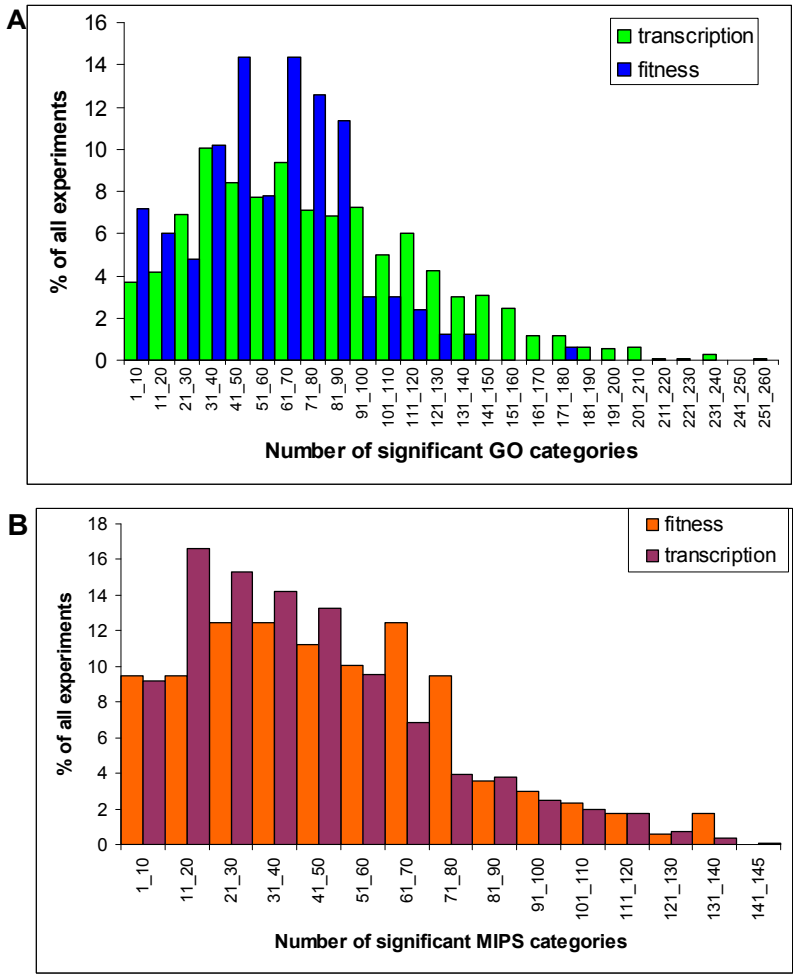

Figure 3. Distributions of significant GO and MIPS groups in fitness and transcription experiments.

For each fitness and transcription experiment we calculated the t-values of all GO and MIPS groups, and the number of significant t-values per experiment was determined. The percentages of experiments with the indicated number of significantly changed groups (in indicated bins) are shown: A-GO-based, B-MIPS-based.

Genes induced in expression or required for fitness in the same environment belong to distinct functional categories

We found that both fitness and expression data show high number of significantly altered functional groups derived from GO and MIPS, thus raising a question about the degree of overlap of these groups in both types of data. There are indications that in *S.*

cerevisiae genes required for fitness/ optimal growth in a new environment seem to be distinct from the genes that are transcriptionally responsive to that environment (Birrell *et al.*, 2002; Giaever *et al.*, 2002; Smith *et al.*, 2006). Yet, it has not been analyzed systematically whether the same cellular functions that determine fitness are transcriptionally regulated under the same stress.

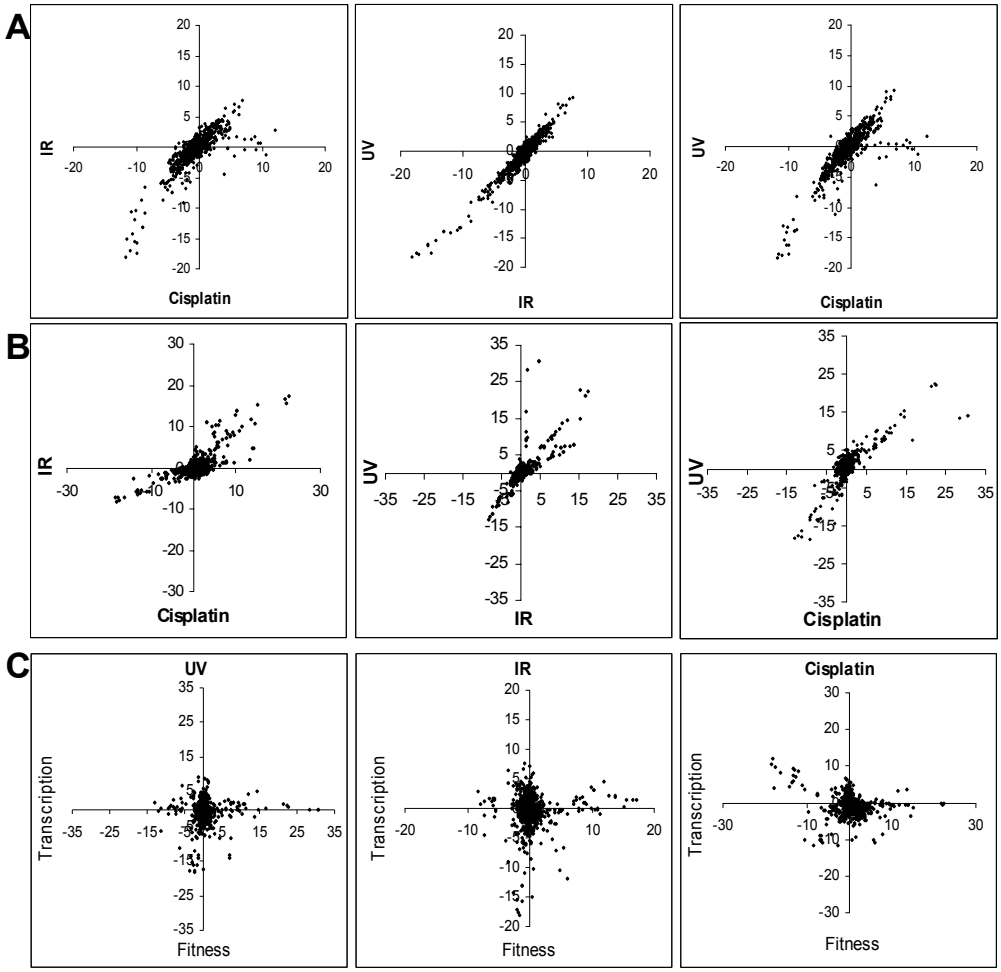

Figure 4. Comparative analysis of t-profiles based on GO groups obtained from fitness and transcription experiments with *S. cerevisiae* cells exposed to DNA-damaging conditions.

T-values for all GO-based groups were calculated for three DNA-damaging conditions, cisplatin, UV, and IR, for both transcription and fitness datasets. The scatter plots of t-values of all GO-based groups are presented;

A- Comparison of expression t-profiles between two conditions

B- Comparison of fitness t-profiles between two conditions

C- Comparison of fitness and transcription t-profiles per individual condition

To examine the degree of similarity of functional categories significantly altered on the level of fitness and transcription to the same stress, we have compared fitness and expression data obtained from treatment of yeast with three conditions causing DNA damage, namely cisplatin, UV, and ionic radiation (IR). Each of the 6 datasets was transformed into a set of t-values for all Gene Ontology groups, and then subjected to pair-wise comparison (Figure 4). Interestingly, all three experiments displayed very high correlation coefficients when their respective expression profiles were compared (r=0.81 for cisplatin and IR, r=0.83 for cisplatin and UV, and r=0.96 for UV and IR, Figure 4A). Similarly, high degree of correlation was observed for cross-stress comparison of fitness profiles (r=0.76 for cisplatin and IR, r=0.83 for cisplatin and UV, and r=0.78 for UV and IR, Figure 4B). Strikingly, there was no correlation of GO groups between the fitness and expression profiles for each individual DNA-damaging stress (r-values = -0.22, 0.04, and 0.06, for cisplatin, IR and UV, respectively, Figure 4C). Similar results were obtained when correlation analysis was performed with expression $\log_2$ ratios of genes and fitness values of mutants (data not shown). Evidently, the DNA-damage caused by these three agents induces almost the same expression response on the level of GO groups, and requires similar GO gene-groups for optimal growth under these stresses. Remarkably, our analysis shows that fitness and expression datasets represent very diverse GO functional groups that can provide very distinct information about the multi-level cellular response to the same type of stimuli.

Hierarchical clustering of fitness profiles reveals general fitness characteristics of *Saccharomyces cerevisiae*

In yeast, a transcriptional regulation program is known that is activated in majority of stress conditions, named the environmental stress response (ESR). The ESR is characterized by simultaneous induction of several hundred of genes regulated by TFs Msn2p, and Msn4p, and repression of a couple hundred of genes containing PAC and/ or rRPE motifs in their promoter region. This transcriptional program was first described by Gasch *et al*.(Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002) and revolutionized our understanding of transcriptional regulation in yeast in response to varying conditions.

The availability of increasing numbers of published fitness profiles led us to question whether they share common features similar to the common expression programs found in a large number of transcription datasets obtained from various stress treatments. To investigate that, we performed global clustering of all fitness profiles of the available fitness experiments based on t-values of groups derived from Gene Ontology.

The results of hierarchical clustering are presented in Figure 5. Yellow represents sensitivity, and blue represents resistance of the Gene Ontology-defined mutant groups in fitness experiments. Our clustering analysis resulted in a number of distinct clusters, out of which only four are discussed here. Cluster 1a represents GO-based mutant groups that show increased resistance to a vast majority of conditions tested in fitness experiments. The

functional groups found in this cluster contain mostly deletions of genes involved in ribosome biogenesis, rRNA metabolism and biosynthesis, and ribosomal protein genes, indicating that deletions of genes playing a key role in cell growth are able to withstand a plethora of stress conditions better than the rest of the mutants' population.

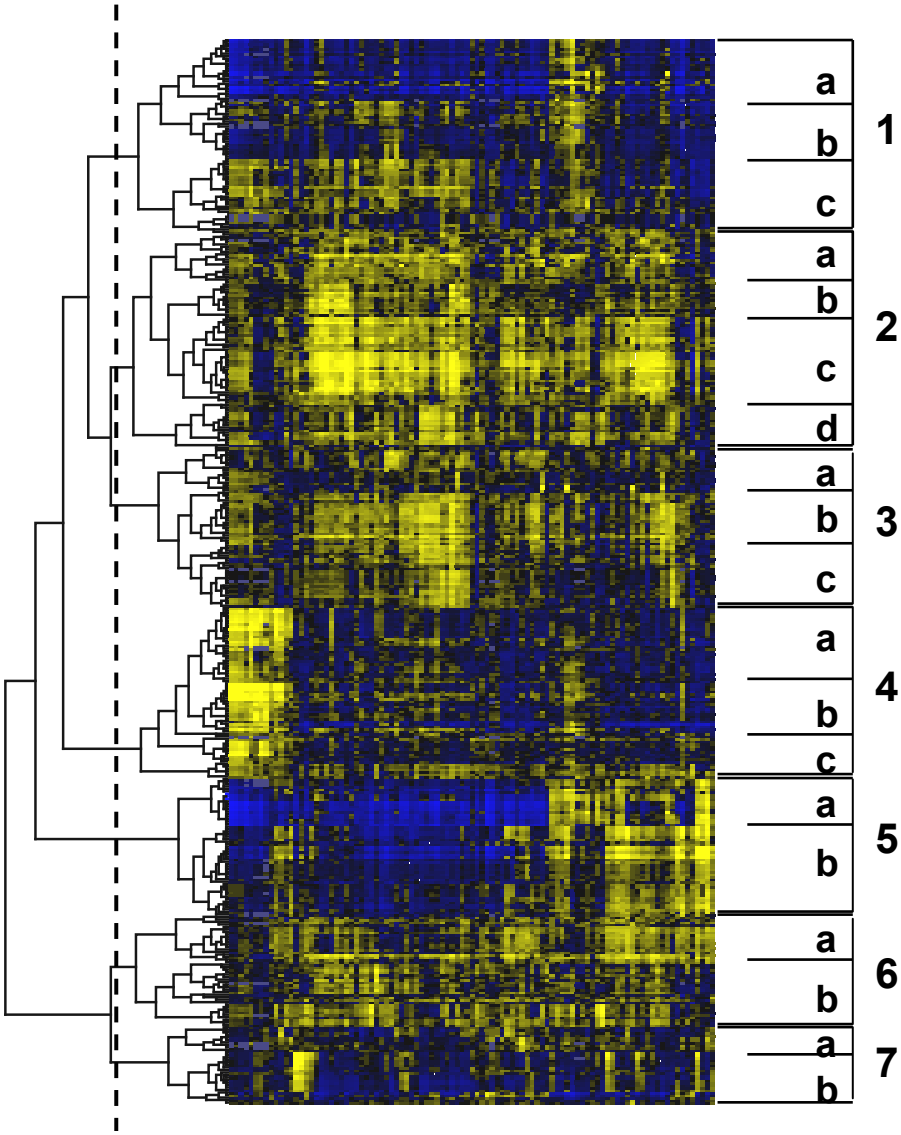

Figure 5. Cluster analysis of GO-based fitness t-profiles.

T-values for the GO-based mutant groups of all available fitness experiments were clustered. Each column represents a single fitness t-profile, and each row represents a single GO-based group. Blue indicates resistant, and yellow indicates sensitive groups. The dashed line indicates the arbitrary cut-off

for determination of individual clusters. Several clusters are discussed in the text (for a complete description of all clusters see supplemental data).

Cluster 2 can be divided into four sub-clusters, out of which we only discuss cluster 2c that contains the groups with the highest sensitivity over the majority of all fitness experiments. Here we identified GO groups representing functions such as vesicle-mediated transport, secretion, Golgi, endosome, and protein localization. The groups found in this cluster are mostly associated with intracellular transport machinery and suggest that the proper functioning of these processes is crucial for growth in diverse stress conditions.

Cluster 3b contains two main types of functional groups, namely those related to intracellular signaling, and to organization of actin cytoskeleton. Interestingly, the mutants in these functions are also found to be hypersensitive to a large number of conditions tested, implicating a more central role for these functions in cellular adaptation to environmental changes. Finally, cluster 4 presents a rather distinct picture where fitness profiles of DNA-damaging agents and conditions show hypersensitivity of the groups found in this cluster, whereas other fitness experiments tend to show resistance or no significance. In this cluster, the functional groups involved in all aspects of DNA metabolism and biosynthesis, such as cell cycle or repair, are found.

Our clustering analysis of GO-based groups over a large number of fitness experiments provides a comprehensive view of functions and processes that are required to maintain cellular fitness in diverse stress conditions, implicating a central role of intracellular transport machinery and actin cytoskeleton in withstanding changes in the environment.

Discussion

The growing number of large-scale fitness data poses a challenge for researchers in data analysis and data integration. In this study, we used T-profiler, a computational method based on t-statistics, to analyze fitness datasets and integrate them with expression data. We first converted all 159 fitness and ~950 expression experiments available through public domain into fitness and expression t-profiles, respectively, which allowed a direct comparison of these two types of genome-wide data.

In contrast to their score in expression data, very few transcriptionally co-regulated groups scored significantly in the fitness profiles. This finding shows that genes whose corresponding deletion mutants share similar fitness characteristics, rarely belong to a transcriptionally co-regulated group, indicating that they are seldom regulated by a single common transcription factor. Conversely, the mutant members of transcriptionally co-regulated gene groups generally do not share similar fitness. These data imply that transcriptional regulation is only rarely reflected in phenotype, and that many post-transcriptional effects disturb the correlation between the transcription and fitness. This is in agreement with several studies that showed very little correlation on the level of individual genes between fitness and expression data of the same stress (Birrell *et al.*, 2002; Giaever *et al.*, 2002; Smith *et al.*, 2006). However, our analysis extends this conclusion from individual genes to the group level.

One significant exception from this general observation is the functional group of ribosomal protein genes, as its behavior in fitness experiments correlates highly with its co-regulated expression by the transcription factors Fhl1p, Rap1p, and Sfp1p. Rap1p is the main transcriptional regulator of RP genes expression, it is constitutively bound to their promoters, and together with Fhl1p, and Ifh1p participates in regulation of RP genes expression (Zhao *et al.*, 2006) Sfp1 is bound to RP genes promoters in the nucleus during exponential growth. It is redistributed to cytoplasm during stress, which coincides with down-regulation of RP genes (Marion *et al.*, 2004). Our results could be explained such that due to RP genes forming a specific functional and expression cluster the behavior of genes in expression can be highly correlated with the behavior of deletion mutants, thus simply, the tight functional category of RP genes is also transcriptionally co-regulated. The 138 genes encoding the ribosomal proteins (RP) of *Saccharomyces cerevisiae* are possibly the most coordinately regulated cluster of functionally related genes in this organism, and in exponentially growing cells RP genes account for nearly 40% of the total number of polymerase II-mediated transcription initiation events. Their transcriptional regulation plays a central role in the control of cell growth through control of protein biosynthesis (Schawalder *et al.*, 2004; Wade *et al.*, 2004; Warner, 1999). Interestingly, our data suggest that, in contrast to ribosomal protein genes, other related genes (*i.e*. sharing fitness properties) are generally under control of more than one transcription factor to allow for robustness of the regulatory system/network, conversely

implying that transcriptionally co-regulated gene groups contain members involved in diverse cellular functions and processes.

Large numbers of significant t-values for GO- and MIPS-based functional groups were found in all fitness data. The discovery that deletion mutants of genes involved in the same functions or biological processes in the cell generally share similar fitness characteristics, supports previous studies (Brown *et al.*, 2006; Smith *et al.*, 2006), and extends them to encompass a much wider range of conditions. We propose that in yeast fitness of a deletion mutant in a specific stress depends on the contribution of the cellular function its respective gene product participates in, to the response to that stress. Comparative analysis of fitness and expression profiles revealed a rather similar distribution of significant GO- and MIPS-based groups in both types of large-scale data. Interestingly, our integrative study of complete GO-based fitness and expression profiles of three DNA-damaging agents revealed that these two types of data represent entirely distinct functional classes. Similar findings were made in a study of the response of yeast to growth in presence of fatty acids (Smith *et al.*, 2006). Together, these results indicate that the response to a certain environmental change leads to transcriptional activation of other functional classes than those that are required for optimal fitness.

Previously, a common transcription program activated in the majority of stress conditions, namely the environmental stress response (ESR), was identified in yeast. Its two most prominent characteristics are: (i) the induction of genes regulated by transcription factors Msn2p, and Msn4p, and (ii) repression of genes containing PAC and/ or rRPE motif(s) in their promoter region (Gasch *et al.*, 2000; Gasch and Werner-Washburne, 2002). In our global fitness analysis we did not identify any significant alterations of the Msn2p and Msn4p regulated groups indicating lack of transcriptional ESR on the level of fitness. This result could be due to a different time scale of transcription and fitness experiments. In yeast transcriptional changes are generally observed within the first two hours of application of stress (1-2 generation times), whereas changes in mutant fitness are observed after 6-16 generations. Otherwise, the absence of Msn2p/Msn4p response in fitness data may imply that different aspects of biological control play a role on the level of transcription and fitness. Indeed, our results show that a different general stress response program, which encompasses several cellular functions, can also be distinguished on the basis of fitness experiments.

First, clustering analysis of GO-based fitness profiles revealed that the functional groups of ribosome biogenesis, structure and assembly (RP genes, rRNA processing and metabolism) are generally more resistant to a majority of stress conditions. Most of the mutants belonging to these categories are slow growers under optimal conditions (Giaever *et al.*, 2002), therefore suggesting that a trade-off between optimal growth rate and the ability to endure stress can take place in these mutants.

Second, the deletions of genes involved in actin cytoskeleton organization, a function which is also involved in maintaining proper trafficking within the cell, were hypersensitive to many conditions, suggesting a key role for this function in survival and adaptation to stress. Finally, we observed hypersensitivity of deletions of genes participating in intracellular transport (vesicle-mediated, endosome, Golgi, and secretion) to most imposed stresses, indicating that these functions may play a central role in response to changes in the environment, and that the intracellular transport network has a low buffering capacity in response to perturbation of its individual components.

In conclusion, we present a simple method for combined analysis of expression microarrays and fitness datasets. We show how a global fitness dataset can be converted into an easily interpretable set of t-values that allow for quantification of the contribution of individual functions to condition-specific responses. Next, our integrative analysis of diverse fitness datasets has led to discovery of key functions in multi-stress responses, and yielded new insights into the multi-level regulation of yeast cellular functions. Finally, we believe that our approach can be successfully used for the integration of various large-scale datasets and provides a platform for solving complex systems biology questions.

Chapter 6

General discussion

General Discussion

Stress is a disruption of homeostasis of an organism through physical stimuli, which in microorganisms very often results in reduction of the rate of growth. Microorganisms continuously encounter stressful conditions such as fluctuations in nutrient availability, external temperature or osmolarity. Additionally, they may suffer from exposure to radiation or to toxic chemicals. Under laboratory conditions, microorganisms are often subjected to genetic manipulations (like: mutation, overexpression or deletion of any given gene) that may result in disturbance of homeostasis, thus stress.

The quest to understand how microorganisms respond to their fluctuating environment, and survive imposed stresses, is not only fundamental for biology but also has an applied value, *i.e.* for the development of novel mild antimicrobial strategies in the food industry. The cellular response of any microorganism to its surroundings is extremely complex and can not be understood or analyzed just by studying a single gene or protein, or even at a single level e.g. transcriptome or proteome. Rather, studying such responses requires a systematic integrative approach, in which results of various genome-wide techniques are integrated to provide a more comprehensive picture of what is going on in the cell.

The aim of this study was to investigate which cellular mechanisms are utilized by fungi to adapt to environmental changes and to survive stress. *Saccharomyces cerevisiae* was chosen as a model fungal organism, because of its ability to grow fast, the ease of its genetic manipulation and the availability of a large number of modern research techniques. To address the complexity of cellular responses of bakers' yeast we chose to employ two large-scale genomic techniques; (i) transcript profiling (Chapter 2 and 3), and (ii) global fitness analysis (Chapter 4 and 5). Chitosan, a polymer of β-1,4-glucosamine with as yet unresolved mode of action was used as the primary stress-generating model compound. In this chapter the results of this thesis are discussed in view of recent scientific developments in the field of yeast functional genomics and stress responses.

One aspect of the cellular response of yeast to any change in its surrounding conditions is the rapid reorganization of its genomic expression program (Gasch and Werner-Washburne, 2002). Transcript profiling is one of the most common genome-wide techniques that allows for screening of global changes in the expression of all genes in a single organism, allowing one to identify general and specific expression programs that are activated in response to any given stress. At the beginning of this study expression microarrays were already a well established tool in bakers's yeast research (Gasch *et al.*, 2000; Hughes *et al.*, 2000; Hughes and Shoemaker, 2001). The use of transcript profiling led to the unraveling of the Environmental Stress Response expression program (Gasch *et al.*, 2000), which is often activated in bakers' yeast grown in other than optimal conditions. The exact timing and degree of activation of the ESR depends on the specificity of the imposed stress condition, and is often accompanied by induction of other condition-specific expression programs. Of

special interest for our study are the Ca^{2+}/ calcineurin pathway and the cell wall integrity pathway.

The Ca^{2+}/ calcineurin pathway was characterized in detail in the genome-wide expression analysis of bakers' yeast exposed to calcium, sodium and/ or the specific inhibitor of this pathway, FK506 (Yoshimoto *et al.*, 2002). This signaling pathway is activated in cell wall mutants, at high extracellular levels of Ca^{2+} and Na^{+}, upon increased temperature, and in case of a stress imposed on the endoplasmic reticulum. It operates via the Crz1p transcription factor (Bonilla *et al.*, 2002; Bonilla and Cunningham, 2003; Cyert, 2003). The activation of this pathway leads to induction of genes involved in various cellular functions, such as signaling, ion transport/ homeostasis, vesicle-mediated transport and cell wall maintenance (Yoshimoto *et al.*, 2002). The corresponding pathway in the opportunistic human pathogen, *Candida albicans*, was found to be essential for the organism to survive plasma membrane stress such as treatment with ergosterol-biosynthesis inhibitors, or addition of an anionic surfactant , such as SDS (Cruz *et al.*, 2002).

The activation of the cell wall integrity pathway either by mutation in cell wall biosynthetic genes or by perturbation of the cell wall with compounds such as Calcofluor White, Zymolyase, Congo Red and caspofungin, is well documented (Boorsma *et al.*, 2004; Garcia *et al.*, 2004; Lagorce *et al.*, 2003; Reinoso-Martin *et al.*, 2003). The cell wall integrity pathway makes use of the Rlm1p transcription factor and leads to increased expression of a number of genes that function in cell wall biosynthesis and cell wall maintenance (Jung and Levin, 1999; Smits *et al.*, 2001).

Interestingly, activation of the transcription factors Crz1p and Rlm1p was also found to play a major role in the transcriptional response of *S.cerevisiae* to moderate concentrations of chitosan (Chapter 2). Additionally, the ESR was induced. The use of T-profiler, a statistical tool developed in our lab for expression analysis, allowed us to focus on transcriptional and/ or functional groups rather than single genes, and provided initial insight into the expression program activated when bakers' yeast encounters chitosan stress. Earlier studies on cell wall stress and cell wall mutants identified activation of similar responses at the level of transcription, indicating that cell wall stress and chitosan stress activate common pathways (Garcia *et al.*, 2004; Lagorce *et al.*, 2003). Although one can not exclude the possibility that chitosan also acts on the cell wall, studies in both yeast and bacteria, as well as on artificial membrane, *i.e.* lipid bilayers (Helander *et al.*, 2001; Yang *et al.*, 2002), clearly indicate the plasma membrane as the primary target of chitosan. This suggests that induction of Crz1p- and Rlm1p-dependent transcription could be a key expression program, accompanied by the ESR, elicited by plasma membrane stress in yeast.

To test this hypothesis an integrative approach was undertaken in which a number of transcript profiles derived from plasma membrane stress treatments or of mutants in genes involved in plasma membrane lipid biosynthesis were analyzed in a comparative way using T-profiler and hierarchical clustering (Chapter 3). We observed induction of gene clusters

mediated both by the Crz1p and Rlm1p transcription factors. Additionally, two studies reported on the expression analysis of plasma membrane stress caused by DMSO and two detergents, LAS and SDS, which affect the structure and fluidity of the plasma membrane (Murata *et al.*, 2003; Sirisattha *et al.*, 2004). In all three stresses, the induction of genes regulated by both transcription factors, Crz1p and Rlm1p, was observed. Thus the key findings of these and our study seem to confirm the idea that indeed the genetic or chemical perturbation of plasma membrane in *S. cerevisiae* results in activation of specific expression program comprising the ESR, the Ca^{2+}/ calcineurin, and the cell wall integrity pathway. Up to date the induction of the cell wall integrity pathway via the transcription factor Rlm1p has been mostly associated with the response to cell wall stress. Recent studies, including chapter 3, clearly indicates that both plasma membrane and cell wall stress share this common transcriptional signature, which could thus be described as a more general response to yeast cell surface perturbation. One common output of the transcriptional response to cell wall stress is the remodeling of the cell wall, which results, among others, in an increase of chitin levels, and can be measured as increased resistance to β-1, 3-glucanase treatment (Boorsma *et al.*, 2004). As expected, the treatment with plasma membrane stressors; chitosan, LAS, and SDS, also resulted in increased b-1,3-glucanase resistance, further corroborating that both plasma membrane and cell wall stress induce common responses. Additionally, the involvement of the Ca^{2+}/ calcineurin signaling pathway in maintenance of the plasma membrane integrity can be tested with the specific inhibitors of the pathway, Cyclosporin A and FK506. Interestingly, several studies in *C.albicans* reported that addition of FK506 or Cyclosporin A to cells treated with plasma membrane stress compounds results in highly detrimental effect on growth, indicating that the Crz1p-mediated pathway plays a crucial role in response to plasma membrane stress (Cruz *et al.*, 2002; Karababa *et al.*, 2006; Marchetti *et al.*, 2000; Onyewu *et al.*, 2003).

As mentioned above, cellular responses to stress can be measured with various techniques, including global expression analysis or the recently developed global fitness analysis. The last decade in yeast genetics brought about the construction of the whole set of yeast deletion mutants both essential (heterozygous) and non-essential (homozygous), which facilitate the global fitness analysis (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). The initial idea behind mutant analysis is such that a sensitive growth phenotype in a specific condition can give indications of the function of the mutated or deleted gene (Sopko *et al.*, 2006). Furthermore, the genome-wide fitness analysis offers cues as to mode of action of unknown compounds or the functions involved in response to various stresses. The mutant collection has been used to elucidate the cellular response to DNA-damaging agents, ergosterol biosynthesis inhibitors, and an anti-cancer drug tamoxifen (Birrell *et al.*, 2002; Brown *et al.*, 2006; Parsons *et al.*, 2006; Wu *et al.*, 2004).

The fascinating finding of several yeast fitness and transcription studies is that generally single genes required for growth under a certain condition are not upregulated

transcriptionally under the same condition (Birrell *et al.*, 2002; Giaever *et al.*, 2002). In a more recent study of bakers' yeast response to growth in the presence of fatty acids this notion was confirmed, interestingly even on the level of functional groups (Smith *et al.*, 2006). The mode of action of our model plasma membrane stress compound, chitosan, was investigated using the yeast deletion collection and T-profiler, adapted for the analysis of fitness datasets (Chapter 4). The main functions identified by global fitness analysis as being vital for counteracting chitosan-induced stress include actin organization, endocytosis, plasma membrane and cell wall maintenance. Interestingly, these functions do overlap to a certain degree with cellular responses to chitosan identified on the transcriptional level. Although global fitness analysis of response to chitosan provides key insights into which functions yeast cells require to combat chitosan, it is still unclear which of these are specific for plasma membrane stress and which are more generally involved in the bakers' yeast battle against stress. Additional global fitness studies, in which the behavior of *S. cerevisiae* mutants is tested upon incubation in the presence of specific membrane and cell wall perturbing compounds may provide a better functional resolution for different categories of stress and thus facilitate the design of novel antimicrobial combinations.

Perhaps the main limitation of all large-scale genomics studies is the lack of data standardization. Although many yeast datasets are available both for transcription and fitness, they differ largely in time-scale of the experiments, degree of applied stresses, and simply techniques used to obtain the data. For example, transcription datasets vary in the time resolution of the experiments from a few minutes to several hours, whereas yeast fitness datasets can be obtained from studies of the growth of individual mutants, colony size data, or microarrays used for scoring the abundance of each mutant, at a given point in time after stress application, through the number of copies of their respective unique DNA tags (Dudley *et al.*, 2005; Ericson *et al.*, 2006; Giaever *et al.*, 2002). Therefore, one of the biggest challenges facing biologists is to manage, integrate and compare large-scale data in order to better comprehend the biology behind it. In Chapter 5 we aimed to address that challenge by showing how using T-profiler for analysis of both yeast fitness and transcription data on the level of functional groups can facilitate integration of large-scale data and extraction of relevant biological information. For example, this analysis showed that functions involved in intracellular transport network are crucial for combating almost any type of stress in *S. cerevisiae*.

As stated previously, understanding how microorganisms respond to stress can greatly facilitate the development of novel drugs and antimicrobials against food spoilage. Chitosan has been found effective against the potent food spoilage fungus *Zygosaccharomyces bailii*, and the human pathogen *Candida albicans* (Chapter 2). The insights into the mode of action of chitosan and the revealed synergistic effects of chitosan with other chemicals (such as miconazole, chlorpromazine, SDS, staurosporine or FK506) or

high temperature (Chapter 2 and 4) offer a valuable platform for further research on the effective antifungal combinations in the fields of food and drug industry.

The understanding of the effect of chitosan on *S. cerevisiae* both on the transcription and fitness level has greatly improved. Moreover, the cellular effects of plasma membrane stress in bakers' yeast have been further elucidated in this as well as other recent studies. Finally the T-profiler analysis of global transcription and fitness data revealed the need to look beyond single levels in any analysis of a (micro)biological system responding to a given environmental condition. Indeed, contemporary Systems Biology aims at quantitatively analyzing and integrating data on transcription, post-transcription, translation, metabolism and fitness levels. It may well be that the combinations of profiles at these "horizontal" levels will comprise the functional, cellular response modules that will prove to be characteristic for a given environmental condition at a given time. Identification of such "vertical genomics" modules constitutes a major challenge for future studies.

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Summary

To understand how living organisms are able to respond to and survive stress, defined as any change in their surrounding environment that leads to disruption of homeostasis, is one of the main goals of biology. Modern techniques enable us to address such questions in a more comprehensive way, as a consequence presenting us with a clearer idea of how a living organism deals with stress. The aim of this study was to investigate the response of *Saccharomyces cerevisiae* to stress, with specific focus on plasma membrane stress, while utilizing various large-scale genomics tools.

In **Chapter 1** *S. cerevisiae* is introduced as a model organism and the available functional genomics tools are discussed. Furthermore, the plasma membrane composition, function and biosynthesis are described, with special focus on the lipid components of the membrane. Next, the definition of plasma membrane stress is provided, which distinguishes three main categories: indirect, direct, and genetic perturbation of the plasma membrane. The indirect perturbation occurs when chemicals inhibiting membrane lipids biosynthesis are used. The direct membrane perturbation refers to cells exposed to compounds whose toxic effect is related to their ability to directly interact with the lipid components of the plasma membrane. Finally, the genetic perturbation of the membrane is described as deletions or mutations of genes involved in membrane lipids biosynthesis, which can lead to changes in lipid composition and plasma membrane integrity. Examples of these three types of plasma membrane stresses and their additional cellular effects are discussed.

In **Chapter 2** chitosan was shown to disrupt the plasma membrane of *S. cerevisiae* and subsequently to lead to leakage of cellular components. Also, the transcriptional response of yeast to mild concentration of chitosan was elucidated. The analysis of yeast expression profile in response to chitosan with t-profiler revealed induction of three main gene groups controlled by transcription factors Crz1p, Rlm1p and Cin5p, as well as the Environmental Stress Response program. The involvement of Crz1p-mediated pathway was confirmed by the synergistic growth inhibitory effect of chitosan with the inhibitor of this pathway, FK506. The participation of Rlm1p-controlled pathway in response to chitosan was further verified by its cellular effect, namely cell wall remodeling, which was measured as increased resistance to β-1,3-glucanase in cells treated with chitosan. The deletion mutant of gene *CIN5* was found hypersensitive to chitosan. Additionally, the activation of expression of genes that belong to functional groups such as “endoplasmic reticulum”, “cell wall”, and “plasma membrane” supported the idea that chitosan causes stress on the level of plasma membrane and induces a cell wall compensatory mechanism. Finally, the inhibitory effect of chitosan was determined on two human pathogens, *Candida albicans* and *Candida glabrata*, as well as a known food-spoilage fungus *Zygosaccharomyces bailii*.

Chapter 3 presents the integrative analysis of expression profiles obtained from yeast treated with plasma membrane stress as defined by treatment with a plasma membrane perturbing compound or by deletion or mutation of a gene involved in membrane lipids

biosynthesis. Two anionic detergents, SDS and LAS, were found to share a similar expression profile with chitosan, in that both compounds led to induction of genes regulated by transcription factors Crz1p, Rlm1p, Msn2p and Msn4p. Additionally, treatment with LAS or SDS showed similar phenotypes like treatment with chitosan. For example, all three compounds induced expression of *FKS2-LacZ*, which is under the dual control of Crz1p and Rlm1p. Also, cells treated with LAS or SDS displayed a synergistic growth defect in combination with the Crz1p-pathway inhibitor, FK506, as well as increased resistance to β-1,3-glucanase, a known consequence of the activation of the cell wall integrity pathway. Interestingly, the expression profiles of all three compounds clustered together with expression profiles of several cell wall mutants and the tested cell wall mutants turned out to be hypersensitive to these agents. Finally, the integrative analysis of expression profiles derived from treatments with various plasma membrane perturbants, mutants of membrane lipid and cell wall biosynthetic genes, revealed an expression program that involves gene groups regulated by Crz1p and Rlm1p. This transcriptional program could be described as a common signature response of *S. cerevisiae* to stresses affecting plasma membrane integrity.

The fitness effect of chitosan on the whole yeast mutant deletion collection was investigated In **Chapter 4**. The fitness datasets were analyzed using t-statistics, by determining the sensitivity to chitosan of deletion strains belonging to functional groups defined by Gene Ontology or through the synthetic lethality screen. The chitosan-hypersensitive groups identified in this study were defective in RNA synthesis and processing, actin cytoskeleton organization, protein N-glycosylation, ergosterol synthesis, endocytosis, and in cell wall formation, indicating that these functions protect and buffer the cell against the harmful effect of chitosan. These results were confirmed by demonstrating that tunicamycin, miconazole, and staurosporine, three compounds that target protein N-glycosylation, ergosterol synthesis, and the cell wall integrity pathway, respectively, exhibited synergistic inhibitory growth effect when combined in chitosan against yeast cells. Additionally, a group of deletion strains characterized by a defective HOG signaling pathway was identified as chitosan-hypersensitive, thus implying that the HOG pathway is required for counteracting chitosan-induced stress. In corroboration with this, activation of the HOG pathway in wild-type cells by hypertonic conditions offered partial protection against chitosan, whereas hypotonic conditions sensitized the cells to chitosan. This study showed that T-profiler analysis of yeast global fitness data allows for identification of specific cellular processes and pathways that are required for survival under stress conditions.

Chapter 5 takes advantage of the existing yeast fitness and transcription datasets in order to elucidate the general response of *S. cerevisiae* to multi-stress conditions. The results show that correlation of group fitness and transcriptional regulation is very weak, with the exception of mutants in ribosomal protein genes. In addition, the functional groups required for growth in a certain condition do not overlap with those that are transcriptionally upregulated in response to the same condition. Next, the general features of yeast fitness in

response to various stresses are revealed; in particular, the overall resistance of deletion mutants in ribosomal protein encoding genes. Notably, groups of mutants involved in vesicle-mediated transport, actin cytoskeleton organization and cellular communication are found to display broad sensitivity to various stress conditions, indicating that these functions play a vital role in yeast adaptation to stress. This chapter presents a comprehensive study of global fitness data and its integration with expression data, which provides new insights into multilevel responses to changing environmental conditions. To conclude, both fitness and expression studies provide complementary information about the complexity of cellular responses and as such should be analyzed in an integrative way.

Chapter 6 places the results of this work in the context of recent literature in the field of yeast stress responses and functional genomics.

Samenvatting

Eén van de belangrijkste uitdagingen in de biologie is het begrijpen hoe levende organismes in staat zijn om te reageren op stress, gedefinieerd als elke verandering in hun omgeving die leidt tot een verstoring van homeostase, en vervolgens te overleven. Moderne technieken stellen ons in staat zulke vragen in een meer complete en brede manier te beantwoorden. Het hoofddoel van het werk beschreven in dit proefschrift was het onderzoeken van de respons van *Saccharomyces cerevisiae* op stress, met speciale aandacht voor het plasma membraan, en gebruikmakende van verscheidene 'large-scale' genomics methoden.

In **Hoofdstuk 1** worden zowel *S. cerevisiae* als model organisme, alsmede de beschikbare functionele genomics methoden besproken. Bovendien worden de samenstelling, functie en biosynthese van het plasma membraan beschreven, met speciale nadruk op de lipide bestanddelen van het membraan. Verder wordt de definitie van plasma membraan stress gegeven, die zich onderscheid in drie categorieën: indirecte, directe, en genetische verstoring van het plasma membraan. Indirecte verstoring vindt plaats wanneer chemicaliën worden gebruikt die de biosynthese van membraan lipiden remmen. Directe membraan verstoring duidt op cellen die onderhevig zijn aan stoffen waarvan het giftige effect verband houdt met hun mogelijkheid om direct een interactie aan te gaan met de lipide bestanddelen van het plasma membraan. Tenslotte wordt de genetische verstoring van het membraan beschreven als deleties of mutaties van genen die betrokken zijn bij de membraan lipide biosynthese, die tot veranderingen in de lipide compositie en plasma membraan integriteit kunnen leiden. Voorbeelden van deze drie typen plasma membraan stress en hun cellulaire effecten worden besproken.

In **Hoofdstuk 2** werd laten zien dat chitosan het plasma membraan van *S. cerevisiae* verstoord en vervolgens leidt tot het lekken van cellulaire componenten. Ook werd de transcriptionele respons van gist op een milde concentratie van chitosan ontrafeld. De analyse van het gist expressie profiel als reactie op chitosan met behulp van t-profiler liet de inductie zien van drie belangrijke gen groepen die gecontroleerd worden door de transcriptie factoren Crz1p, Rlm1p and Cin5p, alsmede het 'omgevings stress reactie programma'. De betrokkenheid van het door Crz1p-geregelde pad werd bevestigd door het synergetisch groei-inhibitie effect van chitosan samen met de remmer van dit pad, FK506. De deelname van het door Rlm1p-gecontroleerde pad als reactie op chitosan, werd verder geverifieerd door het cellulaire effect, namelijk een celwand herinrichting, dat gemeten werd als een verhoogde resistentie voor β-1,3-glucanase in chitosan behandelde cellen. De deletie mutant van het gen *CIN5* is hypergevoelig voor chitosan. Bovendien ondersteunen de activering van de expressie van genen die behoren tot de functionele groepen zoals "endoplasmatisch recticulum", "celwand", en "plasma membraan" het idee dat chitosan stress veroorzaakt op het niveau van het plasma membraan en een celwand compensatie mechanisme induceert. Tenslotte werd het inhibitie effect van chitosan bepaald in de humane pathogenen, *Candida albicans* and

Candida glabrata, alsmede in *Zygosaccharomyces bailii*, een bekend schimmel bederforganisme.

Hoofdstuk 3 presenteert de geïntegreerde analyse van expressie profielen verkregen uit gist behandeld met plasma membraan stress, zoals gedefinieerd door een behandeling met een membraan verstorende stof of door deletie of mutatie van een gen betrokken in membraan lipide biosynthese. Twee anionische detergentia, SDS en LAS, delen een vergelijkbaar expressie profiel met dat van chitosan: beide stoffen leidden tot de inductie van genen gereguleerd door transcriptie factoren Crz1p, Rlm1p, Msn2p and Msn4p. Bovendien gaven een behandeling met LAS of SDS een vergelijkbaar fenotype met dat van chitosan. Bijvoorbeeld, alle drie de stoffen induceerden de expressie van *FKS2-LacZ*, dat zowel door Crz1p als Rlm1p gereguleerd wordt. Cellen behandeld met LAS of SDS vertonen ook een synergistisch groei-defect in combinatie met de Crz1p-pad remmer, FK506, alsmede een verhoogde resistentie voor β-1,3-glucanase, wat een bekende consequentie van de activering van het 'celwand integriteits pad' is. De expressie profielen van alle drie de stoffen clusteren interessant genoeg samen de expressie profielen van verscheidene celwand mutanten en de geteste celwand mutanten bleken bovendien hypergevoelig voor deze stoffen. Tenslotte, de geïntegreerde analyse van de expressie profielen, afgeleid uit de behandelingen met verschillende plasma membraan verstorende stoffen, mutanten van membraan lipide en celwand biosynthese genen, heeft een expressie programma aan het licht gebracht dat gen groepen bevat die gereguleerd worden door Crz1p en Rlm1p. Dit transcriptionele programma kan beschreven worden als een algemene reactie van *S. cerevisiae* kenmerkend voor stressen die de integriteit van het plasma membraan beïnvloeden.

Het effect van chitosan op de fitheid van de gehele gist mutant deletie collectie is onderzocht in **Hoofdstuk 4**. De fitheids datasets zijn geanalyseerd met behulp van t-statistiek, door middel van het bepalen van de gevoeligheid voor chitosan van de deletie stammen die behoren tot de functionele groepen gedefinieerd door 'Gene Ontology' of door de synthetische letaliteits 'screen'. De chitosan hypergevoelige groepen die geïdentificeerd zijn in deze studie hebben een defect in RNA synthese en voortgang, actine cytoskelet organisatie, eiwit N-glycosylering, ergosterol synthese, endocytose, en in celwand formatie. Deze waarnemingen wijzen erop dat deze functies de cel beschermen en bufferen tegen het schadelijke effect van chitosan. Het vertoonde synergistische groei-remmings effect van chitosan met tunicamycine, miconazol, en staurosporine, drie stoffen die respectievelijk de eiwit N-glycosylering, de ergosterol synthese, en het 'celwand integriteits pad' aanvallen, bevestigen deze resultaten. Bovendien is een groep deletie stammen die gekarakteriseerd worden door een defect in het HOG signalerings pad geïdentificeerd als chitosan hypergevoelig. Dit impliceert dat het HOG pad nodig is voor het tegengaan van chitosan geïnduceerde stress. Dit wordt ondersteund door de observatie dat de activering van het HOG pad in wild-type cellen door hypertonische condities een gedeeltelijke bescherming

tegen chitosan opleverde, maar in hypotonische condities de cellen juist gevoeliger maakten voor chitosan. Deze studie heeft laten zien dat de analyse van gist fitheids data met t-profiler het mogelijk maakt om specifieke cellulaire processen en paden, die nodig zijn voor het overleven onder stress condities, te identificeren.

In **Hoofdstuk 5** wordt met behulp van de beschikbare gist fitheids en transcriptie datasets de algemene respons van *S. cerevisiae* op multi-stress condities ontrafeld. De resultaten laten zien dat de correlatie tussen de fitheid van een groep en de transcriptionele regulatie erg laag is, behalve voor mutanten van ribosomale eiwit genen. Bovendien is er geen overlap tussen functionele groepen die nodig zijn voor de groei in een zekere conditie met groepen die transcriptioneel op-gereguleerd zijn als reactie op dezelfde conditie. Ook zijn de algemene kenmerken van gist fitheid als reactie op verscheidene vormen van stress blootgelegd. In het bijzonder laten groepen van mutanten die betrokken zijn bij transport door middel van 'vesicles', actine cytoskelet organisatie en cellulaire communicatie een brede gevoeligheid zien voor verscheidene stress condities. Dit laat zien dat deze functies een essentiële rol spelen in de adaptatie van gist blootgesteld aan stress. Dit hoofdstuk presenteert een uitgebreide studie van fitheids data en de integratie hiervan met expressie data en verschaft nieuwe inzichten in de responsen van verschillende niveaus op veranderende condities in de omgeving. Concluderend geven de fitheids en expressie studies complementaire informatie over de complexiteit van de cellulaire responsen en moeten op een dusdanige geïntegreerde manier geanalyseerd worden.

Hoofdstuk 6 plaatst tenslotte dit werk in context met de recente literatuur op het gebied van de stress reacties van gist en de functionele genomics.

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