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Chapter 3

DNA micro-array mRNA MAPKINASE Quontology STRE post-genomic Rlm1p Rho1 ß1,3-glucanase *Candida albicans* gene groups rRPE Bonferonni cell Wall REDUCE Gene Ontology chitin synthesis consensus motif *Saccharomyces cerevisiae* Slt2 MIPS

Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*

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Abstract

The cell wall perturbants Calcofluor white and Zymolyase activate the Pkc1-Rho1-controlled Slt2p MAP kinase pathway in *Saccharomyces cerevisiae*. A downstream transcription factor of this pathway, Rlm1p, is known to control expression of about 20 cell wall-related genes. Global transcript analysis of Calcofluor white and Zymolyase treatment was performed to determine whether cell wall stress affects transcription of these and other genes. Transcript profiles were analyzed using two recently developed algorithms, viz. REDUCE, which correlates upstream regulatory motifs with expression, and Quontology*, which compares expression of genes from functional groups with overall gene expression. Both methods indicated upregulation of Rlm1p-controlled cell wall genes and STRE-controlled genes, and downregulation of ribosomal genes and rRNA genes. Comparison of these expression profiles with the published profiles of two constitutively active upstream activators of the Slt2p-MAP kinase pathway, viz. Pkc1-R398A and Rho1-Q68A, revealed significant similarity. In addition, a new putative regulatory motif, CCC(N)10GGC, was found. In Zymolyase -treated cells a regulatory site was identified, AT-GACGT, which resembles the AFT/CRE binding site. Interestingly, Sko1p, a downstream regulator of the high osmolarity pathway is known to bind to the AFT/CRE binding site, suggesting a possible role for the Hog1 pathway in the response to cell wall stress. Finally, using REDUCE, an improved version of the Rlm1 binding motif, viz. TA(W)4TAGM, was discovered. We propose that this version can be used in combination with REDUCE as a sensitive indicator of cell wall stress. Taken together, our data indicate that cell wall stress results in activation of various signalling pathways including the cell wall integrity pathway.

*Quontology [81] is the forerunner of T-profiler, although the statistics that are used in both methods slightly differ, the results produced by both methods, are highly comparable.

Introduction

All living organisms must be able to adapt to a wide variety of stress conditions. Usually, this involves changes in gene expression, leading to increased levels and activities of proteins that have stress-protective functions [109]. Fungal cells, for instance, need to maintain their cellular integrity in hostile environments that contain cell wall-degrading enzymes. Specifically, they have to resist glucanases and chitinases that are secreted by plants in response to fungal infection [110]. Cell wall lytic enzymes are also secreted by mycoparasites [111] and by human and other mammalian macrophages [112-114]. Although several cell wall proteins have been shown to protect yeast against certain stress conditions [115-118], the mechanisms used to detect, respond to, and eventually counter cell wall weakening are only starting to be unravelled [119, 120]. Understanding these mechanisms is expected to aid the development of effective antifungal agents.

The cell wall of *Saccharomyces cerevisiae* consists of only four classes of macromolecules, which are all interconnected by covalent bonds: the cell wall proteins (CWPs); ß1,6-glucan; ß1,3-glucan; and chitin [121]. Over 30 genes coding for putative CWPs have been identified. Two main classes of covalently bound CWPs can be distinguished, GPI-CWPs and Pir-CWPs, based on how they are linked to the cell wall glucan [119]. A characteristic set of compensatory alterations in the composition and architecture of the cell walls has been detected when cell wall integrity is compromised. These alterations result in cells that are more resistant to further cell wall degradation. Notably, chitin synthesis and the expression of several cell wall proteins and of the alternative subunit of ß1,3-glucan synthase, *FKS2*, are increased [122-125]. The so-called PKC-SLT2 or cell wall integrity pathway contributes to this process and is essential for the viability of mutants defective in wall biosynthesis, as well as cells challenged with wall-degrading compounds [50, 126, 127]. Two important proteins that control the cell wall integrity pathway are *PKC1* and the *RHO1* GTPase. Rho1p is a regulatory subunit of the 1,3 ß-glucan synthase complex [128] and stimulates glucan synthesis in a GTP-dependent way. Rho1p also binds and activates Pkc1p [129], which in turn stimulates the cell wall integrity pathway. Several putative downstream effectors of the MAPK kinase Slt2p have been reported, including the transcription factor Rlm1p that binds the nucleotide sequence CTA(W)4TAG [48, 49, 130]. Activation of Slt2p through overexpression of a hyperactive allele of MKK1, which encodes the MAP kinase of this pathway, results in changed transcription of more than 20 genes. Most of these genes affect the cell wall and all but one depend on Rlm1p for regulation in response to Slt2p activation [48, 131]. The cell integrity pathway is activated in response to heat and hypotonic stress, during polarized growth, and as a result of cell wall weakening [55, 131-133]. We, and others have shown previously that mild cell wall stress activates Slt2p, which in turn mediates cell wall alterations and the induction of Rlm1p-controlled gene expression [122, 124, 131].

In this study global transcript analysis was used in combination with novel statistical analysis methods. Using these methods, we have found that the response to cell wall stress includes activation of the environmental stress response and the cell integrity pathway. In addition, osmosensing genes are upregulated. Also, an uncharacterized sequence motif was significantly

correlated with changed transcription, suggesting the involvement of alternative regulatory components. Finally, we have further improved the predicted Rlm1p binding motif, which can be used in combination with REDUCE to identify cell wall stress in other transcriptional data sets.

Materials & Methods

Strains and growth conditions

The *S. cerevisiae* strains used in this study are all isogenic to strain FY834 (MAT his3200 ura3-52 leu21 lys2202 trp163). Yeast cells were grown overnight at 28 °C in YEPD (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) to OD595 = 0.25. The incubation was continued for 2 h in the absence or presence of the cell wall perturbing agents Calcofluor white (10 μg/ml) or Zymolyase-100T (0.26 U/ml). Under these conditions no growth inhibition was apparent for stressed cells compared to mock-treated cells. Cells were collected by centrifugation, flash-frozen in liquid nitrogen and stored at -70 °C prior to isolation of RNA.

ß1,3-glucanase sensitivity

The cells were washed and resuspended in 50 mM Tris-HCl, pH 7.4, to a concentration of 1.5 × 10^7 cells/ml. b-Mercaptoethanol was added to a final concentration of 40 mM and the cells were incubated at room temperature for 30 min prior to the addition of 100 U/ml 1,3-glucanase (Quantazyme ylg, Quantum Biotechnologies Inc. Laval, Canada). The decrease in OD595 was followed in time as a measure of cell lysis and was expressed as a percentage of the OD595 prior to enzyme addition.

RNA isolation and cDNA synthesis

Total RNA was isolated from stressed and mock-treated yeast cells using the FastPrepTM system (BIO 101, Inc). RNA was primed for cDNA synthesis by mixing 4 μg total RNA with 2 μg oligo dT (Research Genetics) in 10 μl DEPC-treated water, followed by heating for 10 min at 70 °C and brief chilling on ice. Next, Superscript II reverse transcriptase (300 units), dithiothreitol (3.3 mM) and first strand buffer (all from Life Technologies), a mixture of dNTPs (dATP, dGTP and dTTP, each at 1 mM), and [-33P]-dCTP (90 μCi, 3000 Ci/mmol, ICN) were added to the primed RNA. Elongation was allowed to occur during incubation of the 30 μl mixture for 90 min at 37 °C. Unincorporated nucleotides were separated from the 33P-labelled cDNA probe by passage through a Sephadex® G-50 column. Typically, incorporation efficiencies of about 30% were obtained.

Mini-array filter membrane hybridization

Genefilters® were prehybridized for 2 h in 5 ml MicroHybTM solution containing 1 μg/ml Poly dA (all from Research Genetics). The purified probe was heat-denatured for 3 min at 100 °C and added to the prehybridization mixture to allow overnight hybridization. (Pre)hybridizations were performed at 42 °C in a roller oven. The filters were washed twice at 50 °C in 2 × SSC, 1% SDS, for 20 min and, once at room temperature, in 0.5 × SSC, 1% SDS for 15 min. The washed filters together with a moist support of Whatmann paper were wrapped in plastic film prior to exposure for 5 days to a storage phosphor screen. Data were collected using a phosphoimaging system (STORM Systems, Molecular Dynamics). The membranes were stripped of probe by pouring a boiling solution of 0.5% SDS over the filters and incubating them at room temperature with gentle shaking for 1 h. Stripped membranes were checked for loss of signal before using them again.

Analysis of the hybridization results

The hybridization data were analysed using Pathways™ software (Research Genetics). The filter images were normalized using all data points instead of using only the control spots that contain a mixture of yeast ribosomal genes. This resulted in the best-centred distribution of expression ratios and yielded expression ratios closest to 1 for genes that are expected not to be affected by cell wall stress (for *ACT1*, encoding actin, the ratio was 1.10 ± 0.05; and for *IPP1*, encoding inorganic pyrophosphatase, it was 1.02 ± 0.03). Three separate Genefilters® containing > 98% of all yeast ORFs were simultaneously but separately hybridized with three probes (made from RNA isolated from mock-, Calcofluor white- and Zymolyase-treated cells). After signal detection and stripping, each of the filters was re-hybridized with freshly made probe, eventually resulting in successive hybridizations of each filter with all three probes. Hybridization patterns of different probes on the same filter gave very similar transcript profiles for stressed vs. mock-treated cells compared to the presented results that were obtained by simultaneous hybridization of these probes on separate filters. <3.5% of all spotted genes exhibited more than 1.4-fold repression or induction at least once after Calcofluor with stress, and the same held true for <1.5% of all genes after Zymolyase-stress. Expression ratios are shown only for genes that on average exhibited a change of more than 1.5-fold in three hybridization experiments, and in each separate experiment never less than a 1.4-fold change in expression. Our unpublished data seem to support the notion that expression ratios obtained by microarray analysis, using Research Genetics gene-filters, are likely to underestimate the actual changes in expression. For example, we detected a 2.7-fold induction of *CWP1* expression in cells stressed with Calcofluor white, whereas Northern analysis and expression of a CWP1-lacZ reporter construct indicated an induction of about five-fold (data not shown). It is also noteworthy that although over 98% of all yeast genes are spotted on the membranes, approximately 100 genes are absent, among which are *GAS1*, *KRE6* and *RHO1*, which play important roles in the regulation of cell wall biogenesis. The raw data is available at http://staff.science.uva.nl/boorsma/cellwallstress/

Microarray data analysis

REDUCE

To find upstream elements associated with the observed changes in gene expression, we used REDUCE - a tool developed by Bussemaker *et al*. [88]; see also: http://bussemaker.bio.columbia.edu/reduce). REDUCE uses a statistical approach to find motifs based on single microarray experiments. In addition, no arbitrary cut-offs of gene expression ratios are required. Finally, the statistical significance of the results obtained can be expressed using a single parameter, the so-called z-value. This allows comparing transcriptional responses of groups of genes observed in various microarray experiments. REDUCE stands for regulator element detection using correlation with expression. Based on an unbiased search, REDUCE selects those sequence motifs whose occurrence in the upstream region of a gene correlates with a change in expression. Regions of 600 base pairs located on the coding strand upstream of each ORF were used for this analysis. When the intergenic distance was shorter than 600

base pairs, the upstream region was shortened accordingly. Sequence motifs used for analysis were chosen from a set of oligomers to a length of seven nucleotides. Also, dimers with a length of one to three nucleotides and with a spacing of 1-20 base pairs were used. In addition, sequence motifs from a motif database (SCPD) and sequence motifs found in the YPD database were used for analysis. These motifs can have a high degree of degeneration, e.g. the Rlm1 motif CTA(W)4TAG.

To compare the REDUCE results with those of Quontology (see below) the significance of a specific motif is expressed in a Z-score:

$$Z_\mu = \sqrt{(\Delta X_\mu^2 * G)}$$

where

Z_μ = z-score for a particular motif,
ΔX_μ^2 = delta chi-square for a particular motif

and G = total number of genes.

Quontology

We used the algorithm Quontology [81] to correlate expression changes with functional categories of genes. Quontology is essentially identical to the REDUCE algorithm for scoring promoter elements, but uses manually defined gene categories, e.g. derived from MIPS functional categories replacing the sets of genes sharing a particular DNA motif in their promoter region. For each category, a z-score can be calculated that measures the deviation of the average log-ratio for genes in the category from the genome-wide average, in units of the standard deviation. Pavlidis *et al*. [102] have recently discussed a similar method.

Results

Cells challenged with Calcofluor white or Zymolyase develop glucanase resistance.Various kinds of cell wall damage result in activation of the cell wall integrity pathway. The downstream MAP kinase of this pathway, Slt2p-Mpk1p, becomes rapidly activated by dual phosphorylation in response to mild cell wall stress. Concurrent with Slt2p activation, the cells become more resistant to glucanase digestion and heat shock. Both changes have been shown to depend on *SLT2* [122]. In order to identify transcriptional changes in response to cell wall perturbation, we compared transcript levels of mock-treated yeast cells with those of cells stressed with either Calcofluor white (CFW), a fluorescent dye that hinders normal cell wall assembly, or with Zymolyase, a cell wall-degrading enzyme preparation. To select conditions that allowed adaptation to cell wall stress, the cells were tested for protection against ß-1,3-glucanase after exposure to either Calcofluor white (CFW) or Zymolyase (Figure 1). A 2 h incubation with CFW (10 μg/ml) or Zymolyase (0.26 U/ml) did not significantly inhibit growth but was sufficient to induce a protection mechanism that rendered the cells more resistant to digestion with a recombinant ß-1,3-glucanase than mock-treated cells (**Figure 1**). Therefore, these cells were chosen for global transcript analysis.

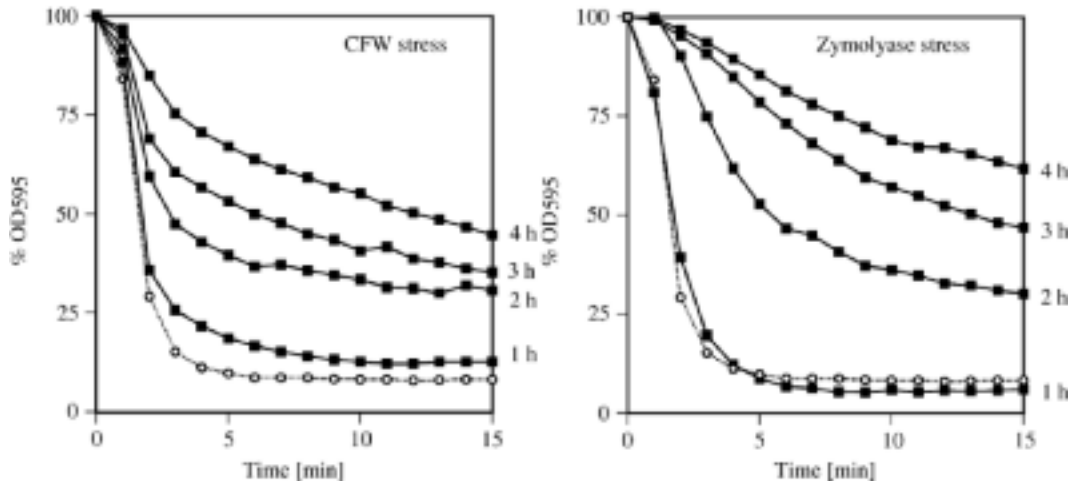

Figure 1. Cells cultured in the presence of Calcofluor white or Zymolyase-100T display a progressive increase in resistance to 1,3-glucanase digestion. YEPD cultures were inoculated with FY834 cells to yield a starting OD595 = 0.25. Cultures were incubated at 28 °C for 1-4 h in the absence or presence of Calcofluor white (10 μg/ml; upper panel) or Zymolyase-100T (0.26 U/ml; lower panel). Cells were washed and digested with a recombinant 1,3-glucanase (Quantazyme ylg), as described in Materials and methods. The decrease in OD595 represents cell lysis and was expressed as percentage of the starting OD595. The results presented are the means of two independent experiments. The dotted lines with open symbols represent cells grown for 4 h in the absence of CFW or Zymolyase

Global expression changes upon cell wall perturbation

RNA isolated from these cells was used for the synthesis of 33P-labelled cDNA and three separate Genefilters® were sequentially hybridized with all three probes. Global transcript analysis showed that 14/18 Slt2p-regulated genes previously identified with mini-arrays [48] were present in our set of genes most responsive to CFW (Table 1). Jung and Levin [48] further showed that these 14 genes all depend on the transcription factor Rlm1p for expression after Slt2 activation and, except possibly for a gene of unknown function (*YIL117c*), code for cell wall-related products. These 14 genes encode: six GPI-CWPs (*YLR194c*, *CWP1*, *SED1*, *PST1*, *CRH1*, *CCW14*), four Pir-CWPs (*PIR1*, *PIR2*/*HSP150*, *PIR3*, *CIS3*), one 1,3-transglucosylase (*BGL2*) and two MAP kinases that have both been shown to interact with Rlm1p (Slt2p/Mpk1p, Mlp1p) [130]. The remaining gene of unknown function (*YIL117c*) has similarity to a gene, *CSI2*, which is believed to be involved in chitin synthesis (*Saccharomyces* Genome Database). In addition to these 14 genes, we detected induction of two genes of unknown function (*SRL3* and *YPL088w*) that are probably induced in a PKC1-dependent manner during polarized morphogenesis [134]. Induction of the remaining 33 genes (i.e. upregulated under CFW-induced stress, but not identified as Slt2p-responsive) suggests that CFW-induced stress triggers other signal transduction pathways in addition to the Slt2p-Mpk1p-dependent cell wall integrity pathway. Six of these genes are known to be induced in response to several stress conditions (*PNC1*, *GPD1*, *HSP12*, *HSP42*, *HOR2*/*GPP2* and *DDR48*). Another six genes of this group have known or likely cell wall-related functions (*GFA1*, *YPS3*, *KTR2*, *YLR042c*, *KRE11* and *ECM4*). Known or suggested functions, among which is transcriptional activation, have also been assigned to another 10 genes (*NMT1*, *YHR209w*, *AFR1*, *YJL108c*, *HXK1*, *ASH1*, *YHR138c*, *FBP26*, *MRP8* and *VRP1*), whereas the function of the remaining 11 genes is unknown. Although all genes upregulated by CFW were to some extent also induced by Zymolyase treatment, expression levels were generally lower (**Table 1**). Possibly, this merely reflects a difference in the severity of the stress conditions. However, transcription of several genes (e.g. *HOR2*, *GPD1*, *DDR48*, *DDR2*, *CWP2*, *YLR042c*) was more strongly affected by Zymolyase-induced stress (Table 1), indicating that the responses to CFW and Zymolyase are similar, but not identical. In summary, the 51 genes most induced by CFW and/or Zymolyase-provoked stress encode 20 cell wall-related products, seven proteins related to general cell stress, 10 proteins of other known or suggested function and an additional 14 proteins of unknown function. The expression of *FKS2* is regulated by the cell wall integrity pathway [48]. In accordance, induction of FKS2-lacZ expression in response to CFW- and Zymolyase-induced cell wall stress depends on *SLT2* [122]. For unclear reasons, we did not detect significant induction of *FKS2* in our array analysis. It cannot be excluded, therefore, that additional genes that are responsive to cell wall stress may have remained undetected.

Table 1. Genes induced by cell wall perturbation. ORFs that gave an induction of 1.4-fold in each separate hybridization by either CFW- or Zymolyase-induced stress and an average induction of 1.5-fold are presented. ORFs depicted in bold were induced 1.5-fold by both CFW- and Zymolyase-induced stress. The results are the mean ± SEM of three hybridizations. Note that expression ratios obtained by Genefilter array analysis are likely to underestimate the actual changes in expression [135]. Arrows indicate ORFs that have been previously been identified as regulated by the MAP kinase Slt2 [48]. **a** CTA(W)4TAG sequence in the upstream sequence. **b** TA(W)4TAG in the upstream sequence. **c** TA(W)4TAGM in the upstream sequence or are candidate PKC regulated genes (arrows between brackets) [134]. Functional categories and gene product are based on the Yeast Proteome Database [136] and the Saccharomyces Genome Database [137]. GPI-CWP, GPI-dependent cell wall protein.

		Fold Changes			
ORF	**Gene**	**CFW**	**Zymolyase**		**Gene product**
Cell wall maintenance					
YLR194C		3.17 ± 0.34	1.74 ± 0.03	↑[b,c]	GPI-CWP
YKL096W	*CWP1*	2.74 ± 0.29	2.52 ± 0.06	↑[a,b,c]	GPI-CWP
YKL161C	*MLP1*	2.68 ± 0.23	1.96 ± 0.08	↑[b,c]	MAP kinase
YKL163W	*PIR3*	2.67 ± 0.21	1.41 ± 0.10	↑[a,b,c]	Pir-CWP
YDR077W	*SED1*	2.48 ± 0.07	1.53 ± 0.34	↑[a,b,c]	GPI-CWP
YKL104C	*GFA1*	2.38 ± 0.21	1.52 ± 0.06		Glucosamine-amino transferase
YDR055W	*PST1*	2.35 ± 0.14	1.45 ± 0.15	↑[a,b,c]	GPI-CWP
YJL159W	*PIR2*	2.16 ± 0.12	1.46 ± 0.22	↑[a,b,c]	Pir-CWP
YLR121C	*YPS3*	2.11 ± 0.09	1.66 ± 0.02		GPI-anchored aspartyl protease
YKL164C	*PIR1*	2.08 ± 0.08	1.31 ± 0.08	↑[a,b,c]	Pir-CWP
YKR061W	*KTR2*	1.90 ± 0.08	1.28 ± 0.09		Mannosyl transferase
YGR189C	*CRH1*	1.88 ± 0.04	1.45 ± 0.04	↑[b,c]	GPI-CWP
YHR030C	*SLT2*	1.87 ± 0.19	1.62 ± 0.07	↑[b,c]	MAP kinase
YJL158C	*CIS3*	1.68 ± 0.11	1.36 ± 0.17		Pir-CWP
YGR282C	*BGL2*	1.65 ± 0.08	1.22 ± 0.08	↑[b]	b1,3-Glucosyltransferase
YLR042C		1.56 ± 0.07	1.91 ± 0.14		GPI-CWP
YGR166W	*KRE11*	1.51 ± 0.05	1.11 ± 0.03		Involved in b1,6-glucan maturation
YKR076W	*ECM4*	1.50 ± 0.02	1.21 ± 0.09		Extracellular mutant 4
YLR390W-a	*CCW14*	1.50 ± 0.03	1.17 ± 0.04	↑[a,b,c]	GPI-CWP
YKL097W-a	*CWP2*	1.37 ± 0.04	1.62 ± 0.04		GPI-CWP
General cell stress					
YGL037C	*PNC1*	2.05 ± 0.12	1.42 ± 0.18		Pyrazinamidase
YDL022W	*GPD1*	2.04 ± 0.08	2.13 ± 0.13		Glycerol-3-P dehydrogenase
YFL014W	*HSP12*	1.88 ± 0.22	1.68 ± 0.06		Heat shock protein
YDR171W	*HSP42*	1.75 ± 0.07	1.38 ± 0.15		Heat shock protein
YER062C	*HOR2*	1.57 ± 0.06	1.61 ± 0.04		Glycerol phosphate phosphatase
YMR173W	*DDR48*	1.53 ± 0.05	1.66 ± 0.10		Heat shock protein
YOL053C-a	*DDR2*	1.38 ± 0.04	1.51 ± 0.06		Heat shock protein
Other functions					
YLR195C	*NMT1*	2.75 ± 0.20	1.50 ± 0.01		N-Myristoyl transferase
YHR209W		2.55 ± 0.27	1.59 ± 0.10		Putative methyl transferase
YDR085C	*AFR1*	2.10 ± 0.22	1.44 ± 0.13		Involved in mating projection
YJL108C	*PRM10*	1.79 ± 0.08	1.51 ± 0.14		Putative permease
YFR053C	*HXK1*	1.70 ± 0.14	1.33 ± 0.16		Hexokinase
YKL185W	*ASH1*	1.65 ± 0.05	1.13 ± 0.03		Transcription factor
YHR138C		1.60 ± 0.11	1.13 ± 0.13		vacuolar membrane fusion
YJL155C	*FBP26*	1.60 ± 0.00	1.18 ± 0.04		Fructose 2,6 bisphosphatase
YKL142W	*MRP8*	1.57 ± 0.10	1.18 ± 0.04		Ribosomal protein
YLR337W	*VRP1*	1.51 ± 0.01	1.10 ± 0.06		Cytoskeletal protein
Unknown function					
YIL117C	*PRM5*	2.35 ± 0.16	1.70 ± 0.09	↑	Similarity to *CSI2*
YPL088W		2.20 ± 0.16	1.60 ± 0.07	(↑)	

YKR091W	*SRL3*	2.04 ± 0.23	1.41 ± 0.08 (↑)	
YKR013W	*PRY2*	1.79 ± 0.11	1.24 ± 0.13	
YBR071W		1.75 ± 0.08	1.32 ± 0.02	
YLR414C		1.75 ± 0.05	1.33 ± 0.05	
YAL053W		1.70 ± 0.03	1.34 ± 0.03	
YDL124W		1.69 ± 0.10	1.31 ± 0.06	
YMR315W		1.67 ± 0.07	1.22 ± 0.02	
YBR053C		1.65 ± 0.08	1.29 ± 0.01	Similarity to regucalcin
YBR214W	*SDS24*	1.64 ± 0.06	1.33 ± 0.03	Nuclear protein
YKL065C	*YET1*	1.63 ± 0.07	1.23 ± 0.09	Transmembrane protein of the ER
YJL161W		1.58 ± 0.06	1.21 ± 0.04	
YJL066C	*HRE252*	1.52 ± 0.02	1.18 ± 0.08	

Transcript analysis of Calcofluor white and Zymolyase transcription profiles by correlation of gene expression with functional groups

We decided to use transcript analysis algorithms specially designed to analyse single transcription profiles. When using these tools, it is not necessary to introduce cut-offs for up- or downregulated genes. These tools also enabled us to compare data obtained from different types of microarrays. To obtain global information about the biological processes that are associated with the transcriptional response, we used a variation of the REDUCE algorithm [88], which is called Quontology [138]. This algorithm compares gene expression of function-related genes, such as the functional categories as defined in the MIPS database (http://mips.gsf.de/proj/yeast/CYGD/db/index.html), with the global expression profile.

The transcription profiles of both Calcofluor white- and Zymolyase-treated cells shared upregulation of genes from five different functional groups (**Table 2**):

1. Stress response genes. Most of these genes are differentially regulated upon various forms of stress.

2. Cell wall-related genes. This most probably reflects the regulation of genes involved in construction and remodeling of the cell wall.

3. Osmosensing genes. This group contains genes that encode proteins partially belonging to or related to the Hog1p osmosensing MAP kinase pathway.

4. The functional group of C-compound and carbohydrate utilization. Regulation of the genes of this functional group could point to an increase in energy demand. It also could point to the redirection of glucose towards incorporation in the cell wall. One of the genes involved in the redirection of glucose flux is *GFA1*, which encodes the glutamine-fructose-6-phosphate amidotransferase, the major controller of chitin synthesis [139]. *GFA1* is significantly upregulated in both CFW and Zymolyase gene expression profiles (**Table 1**).

5. Genes involved in budding, cell polarity and filament forming.

Significant upregulation of the genes from the groups involved in the cytoskeleton, or in pro-

teolytic degradation, is only seen in the gene-expression profile of the Calcofluor white-treated cells.

The functional categories of genes that are, in the majority, downregulated, are (a) the group of genes involved in ribosomal biogenesis, which are mostly ribosomal protein (RP) genes, and (b) the group of genes involved in rRNA transcription and processing. The highly significant Z-scores (**Table 2**) of the functional group of ribosomal biogenesis reveals the strength of this kind of analysis, as genes repressed based on cut-offs only revealed downregulation of three RP genes (data not shown). Downregulation of RP genes is often associated with reduction of growth. Although the mild treatments of both Calcofluor white and Zymolyase did not affect growth in these experiments (data not shown), it cannot be ruled out that the downregulation of ribosomal gene expression is predictive of later growth inhibition. Interestingly, Li *et al.* [140] showed that regulation of RP genes is part of the cell wall integrity pathway, dependent on *PKC1* but not dependent on *SLT2*. Finally, the functional group of genes involved in nucleotide metabolism was downregulated in the Zymolyase experiment, and possible also in the CFW experiment, whereas a subcategory, the group of genes involved in purine ribonucleotide metabolism, was downregulated in the CFW experiment.

Table 2. Quantology analysis of expression profiles corresponding to cell wall perturbation and to two constitutive activators of the cell integrity pathway

	Z-scores			
Functional category (MIPS)	**CFW**	**Zymolyase**	**Pck1-R398A**	**Rho1-Q68H**
Stress response	**7.69** (161)	**7.52** (161)	**7.97** (164)	**7.31** (162)
C-compound and carbohydrate utilization	**7.37** (247)	**5.90** (247)	2.68 (248)	1.10 (247)
Cell wall	**7.34** (36)	**5.30** (36)	**4.97** (36)	3.13 (36)
Osmosensing	**6.84** (16)	**6.95** (16)	**6.25** (16)	**4.81** (16)
Budding, cell polarity and filament formation	**5.76** (163)	**4.08** (163)	2.86 (164)	−0.07 (163)
Proteolytic degradation	**4.77** (156)	1.63 (156)	1.05 (157)	2.79 (157)
Cytoskeleton	**4.27** (100)	2.44 (100)	0.96 (101)	0.04 (100)
Ribosome biogenesis	**−18.22** (194)	**−11.69** (194)	**−17.42** (197)	**−14.23** (197)
rRNA transcription	**−7.08** (99)	**−7.20** (99)	−2.60 (101)	−3.80 (101)
rRNA processing	**−7.01** (60)	**−6.68** (66)	−2.19 (60)	−3.62 (60)
Purine ribonucleotide metabolism	**−4.39** (43)	−3.73 (43)	−1.15 (43)	−0.76 (43)
Nucleotide metabolism	−3.88 (147)	**−4.02** (147)	−1.29 (141)	−1.88 (140)
Additional categories only scoring significantly in the PKC1 or RHO1 mutant				
Unclassified proteins	2.07 (1855)	3.17 (1855)	**4.82** (1847)	**6.77** (1848)
DNA recombination and repair	1.31 (143)	1.36 (143)	**4.02** (139)	1.72 (137)
Mitochondrion	2.89 (314)	1.57 (314)	**−8.60** (317)	**−7.91** (316)

Significant Z-scores (above 4 or below −4) are shown in bold. Between brackets the numbers of ORFs from a category are shown. Categories are derived from the MIPS functional catalogs: all categories (241 in total) were tested, but only the categories with a significant Z-score are shown. CFW, Calcofluor white; Zymolyase, a cell wall degrading enzyme mixture.

Detection of promoter elements by correlation with gene expression

In an attempt to identify regulatory elements that affected gene expression in our experiments, we used the program REDUCE, which correlates the expression ratios obtained by global transcript analysis with the presence of sequence motifs in the promoter regions of all genes [88]. REDUCE is very powerful in discovering putative regulatory elements. In addition, we used a database of previously identified motifs as input in REDUCE. The results of the REDUCE analysis are shown in **Table 3.**

Table 3. REDUCE analysis of expression profiles corresponding to cell wall perturbation and to two constitutive activators of the cell integrity pathway

		Z-scores			
Consensus	**Motif**	**CFW**	**Zymolyase**	**Pkc1-R398A**	**Rho1-Q68H**
TA(W)$_4$TAG	Rlm1	**10.31** (668)	**6.60** (668)	**9.99** (671)	**9.61** (669)
CTA(W)$_4$TAG	Rlm1	**9.13** (103)	**7.06** (103)	**8.68** (103)	**7.36** (104)
AGGGGG	STRE	**8.72** (901)	**8.37** (901)	2.86 (901)	**7.78** (893)
CCCCT	STRE	**6.34** (947)	**6.45** (947)	**5.94** (949)	**8.00** (951)
CCC(N)$_{10}$GGC	Unknown	3.87 (136)	**5.30** (136)	3.03 (136)	1.95 (136)
ATGACGT	ATF/CRE	2.71 (79)	**5.76** (79)	1.96 (78)	1.63 (78)
TATGACG	ATF/CREP	3.27 (80)	**4.06** (80)	2.84 (81)	2.21 (81)
AAAATTT	nRRPE	**−11.19** (1311)	**−11.24** (1311)	**−4.10** (1316)	**−8.31** (1312)
CGATGAG	PAC	**−8.68** (243)	**−8.13** (241)	−0.40 (242)	**−4.21** (242)
CCATACA	Rap1	**−7.95** (259)	**−5.07** (259)	**−5.38** (260)	**−4.02** (259)
Additional categories only scoring significantly in the *RHO1* mutant					
TTCTAGAA	HSE	−0.51 (197)	1.03 (197)	3.91 (195)	**4.97** (194)
AGCCWC	Crz1	2.21 (548)	2.55 (548)	0.88 (549)	**4.54** (546)

REDUCE analysis was performed with 600 base pair upstream sequences. The numbers of ORFs in each category are shown between brackets. Significant Z-scores (above 4 or below −4) are shown in bold. IUPAC symbols: W = A or T, R = G or A. CFW, Calcofluor white; Zymolyase, a cell wall-degrading enzyme mixture. Genes sharing particular motifs can be found at **http://staff.science.uva.nl/~boorsma/cellwallstress/**.

Regulatory motifs that were positively correlated with expression in both CFW and Zymolyase gene expression profiles could be separated into four different groups:

1. The Rlm1p regulatory binding site, TA(W)4TAG or CTA(W)4TAG. The latter motif was initially identified as the Rlm1p regulatory binding site [48], but later it was shown that a truncated version of this motif, TA(W4)TAG, was also capable of regulating genes involved in cell wall function.

2. The STRE element, AGGGG or its reverse complement CCCCT. The STRE elements are known to be bound by the transcription factors Msn2p and Msn4p, and thought to be involved in the general stress response [53].

3. The putative binding site CCC(N)10GGC. This motif was absent in the motif database SCPD (http://cgsigma.cshl.org/jian/) and could thus represent a new regulatory sequence. We used the program FUNSPEC (Robinson, [2002]; http://funspec.med.utoronto.ca/) to test whether the genes sharing this motif are enriched for listed functional categories. When we used a p-value cut-off <0.01 and applied the Bonferroni correction, no enrichment of the listed categories was observed.

4. Interestingly, REDUCE analysis of the Zymolyase expression profile revealed the putative regulatory sequence, ATGACGT. This motif is identified in the motif database SCPD as the ATF/CREB site, which is regulated by the Sko1p transcription factor. Interestingly, with REDUCE a similar motif (TATGACG), which might also reflect the ATF/CRE site, was found in the gene expression profiles obtained after sorbitol treatment [40]. The latter motif is also significantly detected in the Zymolyase gene expression profile. Sko1p acts downstream of the high

osmolarity MAP kinase pathway [53]. This might point to the involvement of the high osmolarity MAP kinase pathway upon cell wall perturbation, although the MAP kinase Hog1p is not phosphorylated under these conditions (data not shown).

Negatively correlated motifs could be separated into two groups:

1. The PAC motif, CGATGAG, and the mRRPE motif, AAAATTT, play a role in the regulation of rRNA transcription genes [141].

2. The Rap1p regulatory motif, CCRTACA. Most of the genes with these motifs are involved in ribosomal biogenesis.

Comparison with the transcription profiles of constitutively active mutants of *PKC1* and *RHO1*

As both REDUCE and Quontology allow analysis of single expression profiles, we compared gene expression profiles of constitutively active regulators of the cell wall integrity pathway to the CFW and Zymolyase profiles. Roberts *et al*. [134] used global transcript analysis to study the biological responses associated with the activation and perturbation of signal transduction pathways. Overexpression of two constitutively active mutants of the cell integrity pathway were used in this study, viz. Pkc1-R398A [142] and Rho1-Q68H [143]. Tables 2 and 3 show the Quontology and REDUCE results, respectively, of these mutants.

These results show remarkable similarities between the transcription profiles of the two mutants and the profiles of the Calcofluor white and Zymolyase treatments. Quontology analysis reveals general upregulation of the genes from the functional groups of stress response, cell wall function (although less significant in the Rho1-Q68H mutant) and osmosensing, and general downregulation of the genes from the functional group of ribosomal biogenesis (Table 2). In addition, the expression of genes with an Rlm1 motif, the STRE elements, the mRRPE and PAC element (although not significant in the Pkc1-R398A profile) and the Rap1p motif show a behaviour similar to that in the CFW and Zymolyase profiles (**Table 3**).

Differences are seen in the genes from the group of unclassified proteins, which are generally upregulated, and the genes from the group of mitochondrial genes, which are generally downregulated in both the Pkc1-R398A and Rho1-Q68H expression profiles. The group of DNA recombination and DNA repair is only upregulated in the Pkc1-R398A expression profile (**Table 2**).

Finally, REDUCE analysis revealed two regulatory motifs in the Rho1-Q68H profile (Table 3); first, the motif sequence, TCTAGAA, which represents the heat-shock element, and second, the motif AGCCWC, which is the regulatory site for the calcineurin-dependent transcription factor Crz1p. The heat shock motif is also found in the Pkc1-R398A profile, although just below the border of significance.

Improved prediction of the Rlm1p regulatory sequence

Table 3 reveals that the Rlm1p regulatory element, in terms of Z-scores, shows on average the strongest response in all four profiles analysed. The regulatory element CTA(W)4TAG, is described by Dodou and Treisman [50] as the consensus sequence for the Rlm1p transcription factor. Later, Jung and Levin [48] and Terashima *et al*. [144] found that the binding site TA(W)4TAG was enough for regulation by Rlm1p. We improved the Rlm1p regulatory site using REDUCE. For this we tested a series of variations of the Rlm1p motif and used the Z-scores as a measure of their biological effectiveness. **Table 4** shows the results of this analysis.

Table 4. Improved prediction of the Rlm1 binding sequences based on REDUCE

Rlm1 binding sequence	Z-score (ORFs) + ranking			
	CFW	Zymolyase	Pkc1-R398A	Rho1-Q68H
CTA(W)$_4$TAG	9.13 (103) **5**	7.06 (103) **3**	8.68 (103) **6**	7.36 (104) **5**
TA(W)$_4$TAG	10.31 (668) **4**	6.60 (668) **6**	9.99 (671) **4**	9.6 (669) **4**
TA(W)$_4$TAGM	**12.03** (369) **2**	**8.49** (369) **2**	**11.75** (372) **1**	**10.45** (372) **1**
CTA(W)$_4$TAGM	8.93 (58) **6**	6.65 (58) **5**	9.44 (58) **5**	7.33 (59) **6**
HTA(W)$_4$TAGM	12.17 (286) **1**	8.53 (286) **1**	11.19 (288) **2**	9.62 (288) **2**
HTA(W)$_4$TAG	10.84 (536) **3**	6.88 (536) **4**	10.04 (538) **3**	9.79 (537) **3**

Z scores above 4 are considered to be significant. The numbers of ORFs in each category are shown between brackets. The ranking of the Z score is shown in bold. IUPAC symbols: H = A or C or T, W = A or T, M = A or C. The sequence with on average best Z score is presented in bold.

Except for the Zymolyase experiment, the Z-scores of the regulatory motif CTA(W)4TAG can be significant improved by removal of the first C, which is in agreement with the finding of Jung and Levin [48] and Terashima *et al*. [144]. This motif, TA(W)4TAG, can in turn be improved by addition of a M (A or C) at the 3 end of the motif sequence in all experiments. Addition of an H (A, C or T) at the 5 end of the sequence motif resulted in only a slight improvement of the Z-scores of the CFW and Zymolyase experiments. Therefore the sequence motif TA(W)4TAGM gives the best overall improvement of the Z-score in all experiments. In **Table 1**, the 14 ORFs regulated by Rlm1p, as identified by Jung and Levin [48], are marked with an arrow. From these 14 ORFs, seven contain the original CTA(W)4TAG motif in the 600 bp upstream region. The motif TA(W)4TAG is shared by 12 ORFs, whereas the motif TA(W)4TAGM is shared by 11 ORFs. Importantly, the group of genes sharing the motif TA(W)4TAGM is about 45% smaller than the group of genes with the motif TA(W)4TAG, and is thus considerably enriched with known Rlm1-dependent genes. We propose that this motif can be used in combination with REDUCE on-line [106] (http://bussemaker.bio.columbia.edu/reduce/) to analyse other expression data as a sensitive indicator of cell wall stress.

Stimulation of stress-activated signal-transduction pathways invariably leads to changes in the transcriptional pattern of the cell and results in the activation of genes involved in stress protection [40, 109]. Interestingly, a stress response can be generally divided into a stress-specific response and a more general response, designated the environmental stress response (ESR), which includes upregulation of STRE-driven genes and downregulation of genes that are involved in rRNA transcription [40, 41]. We have investigated the transcriptional response of yeast cells exposed to low levels of cell wall stress that did not significantly affect growth. Both Calcofluor white, which binds to chitin and interferes with normal cell wall construction

[145], and Zymolyase, a cell wall-degrading enzyme preparation, were used for this purpose. Although the treatments were mild and growth was not significantly affected, both treatments resulted in increased resistance of intact cells to ß1,3 glucanase digestion.

Discussion

Despite their different modes of action, the transcriptional reprogramming that occurred in response to Calcofluor white and Zymolyase was remarkably similar. Analysis of the transcriptional data with Quontology and REDUCE [88] basically revealed the following:

1. Upregulation of genes involved in cell wall functioning. This is seen both with Quontology analysis, which shows significant upregulation of genes from the functional group of cell wall functioning and with REDUCE analysis, which shows upregulation of genes sharing a Rlm1 promoter site in their upstream sequences. This most likely reflects the response upon cell wall stress in order to counteract the weakened cell wall.

2. The ESR, as revealed by upregulation of STRE-driven genes, which are involved in stress protection, and downregulation of genes with a PAC or a mRRPE element, which are involved in rRNA transcription and rRNA processing.

3. Downregulation of genes involved in ribosomal biogenesis either by sharing a RAP1 element or downregulation of the genes of the functional category of ribosomal biogenesis.

4. Upregulation of osmosensing genes and genes involved in budding, cell polarity and filament formation.

5. Upregulation of genes sharing the motif CCC(N) 10GGC in their upstream sequences. When genes sharing this motif were tested by the program FUNSPEC [79], no enrichment of genes in functional groups was observed. This could be explained in two ways: either the motif is a false positive, or the motif represents an authentic regulatory element, but it is shared by genes from various functional groups. Possibly, the current definitions of functional groups are not sufficiently specific.

Differences in transcriptional response of the CFW and Zymolyase treatments are seen in the upregulation of genes involved in proteolytic degradation and the cytoskeleton, which are significantly detected in the CFW expression profile only. Interestingly, REDUCE results reveal a putative regulatory motif (ATGACGT) in the Zymolyase transcription profile that is highly similar to a regulatory sequence (TATGACG), which has been found with REDUCE (data not shown) in osmotic stress-induced transcript profiles [40]. Both motifs resemble the ATF/CREB binding site, which is repressed by the Sko1p transcription factor. Sko1p is a downstream regulator of the high-osmolarity pathway (see below for further discussion).

We compared the expression profiles of cells in which either Pkc1-R398A or Rho1-Q68H were expressed, two constitutive activators of the cell wall integrity pathway to those of the cell wall perturbation experiments. Similar responses were seen in the upregulation of genes involved in cell wall functioning, general stress response (ESR), and ribosomal biogenesis. Interestingly, the osmosensing genes - a group of 16 genes as defined by MIPS [146] - are also upregulated (Table 2). This result, combined with a similar observation in the CFW and Zymolyase

treatments, points to the involvement of the high-osmolarity pathway in case of cell wall stress. Two observations support the possible involvement of the high-osmolarity pathway:

1. Upregulation of the osmosensing genes. Especially in the expression profile of the Pkc1-R398A mutant, almost all components of the Hog1p MAP kinase pathway, including HOG1 itself, are highly upregulated.

2. Identification of the putative Sko1p binding sequence, ATGACGT, which closely resembles the motif TATGACG, which is found in osmotic stress-induced transcript profiles.

Regulation of the high-osmolarity MAP kinase pathway in parallel to the activation of the cell wall integrity pathway might seem counterintuitive. Both signal-transduction pathways allow yeast cells to adapt to changes in the osmolarity of their surrounding medium by regulating the activity of the MAP kinase Hog1 and Slt2, respectively [147]. Hog1 is activated in response to an increase in osmolarity (hyperosmotic stress), which results in increased glycerol synthesis, the osmolyte in yeast, and minimizes loss of water and shrinking of the cells. Although Hog1-dependent induction of STRE-controlled gene expression is partially independent of Msn2/Msn4 [36], most Msn2/Msn4-dependent genes also depend on Hog1 for osmoregulated expression [148]. In contrast to hyperosmotic shock, a sudden decrease in osmolarity (hypo-osmotic shock) leads to an influx of water into the cells and results in glycerol being released from the cells [149]. Recently the function of four protein phosphatases has been elucidated, the protein-tyrosine phosphatases, Ptp2 and Ptp3, and the dual specificity phosphatases Msg5 and Sdp1 [150]. Tyrosine phospatase, PTP2, in particular has an interesting role. Ptp2p has affinity for both Slt2p and Hog1p [151] and it can prevent hyperphosphorylation of Hog1p. Ptp2p has been shown to be, at least partially, under control of the Rlm1p transcription factor, but not of Msn2p/Msn4p [48, 150]. Interestingly, in all four expression profiles the PTP2 transcript is upregulated. These results suggest a tight control of parallel induction of both the cell wall integrity pathway and the HOG pathway. Possibly, Ptp2 could counteract small fluctuations in osmolarity in this way.

Analysis of the Rho1-Q68H mutant revealed two additional motifs, which positively correlate with gene expression: first, the heat shock element (HSE); and second, the regulatory element AGCCWC, which resembles the binding site of Crz1p, a transcription factor controlled by the calcineurin pathway. This element was also found with high significance under conditions of calcium stress [46]. Interestingly, in a recent study of the expression profiles of five cell wall-defective mutants, Lagorce *et al.* [120] proposed, in addition to induction of Rlm1-regulated genes, the involvement of the Crz1 transcription factor. In a cluster of 80 genes, which are induced in all five mutants, they show overrepresentation of the Crz1 bindingsite (AGCCTC). This motif was not detected by REDUCE in the cell wall-perturbant profiles. We analysed two expression profiles, obtained with two strains deleted for *GAS1* and *FKS1*, respectively, two genes with crucial cell wall functions from the Rosetta compendium dataset [59]. In both expression profiles, Crz1 regulatory sites were identified in addition to the Rlm1 binding site (data not shown), confirming the findings by Lagorce and co-workers. The absence of these motifs in the expression

profiles of CFW and Zymolyase could be caused by dose effects. Both cell wall perturbation treatments were mild and cell growth was not affected. Another explanation could be time dependency of the response: the chosen time point (2 h in this study) might be too early or too late to detect this response. Of course, it cannot be excluded that an additional pathway, in addition to the Slt2-Pkc1 pathway, is activated. The Crz1 binding sequence was only found significantly in the transcription profile of the Rho1-Q68H mutant and not in the Pkc1-R398A mutant profile. This might suggest activation of the calcineurin pathway via Rho1p. Finally, we used REDUCE to improve the Rlm1 regulatory binding site. The improved motif TA(W)4TAGM, can be used in combination with REDUCE as a rapid and sensitive indicator for cell wall stress, independent of the type of microarray. When we tested this motif using the microarray data of the five cell wall mutants studied by Lagorce *et al*. [120], indeed in all cases use of the improved motif resulted in a significant Z-score. Furthermore, our motif consistently resulted in higher Z-scores than the original motifs (data not shown). The motif is present in the upstream region of 369 ORFs as detected in the Zymolyase and Calcofluor white gene expression profiles. On the other hand, Jung and Levin [48], could only find about 20 Rlm1p-dependent genes. Possible explanations for this apparent discrepancy are: (a) some DNA regions may not be accessible to Rlm1p due to higher order DNA organization; (b) competing transcription factors may exist; (c) the context around the sequence motif might affect the binding of Rlm1p; and (d) the distance of the motif to the transcriptional initiation site probably also affects transcriptional control by Rlm1p.

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