

Genome-wide Analysis of the Response to Cell Wall Mutations in the Yeast *Saccharomyces cerevisiae**

Received for publication, November 14, 2002, and in revised form, January 30, 2003
Published, JBC Papers in Press, March 18, 2003, DOI 10.1074/jbc.M211604200

Arnaud Lagorce‡§, Nicole C. Hauser¶, Delphine Labourdette‡¶, Cristina Rodriguez**, Helene Martin-Yken‡, Javier Arroyo**, Jörg D. Hoheisel¶, and Jean François‡¶‡

From the ‡Centre de Bioingenierie Gilbert Durand, UMR-CNRS 5504 and INRA 792, ¶Transcriptome-Biochips platform, Genopole de Toulouse, 135 Avenue de Rangueil, 31077 Toulouse, France, the ¶Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, 69120 Heidelberg, Germany, and the **Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain

Perturbations of the yeast cell wall trigger a repair mechanism that reconfigures its molecular structure to preserve cell integrity. To investigate this mechanism, we compared the global gene expression in five mutant strains, each bearing a mutation (i.e. *fks1*, *kre6*, *mnn9*, *gas1*, and *knr4* mutants) that affects in a different manner the cell wall construction. Altogether, 300 responsive genes were kept based on high stringency criteria during data processing. Functional classification of these differentially expressed genes showed a substantial subset of induced genes involved in cell wall construction and an enrichment of metabolic, energy generation, and cell defense categories, whereas families of genes belonging to transcription, protein synthesis, and cellular growth were underrepresented. Clustering methods isolated a single group of ~80 up-regulated genes that could be considered as the stereotypical transcriptional response of the cell wall compensatory mechanism. The *in silico* analysis of the DNA upstream region of these co-regulated genes revealed pairwise combinations of DNA-binding sites for transcriptional factors implicated in stress and heat shock responses (*Msn2/4p* and *Hsf1p*) with *Rlm1p* and *Swi4p*, two *PKC1*-regulated transcription factors involved in the activation genes related to cell wall biogenesis and G_1/S transition. Moreover, this computational analysis also uncovered the 6-bp 5'-AGCCTC-3' CDRE (calcineurin-dependent response element) motif in 40% of the co-regulated genes. This motif was recently shown to be the DNA binding site for *Crz1p*, the major effector of calcineurin-regulated gene expression in yeast. Taken altogether, the data presented here lead to the conclusion that the cell wall compensatory mechanism, as triggered by cell wall mutations, integrates three major regulatory systems: namely the *PKC1-SLT2* mitogen-activated protein kinase-signaling module, the "global stress" response mediated by *Msn2/4p*, and the Ca^{2+} /calcineurin-dependent pathway. The relative importance of these regulatory systems in the cell wall compensatory mechanism is discussed.

Yeast and fungi are surrounded by a cell wall that is a complex structure essential for maintenance of the cell shape, prevention of lysis, and protection against harmful environmental conditions. The yeast cell wall architecture has been determined in detail over the past decade (1). It is a layered structure that is composed of β -1,3- and β -1,6-glucan (50–60% of the cell wall dry mass), mannoproteins (40–50%), and chitin (2%). β -1,3-Glucan and chitin form a fibrillar network to which mannoproteins are anchored, mostly through β -1,6-glucan. Some cell wall proteins, like the PIR family, can be directly linked to β -1,3-glucan (reviewed in Ref. 2). The cell wall is not a rigid structure, since it endures all of the changes that the cell undergoes during division, morphogenesis, and differentiation.

To ensure continuous integrity of the wall in accordance with its plasticity, complex mechanisms must be operating, which need to be strictly coordinated with those governing cell growth. One of these mechanisms, termed the "cell wall compensatory mechanism" (2, 3), can be triggered by several means, including cell wall-weakening mutations (4, 5), cell wall perturbation with chemical drugs (6), and temperature and osmotic shock (7, 8). As reviewed by Klis and collaborators (9), three main responses to cell wall damage have been identified. First, the balance between cell wall polysaccharides is modified, as indicated by hyperaccumulation of chitin. Second, the type of association between β -glucan, mannoproteins, and chitin is changed. For instance, lowering the amount of β -1,6-glucan leads to a larger fraction of the cell wall proteins becoming linked directly to β -1,3-glucan and chitin, concomitant with an increased level of Pir proteins (10). A third response that ensures strengthening of the cell wall is a transient redistribution of cell wall synthesis and the repairing machinery that is normally focused to active growth regions all over the cell.

A major transduction pathway that is essential in the maintenance of the cell wall integrity pathway is the *PKC1-SLT2* MAPK-signaling module (11). This pathway consists of a MAPK¹ kinase kinase, *Bck1p/Slk1p*, a pair of redundant MAPK kinases, *Mkk1/2p*, and a MAPK, *Slt2p/Mpk1p* (11). Activation of this pathway is mediated through a family of plasma membrane-localized sensors, *Wsc1-4p* and *Mid2p* (3, 7, 12), and this results in the activation of the *Slt2p* by dual phosphorylation of its two conserved threonine and tyrosine residues (13). The activated *Slt2p* MAPK activates in turn downstream transcription factors and consequently promotes gene expression. Among the well characterized *Slt2p* targets are *SBF* and *Rlm1p* transcription factors, which control the

* This work was supported in part by European Union Grant QLK3-2000-01537 (to J. F., J. A., and J. H.) and by Fonds de Recherche Hochst Marion Roussel Grant FRHMR 2/9922 (to J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a predoctoral fellowship from the French Ministry of Research and Technology Education.

‡‡ To whom correspondence should be addressed. Tel.: 33-5-61-55-9492; Fax: 33-5-61-55-9400; E-mail: fran_jm@insa-tlse.fr.

¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; DMF, dimethylformamide; ESR, environmental stress response; MIPS, Munich Information Center for Protein Sequences.

by Hauser *et al.* (33), and verified for purity and concentration using an Agilent 2100 Bioanalyser (Agilent Technologies). The complex sample was generated by first strand cDNA synthesis (34). Some 25 μg of total RNA were mixed with 0.5 μg of oligo(dT)₁₅ (Invitrogen) in diethylpyrocarbonate-treated water. The sample was heated to 70 °C for 10 min and subsequently cooled to 43 °C. Reverse transcription was performed in a total volume of 30 μl using SuperScript™II (Invitrogen) in the presence of a 0.25 mM concentration each of dATP, dGTP, and dTTP, 1.66 μM dCTP, and 30 μCi of [^{33}P]dCTP (Amersham Biosciences). After 30 min at 42 °C, another 1 μl of SuperScript™II was added, and the reaction mixture was incubated at 42 °C for 30 min. This step was followed by an alkaline hydrolysis of the RNA in the presence of 1 μl of 1% SDS, 1 μl of 0.5 M EDTA, and 3 μl of 3 M NaOH for 30 min at 60 °C and subsequently incubated for 15 min at room temperature. The solution was then neutralized with 10 μl of 1 M Tris-HCl, pH 8.0, and 3 μl of 2 M HCl. After the addition of 5 μl of 3 M sodium acetate, pH 5.3, 5 μl of tRNA (10 $\mu\text{g}/\mu\text{l}$), and 60 μl of isopropyl alcohol, the cDNA was precipitated at -20 °C for 30 min, pelleted by centrifugation, and resuspended in 100 μl of water. The incorporation of ^{33}P into the cDNA was checked by scintillation counting. For consistent results, incorporation must be above 70% of the initial radioactivity. The arrays were prehybridized for at least 2 h in 20 ml of 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA at 65 °C. cDNA samples were denatured for 5 min at 100 °C and added directly to the solution. The hybridization was carried out for 20 h at 65 °C. Subsequently, the DNA arrays were briefly rinsed in a solution containing 40 mM sodium phosphate, pH 7.2, and 0.1% SDS, followed by two 20-min washes in the same buffer at 65 °C. The arrays were exposed to phosphor screens for 2–3 days. Signal detection was performed by a Storm 860 phosphorimager (Amersham Biosciences). Experiments with each mutant strain and its isogenic wild type were repeated four times.

Northern Blot Analysis—Total RNA was separated in formaldehyde-agarose gels (25 $\mu\text{g}/\text{lane}$) as described by Siderius *et al.* (35) and transferred to Nylon-N⁺ filters (Amersham Biosciences). Probes were obtained by a PCR amplification of complete open reading frames on genomic DNA with the corresponding commercial oligonucleotide primers (purchased from Eurogentec, Belgium). Hybridization was carried out in 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA at 65 °C overnight. The filters were first washed once in 0.1% SDS, 0.01 \times SSC at room temperature and then washed twice for 10 min in 0.1% SDS, 0.01 \times SSC at room temperature. Signal quantification was performed with the phosphorimager, using ImageQuant software (Amersham Biosciences), and values were reported relative to *PDA1* transcription levels used as an internal standard.

Detection of Dually Phosphorylated Slt2p—Yeast cells were grown overnight in YEPD medium at 24 °C to midexponential phase. The cultures were then diluted in the same medium to A₅₉₅ of 0.3, grown for 3 h at 24 °C, and then processed identically as described in Ref. 13. Detection of the dually phosphorylated Slt2p was made with anti-phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴) antibody (New England Biolabs) at a 1:2000 dilution. The amount of Slt2 protein on the same membrane was measured with anti-Gst-Slt2p antibody (36) at a 1:1000 dilution.

Data Acquisition and Evaluation—Image analysis and quantification of the array hybridizations were done using A.I.S. 5.0 software (Imaging Research Inc.), which applies a predefined grid on the array of signals and performs spot quantification in a 16-bit gray scale format. Data from the quantification are then exported and uploaded in M-CHIPS, a microarray data warehouse and analysis tool package (Ref. 37; available on the World Wide Web at www.dkfz-heidelberg.de/tbi/services/mchips/). Signal intensities of repeated hybridizations were normalized, and significance levels were assessed by two stringency criteria (38). The highly stringent “min-max separation” is calculated by measuring the minimum distance between all data points of two strains. The less stringent criteria, called “standard deviation separation,” is defined as the difference of the means of the two data sets diminished by one S.D. value. A color code was applied to each value, indicating whether the variation was significant by either of these two different criteria. The whole set of data is available on the World Wide Web at www.dkfz.de/funct_genome/yeast-data.html.

Computational Treatment of Gene Expression Profiling—Transcript profiles of genes whose expressions were changed significantly by a factor of ≥ 2 were grouped by a two-dimensional hierarchical clustering. Gene similarity metric and the hierarchical clustering algorithm based on average linkage were used according to Eisen *et al.* (39). A modified version of Sherlock software was used for cluster visualization and distance calculation (40). To identify regulatory motifs in DNA noncoding sequence of a gene, a 700-bp promoter sequence upstream of the start codon of any gene of interest was retrieved from the SGD data

base (41). Promoter analysis was carried out using MatInspector (42), DNA-Pattern, and Oligo-analysis software (43). The MatInspector software interrogates the TRANSFAC data base containing a large library of predefined matrix descriptions for protein-binding sites (44). This program assigns a quality rating to matches and allows quality-based filtering and selection of matches. DNA-Pattern searches for all occurrences of a pattern within DNA sequences; the pattern can be entered as a simple nucleotide sequence or may include degenerate nucleotide codes. To overcome the limitation of these two programs that only identify previously characterized DNA motifs, the Oligo-analysis software developed by Van Helden *et al.* (43) was used. It allows the identification of overrepresented short motifs (short oligomer sequences). It calculates total occurrence counts and frequencies for each oligonucleotide in the input set, and it reports those that emerge more frequently than would be expected on a random basis. A visualization of promoter analysis using these software tools can be found on the World Wide Web at bio71.gba.insa-tlse.fr/jmflab/.

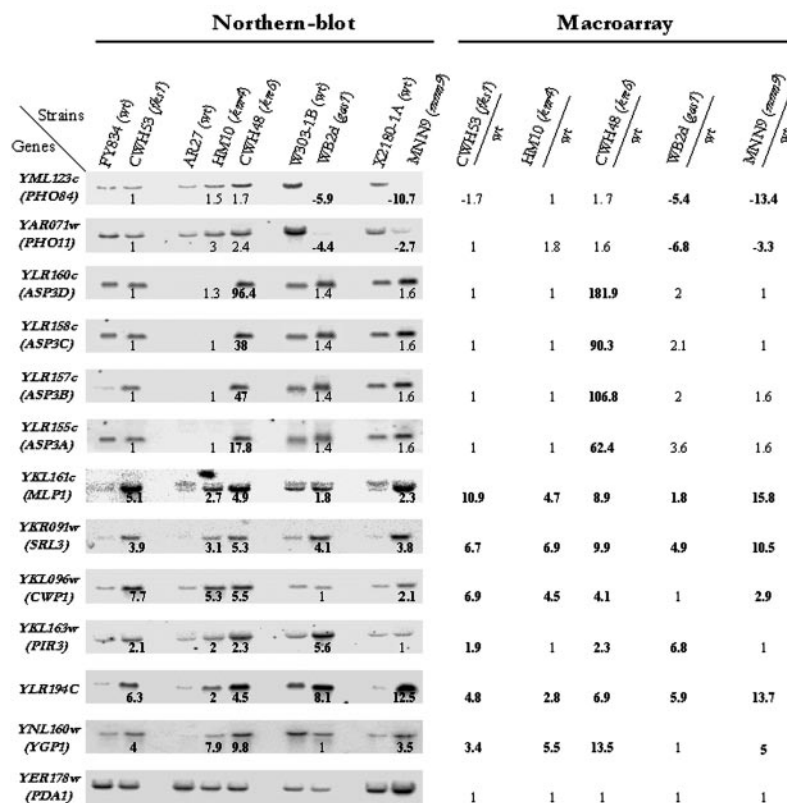
RESULTS

Global Expression Changes in Cell Wall Mutants Using Polypropylene High Density Filters—To get a comprehensive view of the effect of cell wall damage on yeast physiology, we examined on a genome-wide scale changes in gene expression induced by mutations that affect different aspects of the cell wall construction. To this end, we chose mutations in the following genes: *FKS1*, which encodes the major β -1,3-glucan synthase catalytic subunit (45); *KRE6*, which encodes a protein required in the synthesis of β -1,6-glucan (46, 47); *MNN9*, whose defect leads to a truncation of the mannan structure on cell wall proteins (48); *GAS1*, which encodes a GPI-anchored cell surface protein with β -1,3-glucanoyl transferase activity (18, 49, 50); and *KNR4*, whose product connects the *PKC1-SLT2* MAPK pathway with cell proliferation (51).² The DNA macroarrays, which bear 6116 yeast open reading frames (see, on the World Wide Web, www.dkfz.de/funct_genome/YEAST-filters/YGA_genelist.html for a full description) were originally developed within the EUROFAN project (33). These arrays, made on polypropylene membranes, have major advantages over those made on nylon membranes, since their performance with respect to signal intensity, signal-to-noise ratio, and spot sharpness was superior by a factor of 2-fold (data not shown). Moreover, DNA arrays on polypropylene could be reused more than 10 times with an extremely low residual background and with no significant loss of signal intensity between reuses. Thus, we routinely carried out four independent hybridizations for each mutant strain. Since all yeast open reading frames are present in duplicate on the arrays, this yields eight data points per gene. After spot quantification and normalization, the data sets for each gene were analyzed using statistical procedures developed earlier (38). This methodology is straightforward, since changes in expression of genes that were even lower than 2-fold could be statistically confident, and meets the criteria that were recently laid down for international standardization and quality control of microarray experiments at the M.I.A.M.E. convention (53).

According to this analysis, cell wall mutations resulted overall in transcriptional changes of about 300 genes (*i.e.* 5% of the yeast genome). A complete list of numerical and graphical images of the raw data can be found online at www.dkfz.de/funct_genome/yeast-data.html. In summary, 206 genes were up-regulated, and among them 75% showed a significant up-regulation of 1.5–3-fold. 24% were 4–10-fold activated, and 1% increased their transcript level by more than 10-fold, with a maximum of 90-fold for *YLR160c/ASP3D* encoding a nitrogen catabolite-regulated cell wall asparaginase. The remaining 84

² H. Martin-Yken, H. Martin-Brevia, A. Dagkessamanskaia, F. Basmaji, A. Lagorce, M. Molina, and J. Francois, submitted for publication.

FIG. 1. Comparison of gene expression by DNA arrays and Northern blots hybridization. Northern blots (from a larger set, 13 transcripts are shown) were carried out with RNA isolated from cell wall mutants (*fks1*, *knr4*, *kre6*, *gas1*, and *mnn9*) and their isogenic wild type. For Northern blot analysis, the expression levels indicated under the bands are the -fold change of the transcript between mutants and their isogenic wild types. *PDA1* mRNA was used as loading control to normalize the transcript levels before calculating the ratio. *PDA1* encodes a subunit of the pyruvate dehydrogenase complex.



genes were down-regulated genes, with more than 95% of them showing a 1.3–4-fold transcriptional reduction. In addition to this overall response, a small number of genes (27 of 290) had their expression pattern specifically altered in response to a single mutation, with the *gas1* mutation showing the strongest pattern specificity (see supplemental material on the World Wide Web at bio71.gba.insa-tlse.fr/yeastgen/cellwall.php3). The 300 differentially expressed genes identified in our analysis are thus far above the 22 genes identified in an earlier genomic study carried out with a *fks1Δ* mutant (54). This difference can be explained by the fact that we used five mutants instead of one, by a higher quality of the DNA arrays, and by the repeatability of the experiments, which provides highly reliable and accurate genome-wide data.

To confirm the reliability of these transcriptional changes, we examined by Northern blot hybridizations the expression levels of some selected genes that exhibited small and large transcriptional changes on polypropylene DNA arrays. As illustrated in Fig. 1, the transcript abundances detected by Northern analyses correlated correctly with the median values from the normalized DNA array data. Moreover, these transcriptional changes were also in agreement with previous expression data on these mutants (54, 55). In quantitative terms, the expression changes with the DNA arrays method resulted in values that were higher by a factor of 1.3–2 than those from the Northern blots. More interestingly, this experiment showed striking differences in the expression of selected genes between the different “wild type” strains. This is particularly the case for the *ASP* gene family, whose expression appeared to be strongly activated upon *kre6* mutation, while being extremely weak in the isogenic *AR27* strain. In contrast, the expression of this gene family was barely activated by mutation of *MNN9* and *FKS1*, probably due to the fair expression of this gene family in the corresponding isogenic strains (Fig. 1).

Functional Classification of Expressed Transcripts in Response to Cell Wall Mutations—As a first step for data inter-

pretation, the differentially expressed genes were grouped into the 13 functional categories according to the MIPS nomenclature (56). Our procedure was to express the number of up- and down-regulated transcripts in each of the categories as a percentage of the total number of differentially expressed genes from each cell wall mutant and to compare this functional classification with the functional catalogues of genes described in MIPS. This procedure emphasized the major gene expression remodeling on a genomic scale (Fig. 2). Clearly, cell wall defects bring about a significant enrichment in transcripts of genes encoding products involved in central metabolism, generation of energy, cell rescue, and, to a lesser extent, ionic homeostasis and cellular communication. In contrast, genes encoding products involved in protein synthesis, transcription, and cell division were significantly underrepresented, which is consistent with the fact that cell proliferation is impaired by cell wall mutations (9). The up-regulation of the machinery involved in energy and metabolic activities was actually not unexpected, because the production of cell wall components mobilizes about 20% of the carbon flow in the cell, and hence, cell wall perturbations may further enhance carbon and energy mobilization to efficiently combat cell wall weakening.

As reported in other genome-wide studies (20, 21, 54, 57), we found that most of the induced genes that belong to the carbon and energy metabolism family according to MIPS criteria do not have obvious role under “standard laboratory conditions.” This is the case for *GLK1* encoding a glucokinase, which is typically up-regulated in response to a large variety of stresses and whose deletion does not produce any phenotype (20, 58). Another interesting example is the transcriptional activation of *PFK26* and *FBP26* encoding enzymes that are respectively responsible for the synthesis and the degradation of fructose 2,6-bisphosphate. This metabolite is an important biofactor in promoting glycolysis and inhibiting gluconeogenesis in mammals (59), whereas such a role in yeast is less apparent (60). It was recently shown that hypotonic stress inactivates Pfk26p by

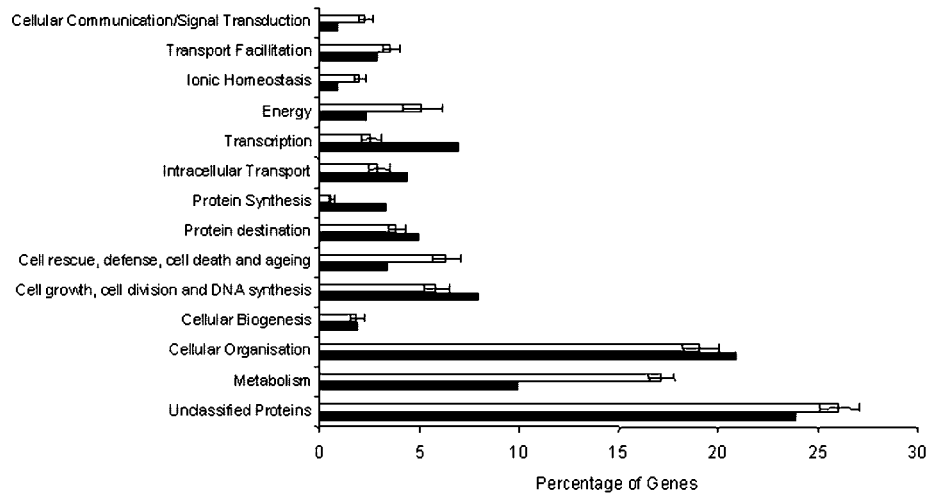


FIG. 2. Distribution of the differentially expressed genes in the five mutants according to the functional categories. Solid histograms represent the functional catalogue of 6200 genes according to the MIPS classification (56). The open histograms represent the distribution of the 300 differentially expressed genes from cell wall mutants in the functional categories of MIPS. The percentage of each category was calculated as the number of up- and down-regulated genes from each of the 13 categories divided by the total number of differentially expressed genes in the cell wall mutants. The bars indicate the S.D. between the five cell wall mutants.

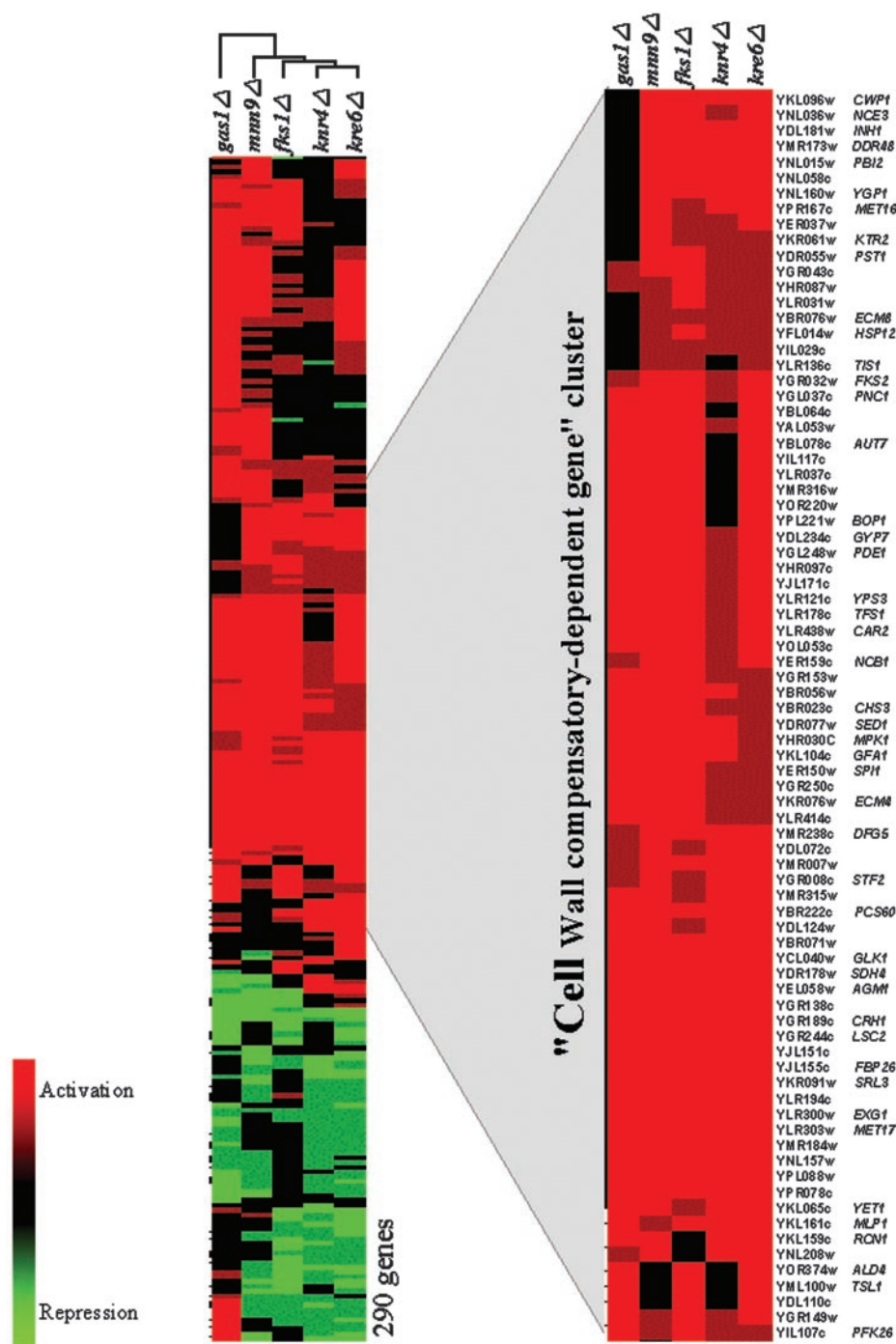
Pkc1p-dependent phosphorylation (61). It was then proposed that this inactivation could lead to a redirection of glucose 6-phosphate to cell wall β -glucan synthesis. Consistent with this suggestion, we recently found that cell wall mutants contained higher glucose 6-phosphate and glycogen levels than their isogenic wild types (55). An increase of *FBP26* gene expression is probably also in accordance with a reduction in glycolysis, since this effect should further lower fructose 2,6-bisphosphate concentration and hence reduce phosphofructo-1-kinase activity. Whereas the glycolytic flux is reduced, the activity of the tricarboxylic acid cycle appears to be enhanced in response to cell wall defects, as suggested by the up-regulation of *ALD4*, *CIT1*, *IDH2*, *SDH4*, and *LSC2*, which encode enzymes of the tricarboxylic acid cycle. The increased carbon flux through this pathway is probably due to a decreased glucose repression effect resulting from a reduction of the glycolytic flux (62). This enhancement of the tricarboxylic acid cycle would also result in an increased ATP production, as indicated by the activation of *COX5B* (which encodes a cytochrome *c* oxidase component (63)), *AAC1* (which codes for an ATP/ADP carrier protein (64)), *YER053c* (encoding a homologue of the mitochondrial phosphate protein carrier (65)), *INH1* (encoding an inhibitor of the ATP hydrolysis by mitochondrial ATP synthase (66)), and *SFT2* (encoding a mitochondrial ATP synthase stabilizer (66)). This possible increase in energy generation would be consistent with up-regulation of genes encoding proteins with protective function and chaperones, which consume ATP to maintain structural integrity of proteins and eliminate undesired proteins in response to cell perturbations (67).

Although not evidently highlighted by our functional classification analysis, ~10% of the differentially expressed genes (33 of 290 genes) are directly involved in the construction and remodeling of the cell wall (see Table II). As indicated in Table II, this set of genes included those involved in chitin synthesis (*GFA1*, *AGM1*, and *CHS3*). Up-regulation of these genes agrees with the recent demonstration that the hyperaccumulation of chitin in these mutants was due to an increase in the flux of the chitin pathway (55). This group also contains genes implicated in β -glucan and mannan synthesis, like *FKS2*, which encodes the minor β -1,3-glucan synthase catalytic subunit (6, 29, 68); *KRE6* and *KRE11*, which are involved in the synthesis of β -1,6-

TABLE II
List of genes implicated in cell wall biogenesis, and whose expression is increased in response to cell wall mutations

ORF/Genes	Cell Wall mutations	Average Δ /wt ratio	Description of the gene product
YBR023c/ <i>CHS3</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.3	Chitin synthase 3
YBR076w/ <i>ECM8</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	2.7	Involved in cell wall biogenesis and structure
YDR055w/ <i>PST1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	4.5	Putative GPI-dependent cell wall protein
YDR077w/ <i>SED1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	4.5	Abundant cell wall glycoprotein, null mutant exhibits increased sensitivity to zymolyase
YEL058w/ <i>AGM1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.2	Phosphoacetylglucosamine mutase, chitin biosynthesis pathway
YER150w/ <i>SPI1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.7	Cell wall protein, stationary phase induced
YGR032w/ <i>FKS2</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	5.1	beta 1-3 glucan biosynthesis
YGR166w/ <i>KRE11</i>	$\Delta fks1, \Delta kre6, \Delta gas1, \Delta mmn9$	2.4	beta 1-6 glucan biosynthesis
YGR189c/ <i>CRH1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	3.8	Cell wall organisation
YGR282c/ <i>BGL2</i>	$\Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.2	Cell wall endo-beta-1-3-glucanase
YHR030c/ <i>MPK1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.6	Ser/Thr kinase (cell wall integrity pathway)
YHR136c/ <i>SPL2</i>	$\Delta knr4, \Delta kre6$	4.8	Cell wall protein
YJL165c/ <i>HAL5</i>	$\Delta gas1$	3.5	Ser/ Thr protein kinase, involved in cell wall biogenesis and architecture
YJR106w/ <i>ECM27</i>	$\Delta gas1, \Delta mmn9$	5.0	cell wall biogenesis and architecture
YKL096w/ <i>CWP1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	4.6	Cell wall mannoprotein
YKL104c/ <i>GFA1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.9	Glucosamine-fructose-6-phosphate-transferase, involved in chitin biosynthesis
YKL161c/ <i>MLP1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	8.4	Putative ser/ thr kinase with similarity with Mpk1p
YKL163w/ <i>PIR3</i>	$\Delta fks1, \Delta kre6, \Delta gas1$	3.7	Member of PIR family protein, cell wall structural protein
YKR061w/ <i>KTR2</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	4.3	Mannosyltransferase
YKR076w/ <i>ECM4</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.5	cell wall biogenesis and architecture
YLR121c/ <i>YPS3</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	5.8	GPI-anchored aspartic protease 3
YLR155c/ <i>ASP3A</i>	$\Delta kre6, \Delta gas1, \Delta mmn9$	22.5	Nitrogen catabolite-regulated cell wall L-asparaginase II
YLR157c/ <i>ASP3B</i>	$\Delta kre6, \Delta gas1, \Delta mmn9$	34.8	Nitrogen catabolite-regulated cell wall L-asparaginaseII
YLR158c/ <i>ASP3C</i>	$\Delta kre6, \Delta gas1$	46.2	Nitrogen catabolite-regulated cell wall L-asparaginaseII
YLR160c/ <i>ASP3D</i>	$\Delta kre6, \Delta gas1$	92.0	Nitrogen catabolite-regulated cell wall L-asparaginaseII
YLR194c	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	6.8	Unknown function and unknown phenotype
YLR300w/ <i>EXG1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	1.9	Exo beta 1-3-glucanase
YLR342w/ <i>FKS1</i>	$\Delta gas1, \Delta mmn9$	2.0	Beta 1-3 -D-glucan synthase
YMR238w/ <i>DFG5</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta gas1, \Delta mmn9$	2.8	Protein involved cell polarity, cellular elongation and possibly in processing of GPI-dependent cell wall proteins
YMR305c/ <i>SCW10</i>	$\Delta kre6, \Delta gas1, \Delta mmn9$	3.3	Member of the glucanase gene family, soluble cell wall protein
YNL058c	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	2.0	Similarity to YIL117c
YNL160w/ <i>YGP1</i>	$\Delta fks1, \Delta knr4, \Delta kre6, \Delta mmn9$	6.8	Cell wall glycoprotein synthesized in response to nutrient limitation
YPR159w/ <i>KRE6</i>	$\Delta gas1, \Delta mmn9$	2.4	synthesis of beta 1-6 glucan

FIG. 3. Hierarchical clustering of differentially expressed genes in the five cell wall mutants versus the isogenic wild type. The columns give the cell wall mutation (*gas1*, *mnn9*, *fls1*, *knr4*, and *kre6*). The red and green squares represent genes whose expression was statistically at least 2-fold higher or lower, respectively, than in the wild type strains, and the black squares indicate no significant change in expression. On the right is shown the cluster of genes that are up-regulated in all of the cell wall mutations, which were used for promoter analysis.



glucan (46, 47); and *KTR2*, encoding a mannosyltransferase (69), as well as genes (*ECM4*, *ECM8*, *ECM27*, *YPS3*, and *DFG5* (70–72)) whose role in the cell wall construction has been inferred from genetic studies. Interestingly enough, we also identify several genes, which code for proteins that have a structural role in the architecture of the cell wall. This is the case for *PIR3*, *PST1*, *SED1*, *YGP1*, and *SPI1*, also known to be induced under poor nutritional conditions (54, 73–75). Another very important subset of up-regulated genes are those encoding putative β -glucanase activity (*BGL2*, *CRH1*, and *SCW10* (76, 77)) and β -1,3-glucanase (*EXG1* (78)). This finding is in accordance with biochemical data showing that cell wall damage provokes an increase in the cross-linking between cell wall polymers and in particular between chitin and β -1,6-glucan (reviewed in Ref. 2).

Hierarchical Clustering Identifies a Stereotypical Transcription Response to Cell Wall Damage—To identify potential regulatory pathways that mediate the cell wall compensatory mechanism, we made use of a two-dimensional hierarchical clustering algorithm that organizes co-regulated transcripts on a vertical axis and cell wall mutants with similar expression profile on a horizontal axis (39). This analysis gave rise to two major features (Fig. 3). First, the two-dimensional clustering method isolated on the vertical axis one single patch of red (gene induction) containing a set of 79 genes that were induced in the five cell wall mutants, whereas no contiguous patch of green color was obtained for down-regulated genes (Fig. 3). Among the 79 up-regulated genes, we retrieved few genes (*i.e.* those marked in black) that did not display reproducible changes in one mutant and hence were excluded by our statis-

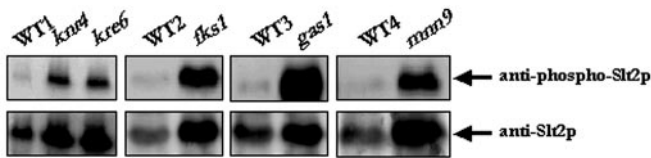


FIG. 4. Activation of PKC1-SLT2 pathway in the cell wall mutants was determined by the level of dually phosphorylated form of Slt2p. WT1, AR27; WT2, FY834; WT3, W303-1B; WT4, X2180-1. Yeast cells grown overnight in YEPD at 24 °C were diluted in the same medium to A_{595} of 0.3, grown again for 3 h at 24 °C before cells were collected for preparation of protein extracts. Top panel, immunoblot assays of cell extract with anti-phospho-p44/42 MAPK antibodies. Bottom panel, anti-Slt2 immunoblot assay of the same sample with anti-GST-Slt2 antibodies.

tical criteria, whereas they showed very reproducible activation in the four other mutants. This clustering analysis led to the notion that the cell wall compensatory mechanism is based on a gene activation process, and the term “cell wall compensatory-dependent gene cluster” was coined for this group of up-regulated genes (Fig. 3). Note that, due to the gene similarity metric calculation used in the hierarchical clustering algorithm (39), a few sets of genes whose expression also changed in all mutants were spuriously dispersed all over the clustering representation. This was the case for *BGL2*, encoding a β -glucosyl transferase (77), and *AFR1*, encoding a protein involved in morphogenesis (79), as well as *YBR230c*, *YHR138c*, *YMR191w*, and *YOR289w*, which encode proteins with still undefined function. The second feature is related to the dendrograms on the horizontal axis, which depicts the relationships between cell wall mutations. Since the branch lengths give the degree of similarity between mutants based on their expression patterns, this indicates that *kre6* and *knr4* mutants show the closest relationship, which is compatible with their weakest cell wall defect phenotype, whereas *gas1* mutant showed the greater distance from the other mutants, consistent with the fact that it shows the more pronounced cell wall defect phenotype (18, 49, 50). Same conclusions could be drawn using the correspondence analysis clustering developed elsewhere (see Ref. 80 and supplemental data on the World Wide Web at www.dkfz.de/funct_genome/yeast-data.html).

Detailed analysis of the “cell wall compensatory-dependent gene cluster” (Fig. 3) showed that, besides a large proportion (30%) of genes with unknown function, the two major fractions of induced genes are genes encoding enzymes of the energy and metabolic pathways (19 of 79) and genes implicated in cell wall construction and remodeling (16 of 79; see Table II). Therefore, there seems to be a stereotypical response to cell wall damage that leads to the transcriptional activation of a set of genes that could support cell wall reorganization necessary for cell survival. Moreover, a relevant feature of this response was the 2.5–4-fold increase in the transcripts of the *SLT2/MPK1* gene encoding the MAPK of the PKC1-dependent signaling pathway (11). Previous works have shown that the activation of the Slt2p-mediated signaling pathway in response to cell wall damage is manifested by increased levels of the dually phosphorylated (Thr¹⁹⁰ and Tyr¹⁹²) form of Slt2p (13, 36). In agreement with these data, we showed in Fig. 4 that the phosphorylated form of Slt2p was increased in all five mutants. Moreover, immunoblot analysis using an anti-Slt2p antibody (36) also indicated an increase in the total amount of Slt2 protein (Fig. 4). Therefore, it seems that activation of the PKC1-SLT2 pathway in these cell wall-defective mutants included not only the activation of Slt2p by phosphorylation but also an increase in the amount of this protein as a consequence of the transcriptional induction of the corresponding gene. Finally, two other putative reporters of the PKC1 pathway, namely *MLP1* encod-

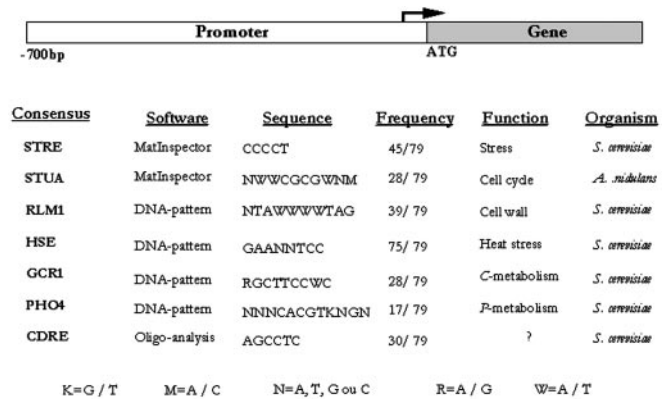


FIG. 5. Identification of DNA regulatory motifs in the promoter region of the 79 co-regulated genes. Analysis was carried out with 700 base pairs upstream of the ATG of each gene using MatInspector, DNA-pattern and Oligo-analysis softwares. For the sake of clarity, we have used capital letters to assign the DNA binding motif of the corresponding transcription factors.

ing a putative serine/threonine kinase with high similarity to Slt2p and *YLR194c* encoding a hypothetical protein, were found to be strongly activated in the cell wall mutants. The potent transcriptional activation of *MLP1* and *YLR194c* supports the previous suggestion (81) that these two genes play a role in cell wall biogenesis.

Promoter Analysis of Co-regulated Genes Identifies Three Major Signaling Pathways Implicated in the Cell Wall Compensatory Mechanism—Promoter analysis of co-regulated genes should identify common motifs that may give some clues to the regulatory systems implicated in the cell wall repair mechanism. To this end, we searched for DNA motifs in the 700-bp noncoding upstream sequence of the 79 co-regulated genes using MatInspector (42), DNA-pattern (43), and Oligo-analysis (43). These algorithms have the common ability to localize regulatory motifs that emerge more frequently in the whole genome than would be expected on a random basis. Using the first two programs, which can identify DNA sequence that matches annotated DNA motifs reported in data bases (44), we found consensus sequences corresponding to binding sites of the transcription factors encoded by *RLM1*, *MSN2/MSN4*, *HSF1*, *GCR1*, and *PHO4* (Fig. 5). The identification of the *RLM1* binding site actually validated our computational analysis, since this gene encodes a transcriptional factor, which is a substrate of the Slt2p-mediated cell integrity pathway that activates several cell wall-related genes (16, 17). The finding of the other consensus sequences was also consistent with the functional classification reported in Fig. 2. *GCR1* and *PHO4* encode transcription factors that control expression of genes involved in glycolysis and phosphate metabolism (82, 83). *MSN2/4* and *HSF1* code for transcription factors that bind to STRE and HSE motifs, respectively (84, 85), and activate genes encoding protein chaperones and implicated in metabolic stress response (23, 67). Less obvious was the identification of a consensus sequence that is recognized by the fungus *Aspergillus nidulans* transcription factor encoded by the *stuA* gene. The closer *Saccharomyces cerevisiae* homologues of *stuA* are *SOK2* or *PHD1*, which code for proteins that share 82% identity within a 104-amino acid region (86). Sok2p, Phd1p, and the product of *stuA* are members of a protein family that harbors a conserved APSES (ASM-1, Phd1p, Stuap, EFGTF-1, and Sok2p) domain (86). These proteins are thought to be implicated in the control of cell development and filamentous growth (87, 88). Alternatively, this consensus could be the binding site of Swi4p, since it matches almost perfectly the CGCGAAA consensus that was recently redefined for this transcription

TABLE III
Representation of nucleotides in the CDRE present in 30 of 79 co-regulated genes in the cluster

W, A/T; M, A/C; Y, C/T; H, A/C/T. The letter probability of the CDRE binding motif was derived with the pattern-finding MEME program (90).

A	11	10	31	0	0	0	0	7	10
C	5	11	0	0	31	31	0	31	9
G	5	6	0	31	0	0	0	5	5
T	10	4	0	0	0	0	31	0	10
Consensus	W	M	A	G	C	C	T	C	Y

factor (89). Using the Oligo-analysis algorithm, we discovered a 6-bp 5'-AGCCTC-3' motif in 30 of the 79 genes of the cluster. Remarkably, this motif turned out to be identical to the recently identified core consensus DNA binding site of Crz1p, a transcription factor that appears to be the major effector in Ca²⁺/calcineurin-regulated gene expression (22). In this study (22), the motif termed CDRE (for calcineurin-dependent response element) was identified by electrophoretic mobility shift and methylation interference methods. As indicated in Table III, the letter matrix probability of the DNA-binding motif derived by the MEME algorithm (90) suggested that any base substitution would result in the loss of the binding of the Crz1p factor. This *in silico* analysis was consistent with the finding of Yashimoto *et al.* (22), who showed that the AGCC core of the CDRE motif was indispensable for the binding of Crz1p.

The identification of seven distinct regulatory motifs in the group of similarly expressed genes suggests a coordinated action of multiple transcription factors for gene activation. Therefore, we were prompted to quantify the frequency of genes containing in their promoter a combination of two, three, or more DNA motifs. As illustrated in Table IV, 75% of the genes that possess STRE in their promoter also harbor the HSE motif. In addition, pairwise motif combinations of RLM1 with either STRE or HSE motifs are found in more than 50% of the genes in the cluster, and 14 of 79 co-regulated genes have both the STUA and the RLM1 binding sites. This suggested that the Slt2p-mediated cell wall integrity pathway and the regulatory systems mediated by Msn2p/4p and Hsf1p transcription factors are working together in the regulation of the cell wall compensatory mechanism. There was also a significant proportion of genes in the cluster containing CDRE with RLM1, STRE, or HSE motifs in their promoters, which may give a preliminary clue that this CDRE motif has a regulatory function in the cell wall repair mechanism. Finally, a significant number of genes (between 15 and 20%) exhibit a triple combination of HSE-STRE-RLM1, STRE-HSE-CDRE, or GCR1-HSE-RLM1, which suggests cross-talks between these different regulatory systems to allow rapid and effective adaptation to cell wall damage.

To get some additional evidence on the relationship between the different regulatory systems controlling the cell wall compensatory mechanism, we examined the effects of combined mutations in genes encoding these transcription factors on the sensitivity to cell wall interfering drugs. Whereas deletion of *MSN2*, *MSN4*, *PHO4*, or *SOK2* alone did not reveal major cell wall defects, the loss of *RLM1* function led to marked phenotypes (*i.e.* resistance to 25 µg/ml Calcofluor White and 0.1 mg/ml Congo red and hypersensitivity to 0.012% SDS or to 4 mM caffeine) (data not shown). Simultaneous deletion of *MSN2* and *MSN4* made this strain more resistant to SDS than the isogenic wild-type strain (data not shown). However, combining *rlm1* with *msn2/msn4* mutations led to mutant cells that display the *rlm1Δ* phenotype on Calcofluor White and that of *msn2/msn4* mutations on SDS (data not shown), suggesting

TABLE IV
Combinatorial analysis of promoter elements

The numbers in the table expressed the frequency of combination between two or three motifs within 700 bases of the translation start site of the 79 co-activated genes. For the sake of clarity, we have used capital letters to assign DNA-binding motifs (RLM1, STRE, STUA, HSE) that are bound by the corresponding transcription factors (Rlm1p, Msn2/4p, Stua1p, and Hsf1p).

Motifs	RLM1	GCR1	PHO4	STUA	STRE	HSE
CDRE	16	10	5	13	18	24
RLM1		12	10	14	18	28
GCR1			7	8	20	23
PHO4				6	8	15
STUA					13	31
STRE						34
HSE						

Motifs	CDRE/GCR1	CDRE/PHO4	CDRE/STUA	CDRE/STRE	CDRE/HSE
RLM1	5	3	7	8	10
GCR1		1	3	8	7
PHO4			3	3	2
STUA				4	9
STRE					12

Motifs	RLM1/PHO4	RLM1/STUA	RLM1/STRE	RLM1/HSE
GCR1	3	1	7	6
PHO4		4	5	1
STUA			4	8
STRE				9

Motifs	GCR1/STUA	GCR1/STRE	GCR1/HSE
PHO4	0	2	2
STUA		4	7
STRE			13

Motifs	PHO4/STRE	PHO4/HSE
STUA	3	2
STRE		3

Motifs	STUA/HSE
STRE	11

that the two regulatory systems contribute to the cell wall repair by two separate routes.

Activation of Genes Involved in the Cell Wall Compensatory Mechanism in Other Experimental Conditions—The data reported above suggest that cell wall mutations trigger at least three major transcriptional responses (*i.e.* the *PKC1-SLT2* MAPK signaling cascade, the global stress system mediated by Msn2p/Msn4p and Hsf1p transcription factors, and the Ca²⁺/calcineurin-mediated pathway). For this reason, we were interested in comparing our data on the transcriptional changes induced by cell wall mutations with transcriptomic data reported on gene expression in response to several stresses (20, 21), activation of the MAPK cascade, or the presence of calcium (17, 22, 81). As shown in Table V, 48 genes induced by overexpression of a *PKC1* allele under the *GAL4* promoter (81) were also found in our set of 79 co-regulated genes, and the extent of gene induction in both conditions was roughly similar. In addition, inspection of the promoter regions of these genes induced by the *PKC1-SLT2* signaling pathway revealed that only 50% of them harbor an RLM1 consensus sequence in their promoter. This observation reinforces the notion that other transcriptional factors are implicated in gene activation by this signaling pathway (29). We also found that among the 79 genes up-regulated by cell wall damage, 50 of them were also induced in the heat shock experiments described by Gash *et al.* (20). However, the most striking result came from the comparison with genes activated in response to dithiothreitol, since 64 of the 79 genes in our cluster were also activated in the presence of this chemical (20). This comparison is particularly relevant, since Gasch and co-workers (20) postulate that dithiothreitol treatment causes cell wall defects, possibly by altering cell wall

TABLE V

List of genes in the cell wall compensatory cluster: Identification of DNA motifs in the promoter region and presence of genes from this cluster in previously published genome-wide analysis

ORF name	Gene	DNA motifs in the noncoding upstream region ^a (-700 bp from ATG)		Induction ^b							
		Other motifs	CDRE motif	Our study	Calcium (22) ^f	GAL- <i>PKC1</i> (81)	Dithiothreitol (20)	Heat shock (21)	<i>ftsI</i> mutation (54)	<i>MKK1</i> ^{S386P} (17) ^d	
YAL053w		STRE*3/PHO4*2/RLM1*2/GCR1/	CDRE	++	+++					++	
YBL064c		STRE/STUA/HSE*2	++			+++	+++				
YBL078c	<i>AUT7</i>	STRE/GCR1/RLM1	CDRE	++		++			++		
YBR023c	<i>CHS3</i>	STUA/RLM1/HSE*3		++		++				+	Induced
YBR056w		STRE/HSE	++			+++	++				
YBR071w		STUA*2/RLM1/HSE	CDRE	++	+++	+++				+	
YBR076w	<i>ECM8</i>	STUA/HSE/GCR1		++							
YBR222c	<i>PCS60</i>	STUA/RLM1/HSE*2	CDRE	++		++					
YCL040w	<i>GLK1</i>	STRE*2/STUA/PHO4*2/RLM1	CDRE	++			+++	+++			
YDL072c		STRE/STUA*3/HSE	CDRE	++		++	+	+			
YDL110c		STRE*2/GCR1*2/RLM1/HSE*2		++			++	+++			
YDL124w		STRE*2/STUA/GCR1/HSE*2	CDRE*2	++		++	+++	+++			
YDL181w	<i>INH1</i>	STRE/GCR1*2/HSE*2	CDRE	++			+++	+++			
YDL234c	<i>GYP7</i>	STRE*2/GCR1/HSE/RLM1	CDRE	++	+++		+				
YDR055w	<i>PST1</i>	PHO4*2/RLM1*2/HSE		++		+++	+++	++		++	Induced
YDR077w	<i>SED1</i>	RLM1*3/HSE		++		+++	+++	++			Induced
YDR178w	<i>SDH4</i>	STRE/HSE*2		++			++	++			
YEL058w	<i>AGM1</i>	STRE*2/RLM1*3/HSE	CDRE	++		++					
YER037w		STRE*3/HSE		++			++	++			
YER150w	<i>SPI1</i>	STRE/RLM1/HSE*4	CDRE	++		++	+++	+++			
YER159c	<i>NCB1</i>	STRE/GCR1/HSE*5		++		++					
YFL014w	<i>HSP12</i>	STRE*4/RLM1/HSE		++		+++	+++	+++			
YGL037c	<i>PNC1</i>	STRE*4/RLM1	CDRE	++		++	+++	+++			
YGL248w	<i>PDE1</i>	STRE/RLM1/GCR1/HSE		++		+++	++				
YGR008c	<i>STF2</i>	STRE/HSE*2		++			+++	+++			
YGR032w	<i>FKS2</i>	STUA/PHO4/HSE/RLM1	CDRE	+++		++	+++	+		++	Induced
YGR043c		STRE/HSE*2		+++		+++	+++	+++			
YGR138c		GCR1/RLM1/HSE		++			+				
YGR149w		STRE/RLM1/HSE		++			+	++			
YGR153w		STUA*2/HSE	CDRE	++		++					
YGR189c	<i>CRH1</i>	STUA*3/GCR1/RLM1*2/HSE*4	CDRE	++	+++	+++	++			++	Induced
YGR244c	<i>LSC2</i>	STRE/RLM1/HSE		++			++	++			
YGR250c		HSE*2	CDRE	++			+	+++			
YHR030c	<i>MPK1</i>	HSE		++		+++	++	+		++	Induced
YHR087w		STRE*3/STUA/RLM1/HSE		++		+++	+++	+++			
YHR097c		STRE/GCR1/HSE	CDRE	++	+++	++	+++	+++			
YIL029c		RLM1/GCR1/HSE		++							
YIL107c	<i>PFK26</i>	STUA/STRE/PHO4/RLM1/HSE		++		++	++	++			
YIL117c		PHO4/HSE2/RLM1*2		++		+++	+++	++			Induced
YJL151c		STRE*2/PHO4/RLM1/HSE		+			+				
YJL155c	<i>FBP26</i>	STRE/GCR1/HSE		++		++	++	+			
YJL171c		STUA*2/RLM1/HSE	CDRE*2	++	+++	++	+				
YKL065c	<i>YET1</i>	STRE*2/HSE		++		++	+	+			
YKL096w	<i>CWP1</i>	HSE		++		+++		+++		++	Induced
YKL104c	<i>GFA1</i>	GCR1/RLM1/HSE		++		+++	+			+	
YKL159c	<i>RCN1</i>	STUA/PHO4/HSE*2		++	+++		+				
YKL161c	<i>MLP1</i>	STUA/GCR1/HSE*2		+++		+++	++	+			Induced
YKR061w	<i>KTR2</i>	RLM1/HSE2	CDRE	++		+++	++			+	
YKR076w	<i>ECM4</i>	HSE	CDRE	++			+++	++			
YKR091w	<i>SRL3</i>	STRE/STUA/RLM1*2/HSE		+++		+++	+++	+++			
YLR031w		HSE*4		++		++					
YLR037c		RLM1/HSE*2		++		++	+				
YLR121c	<i>YPS3</i>	STRE*2/STUA/HSE	CDRE	+++		+++	+++			++	
YLR136c	<i>TIS1</i>	STRE/PHO4/HSE	CDRE	++	+++		++	+			
YLR178c	<i>TSF1</i>	STRE/STUA/HSE*2		+++			+++	+++			
YLR194c		STUA/RLM1*2/HSE2	CDRE	+++	+++	+++	+++	+		++	Induced
YLR300w	<i>EXG1</i>	STUA/PHO4/RLM1		+		+++					
YLR303w	<i>MET17</i>	GCR1/PHO4/HSE		++	+++	++	+++	+			
YLR414c		GCR1/STUA/HSE	CDRE	++	+++	+++	++				
YLR438w	<i>CAR2</i>	STRE/HSE	CDRE	++				+++			
YML100w	<i>TSL1</i>	STRE*6/STUA/HSEGCR1		++			+++	+++			
YMR007w		STUA/RLM1/HSE		++	+++		+++				
YMR173w	<i>DDR48</i>	STRE*2/PHO4/HSE3		++			+++	++			
YMR184w		STRE		++		++	++				
YMR238w	<i>DFG5</i>	RLM1*3/HSE3		++	+++	+++	++				Induced
YMR315w		STRE*2/GCR1/RLM1*2/HSE	CDRE	++		++	+++	++			
YMR316w		GCR1/HSE		++		+++	+++	+			
YNL015w	<i>PB12</i>	STRE/STUA/PHO4/HSE2		++			+++	+++			
YNL036w	<i>NCE3</i>	PHO4*3/GCR1/HSE		++		++	+++	+++			
YNL058c		RLM1/HSE3	CDRE	++		++					Induced
YNL157w		STRE*2/STUA/GCR1/HSE2		+							
YNL160w	<i>YGP1</i>	STRE/HSE3	CDRE	+++			+++	+++			Induced
YNL208w		STRE/PHO4/HSEGCR1/RLM1		++	+++	++	+++	+			
YOL053c		STRE*4/HSE		+++							
YOR220w		STRE*2/GCR1/HSE	CDRE*2	++	+++	++	+++	++			
YOR374w	<i>ALD4</i>	STUA/STRE*2/HSEGCR1		++			+	+++			
YPL088w		PHO4/RLM1/GCR1/HSE		+++		+++	+++	+			
YPL221w	<i>BOP1</i>	STRE/HSE		++	+++	+++	+			+	
YPR078c		STUA/RLM1/HSE		++			+++	+			
YPR167c	<i>MET16</i>	PHO4/GCR1/HSE2		++	+++	++	+				

^a Asterisks indicate more than one copy in the 700-bp DNA noncoding sequence of the gene.

^b +, <2-fold induction; ++, 2-5-fold induction; +++, >5-fold induction.

^c List of genes from Ref.22 that were up-regulated after 30-min incubation in response to 0.2 M CaCl₂.

^d No value was given in this paper (17), except that expression of these genes was at least 2-fold increased.

TABLE VI
Functional analysis of the CDRE motif in the *FKS2* promoter

The CDRE motif in the promoter of the *FKS2-lacZ* construct was modified by PCR-mutagenesis or introduced into six copies, as detailed under “Experimental Procedures” to yield *FKS2(Δcdre)-lacZ* and *FKS2(CDRE)₆lacZ* constructs, respectively. Transformed cells were cultivated in SD medium supplemented with auxotrophic requirements to A_{600} of 0.5–1.0. To half of the culture, CaCl_2 (0.2 M final concentration from a 2 M stock solution) or Calcofluor White (CFW; 50 $\mu\text{g/ml}$ final concentration) was added, and cultures were sampled after 30 min (for CaCl_2 -treated cells) or 4 h (for Calcofluor White-treated cells) to measure β -galactosidase activity. The activity of β -galactosidase is expressed as nmol of *o*-nitrophenyl- β -D-galactoside hydrolyzed per min and per mg of protein, and values reported are the mean values from three independent experiments, with an S.E. of 10%. ND, not determined.

Strain	Genotype	Treatment		β -Galactosidase activity		
		CFW	CaCl_2	<i>FKS2-lacZ</i>	<i>FKS2 (Δcdre)-lacZ</i>	<i>FKS2 (CDRE)₆lacZ</i>
		$\mu\text{g/ml}$	mM			
BY4741	Wild type	0	0	1.60	1.00	4.10
BY4741	Wild type	0	200	2.20	1.60	35.60
BY4741	Wild type	50	0	56.0	ND	ND
BYcrz1	<i>crz1Δ</i>	0	0	1.70	0.90	1.22
BYcrz1	<i>crz1Δ</i>	50	0	11.6	5.11	13.5
BYcrz1	<i>crz1Δ</i>	0	200	2.10	0.90	0.90
W303-1B	Wild type	0	0	4.4	3.1	19.0
W303-1B	Wild type	50	0	175	45	1100
W303-1B	Wild type	0	200	3.5	1.8	59.6
WB2d	<i>gas1Δ</i>	0	0	305	75	3297
JF1409	<i>rlm1Δ</i>	0	0	0.9	0.8	2.1
JF1409	<i>rlm1Δ</i>	50	0	1.7	1.4	5.6
JF1409	<i>rlm1Δ</i>	0	200	1.1	1.2	2.4

disulfide linkages. These cell wall defects in turn trigger induction of STRE-responsive genes by the so-called “environmental stress response” (ESR) program. Finally, we searched for genes in the cell wall compensatory cluster, which possess a CDRE motif and were also found by Yoshimoto *et al.* (22) to be activated by high calcium concentration. Surprisingly, the overlap between the two analyses was relatively low, since only 12 of the 31 genes in our cluster were also found in the Ca^{2+} /calcineurin cluster described in Ref. 22. This low correlation may be due to the fact that the genes in our cluster have other *cis*-acting elements that are responsible for their transcriptional activation in response to cell wall damage. As an example, *FKS2* expression was strongly increased in response to cell wall mutations but was barely affected upon calcium addition (22).

Exploring the Function of the CDRE Motif in the Cell Wall Compensatory Mechanism—The CDRE motif (AGCCTC sequence) was recently found to activate gene expression in response to Ca^{2+} /calcineurin-dependent signaling (22). To investigate the function of the CDRE motif in response to cell wall damage, we made use of reporter constructs carrying the *FKS2* promoter, which contains one copy of this motif 491 bp upstream of the ATG codon, fused to *lacZ*. Strains carrying reporter constructs bearing inactive or multicopy CDRE motifs in the promoter of *FKS2* were also generated, and levels of β -galactosidase in response to cell wall damage were evaluated. As shown in Table VI, the *FKS2-lacZ* gene fusion showed no significant increase in β -galactosidase activity after 30-min incubation with 200 mM calcium. This is consistent with the data of Yoshimoto *et al.* (22), who also reported a weak activation of *FKS2* upon calcium treatment. Interestingly, introduction of six copies of CDRE in the *FKS2-LacZ* construct resulted in a 10-fold rise in the expression of this gene fusion in response to 200 mM calcium, and this activation was completely lost in a *crz1Δ* strain lacking the transcription factor that binds CDRE. The addition of 50 $\mu\text{g/ml}$ Calcofluor White to wild type cells or loss of *GAS1* function, respectively, resulted in a 40–100-fold activation of the *FKS2-lacZ* construct. This activation induced by cell wall damage was even stronger when the promoter of the *FKS2-lacZ* gene fusion contained six CDRE copies. On the other hand, the activation by Calcofluor White or in response to *GAS1* deletion was five times lower when the CDRE was replaced by an unrelated AGATCT sequence or when the Calco-

fluor White experiment was carried out in a *crz1Δ* strain. These data are therefore in favor of an involvement of the calcium signaling in the cell wall compensatory response. To evaluate further the relative importance of this signaling *versus* the *PKC1* pathway mediated by Rlm1p, we measured the effect of calcium on the expression of *FKS2* in a *rlm1Δ* strain. As shown in Table VI, the loss of *RLM1* function led to a residual 2-fold increase in the levels of β -galactosidase from the different *FKS2-lacZ* constructs upon incubation with 50 $\mu\text{g/ml}$ Calcofluor White. More interestingly, the effect of high calcium concentration to activate the *FKS2 (CDRE)₆ lacZ* gene fusion in wild type cells was almost completely abolished in *rlm1Δ* cells. These results illustrate that Ca^{2+} /calcineurin and *PKC1-SLT2* signaling pathways collaborate to induce *FKS2* expression in response to cell wall damage through Crz1p and Rlm1p transcription factors, respectively.

DISCUSSION

Cell wall weakening can be induced by different means, including cell wall-perturbing drugs, harsh environmental conditions, or mutations in cell wall-related genes (reviewed in Ref. 91). These stresses induce a compensatory mechanism that results in a reshaping of the cell wall architecture in order to combat cell lysis (2, 3). To obtain a comprehensive view of this mechanism, we examined the expression profiles of mutants affected in different processes of the cell wall construction, namely the biosynthesis of β -1,3- and β -1,6-glucan, the mannosylation of wall proteins, the cross-linking between cell wall components, and the coordination between cell wall synthesis and cell growth. Overall, a set of 300 genes that displayed transcriptional changes between >1.5 and >50-fold was selected based on solid statistical data analysis. Functional classification analysis of these differentially expressed genes indicates that cell wall mutations lead to an enrichment of gene categories involved in metabolism, energy generation and protective functions as chaperones, and to a lower representation of genes encoding transcription factors and products involved in protein synthesis and cell growth. This reshaping of functional categories in response to cell wall mutations is therefore remarkably comparable with the remodeling of genome expression in response to environmental stress, including changes in temperature, pH, osmolarity, salinity, ethanol shock, etc. (20, 21, 57).

Using the hierarchical clustering algorithm developed by Eisen *et al.* (39), we were able to isolate a set of 79 co-regulated genes from a comparison of expression patterns of the five cell wall mutants. This cluster may therefore represent the signature of the cell wall compensatory mechanism. Interestingly, an important number of activated genes in the cluster encode proteins implicated in cell wall biogenesis, including chitin synthesis (Gfa1p and Chs3p), β -glucan remodeling (Crh1p, Bgl2p, Scw10p, and Exg1p), cell wall strengthening (Pir3p and Cwp1p), and cell surface protection (Spi1p and Sed1p; see Ref. 2 for extensive discussion). The increased chitin content in cell wall mutants was shown recently to be due to a concurrent increase of both Gfa1p and Chs3p activity (55). This higher level of chitin together with increased cross-linkages between this component and β -glucan probably contributes to a strengthening of the cell wall (92, 93). Moreover, a higher number of cross-links between cell wall polymers is likely to occur as indicated by up-regulation of genes that encode glucosylglucanolytransferases (*CRH1*, *BGL2*, and *SCW10*). However, the definitive proof of this assumption will await full biochemical characterization of these putative cross-linking enzymes. This genome-wide analysis of cell wall mutants also uncovered several up-regulated genes that could be implicated in cell wall biogenesis, such as genes that encodes nitrogen-catabolite cell wall asparaginase or *YLR194c*, which is thought to encode a GPI-anchored protein (54).

Our genomic data was also very informative with respect to activation of cell integrity, since *SLT2*, encoding the last kinase of the *PKC1*-MAPK-signaling module, was among the up-regulated genes of this cluster, and this up-regulation was accompanied by increased levels of Slt2p. Moreover, the phosphorylated form of Slt2p was more abundant in cell wall mutants than in their isogenic wild type. Thus, both effects led to a potent activation of the Slt2p-mediated pathway in response to cell wall mutations, which can explain in part, although not exclusively (see below), the increased expression of cell wall-related genes. Since a previous work (94) has shown that loss of *RLM1* abolished transcriptional activation of *SLT2* upon a thermal stress, it is also possible that up-regulation of this gene in response to cell wall mutations is mediated by Rlm1p. However, this effect could be indirect, since, surprisingly, we could not find any RLM1 motif in the promoter of this gene, using the Rlm1p canonical binding consensus (NTAWWWWTAG) from the TRANSFAC data base (44).

The *in silico* analysis of the promoter region of co-regulated genes led to two remarkable results. First, five consensus sequences that bind transcription factors implicated in stress and heat shock responses (Msn2p/Msn4p and Hsf1p), in the cell wall integrity pathway (Rlm1p), and in the carbon and phosphate metabolism (Gcr1p and Pho4p) were identified. This result was not surprising due to the reshaping of the functional categories in response to cell wall mutations. We also uncovered the N(A/T)(A/T)ACGCG(A/T)N(A/C) consensus corresponding to the *A. nidulans* StuAp binding site (86), since it is present in the TRANSFAC data base (42, 44). The yeast proteins showing the highest identity with the *A. nidulans* transcription factor are Sok2p and Phd1p, which are implicated in activation of genes during pseudohyphal differentiation (88). This finding is quite puzzling because there are no data indicating an involvement of either Sok2p or Phd1p in cell wall construction, even during pseudohyphal growth. Moreover, we found an extremely low overlap between our genomic data and transcriptomic data from a *sok2/sok2* diploid mutant (87). Although we do not exclude a role of Sok2p in controlling expression of cell wall genes, another more likely possibility is that Swi4p, a protein that forms with Swi6p the SBF transcription

factor (14), binds to the N(A/T)(A/T)ACGCG(A/T)N(A/C) consensus and then activates cell cycle-regulated gene expression at the G₁ to S transition (15). This suggestion rests on the fact that Swi4p shares similarity within the APSES domains of StuAp, Sok2p, and Phd1p (86) and that the more probable binding site of the SBF factor is the CGCGAAA consensus (89), which is remarkably similar to the *StuAp* consensus. This alternative explanation is also consistent with previous results showing that Swi4p can be activated by Slt2p-dependent phosphorylation (15) and regulates expression of some cell wall-related genes (95). The second important result that illustrates the powerful input of computational analyses in functional genomics was the uncovering of a 6-bp motif, AGCCTC, that was recently isolated from a genome-wide analysis of the response to a high calcium concentration. This motif actually corresponds to the binding motif of Crz1p, the major effector of calcineurin-regulated gene expression in yeast (22). The significance of this motif, termed CDRE (calcium-dependent regulatory element) is high, since it is present in 30 of 79 genes of the cluster, and its occurrence within 700 bp of the translation start of the 6200 yeast genes is about 10%. This finding suggests a role of the calcium signaling in the cell wall compensatory mechanism. We verified this idea as we showed that activation of *FKS2*, a well established reporter gene of cell wall defects (6, 17, 29), in response to Calcofluor White or in a *gas1* mutant was reduced upon the removal of the CDRE element present in the promoter of this gene. Interestingly, two recent papers reported the implication of calcium signaling in the tolerance of yeast to antifungal azoles (96) and in transcriptional induction of genes in response to alkaline pH (97). Altogether, these data demonstrate a major contribution of the calcium signaling in adaptation of yeast cells to various stresses. They also raise the question of how these different stresses perturb the intracellular levels of calcium to activate this signaling system.

Our effort to dissect the complexity of the cell wall repair mechanism led us to propose that this mechanism may integrate the following three major regulatory systems: the *PKC1*-*SLT2* MAPK signaling module, the Ca²⁺/calcineurin-dependent signaling pathway, and the general stress response system. Previous genomic analysis already implicated these signaling pathways in cell wall synthesis and maintenance. However, these three signaling pathways probably cooperate to maximize the efficacy of the yeast cell to combat cell lysis and would also contribute to antifungal resistance. This idea is nicely illustrated by analysis of *FKS2* expression, which shows cooperation of the *PKC1*-dependent and calcium/calcineurin-dependent signaling pathways for optimal activation of this gene in response to cell wall drugs. Another illustration of cross-talks between signaling pathways was that *fsk1* and *gas1* cells were reported to be more resistant than wild type cells to death after 15 min of heat shock at 50 °C (6). This higher resistance may now be explained in part by a preadaptation of these mutants to stress conditions, as a result of the co-occurrence of STRE or HSE motifs with RLM1 in the promoter of 46 of the 79 genes in the cluster.

A critical question is whether the regulatory systems mediated by *PKC1*, calcium/calcineurin, and the general stress factors are entangled or are sequentially activated in response to cell wall damage. According to Jung and Levin (17), who carried out a genomic study in yeast expressing an activated form of Mkk1p kinase, the HSE- and STRE-dependent signaling pathways obscure the "true" transcriptional activation of genes by the *PKC1*-dependent cell wall integrity pathway. However, neither their study nor ours, which used cell wall mutants, could respond to this question, since these regulatory pathways

were probably already set in a permanently activated form in these mutants. Gasch *et al.* (20) provided preliminary arguments in favor of a sequential activation of these regulatory systems by analyzing gene expression patterns in response to dithiothreitol treatment. They found that this chemical induced a three-stage response, starting with the induction within 30 min after exposure of genes encoding protein chaperones, protein-disulfide isomerases, and proteins involved in the redox potential, followed by late induction of cell wall-related genes and, hours later, by activation of genes that belong to the general stress response (ESR). Since the latter event was largely dependent on Msn2p/Msn4p factors, these authors postulated that the ESR is a secondary response triggered by accumulation of cell wall defects in response to dithiothreitol. Further time course experiments carried out on a genome scale with specific cell wall-perturbing agents, such as Calcofluor White, Congo red, and other antifungal drugs, could help us in the investigation of the molecular link between the PKC1-dependent pathway and the ESR system.

Acknowledgments—We thank Drs. Enrico Cabib (National Institutes of Health, Bethesda, MD), Laura Popolo (University of Milano), Frans Klis (University of Amsterdam), Wolfram Horz (University of Munich), and David E. Levin (The Johns Hopkins University, Baltimore, MD) for kind provision of yeast strains and/or plasmids. We kindly thank Dr. Sylvain Jason (Unité Biométrie INRA, Auzeville, France) for initial help with hierarchical clustering of genomic data, Dr. Jean-Luc Parrou for expertise on recombination-mediated PCR-directed plasmid construction *in vivo* in yeast, and Dr. Kurt Fellenberg for assistance with the M-ChiPS data base. We are also indebted to Dr. Humberto Martín and Dr. María Molina (UCM, Madrid) for providing the anti-Slt2p antibodies and advice on Slt2p activation experiments. We acknowledge Dr. Carol Munro (Aberdeen University, UK) for proofreading of the manuscript.

REFERENCES

- Orlean, P. (1997) *The Molecular Biology and Cellular Biology of the Yeast Saccharomyces*, pp. 229–362, Cold Spring Harbor Laboratory Press, NY
- Klis, F. M., Mol, P., Hellingwerf, K., and Brul, S. (2002) *FEMS Microbiol. Rev.* **26**, 239–256
- Popolo, L., Gualtieri, T., and Ragni, E. (2001) *Med. Mycol.* **39**, 111–121
- Dallies, N., Francois, J., and Paquet, V. (1998) *Yeast* **14**, 1297–1306
- Bickle, M., Delley, P. A., Schmidt, A., and Hall, M. N. (1998) *EMBO J.* **17**, 2235–2245
- De Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F. M. (2000) *Microbiology* **146**, 2121–2132
- Verna, J., Lodder, A., Lee, K., Vagts, A., and Ballester, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13804–13809
- Garcia-Rodriguez, L. J., Duran, A., and Roncero, C. (2000) *J. Bacteriol.* **182**, 2428–2437
- Smits, G. J., van den Ende, H., and Klis, F. M. (2001) *Microbiology* **147**, 781–794
- Kapteyn, J. C., Van Egmond, P., Sievi, E., van den Ende, H., Makarow, M., and Klis, F. M. (1999) *Mol. Microbiol.* **31**, 1835–1844
- Heinisch, J. J., Lorberg, A., Schmitz, H.-P., and Jacoby, J. J. (1999) *Mol. Microbiol.* **32**, 671–680
- Rajavel, M., Philip, B., Buehrer, B. M., Errede, B., and Levin, D. E. (1999) *Mol. Cell. Biol.* **19**, 3969–3976
- Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000) *J. Biol. Chem.* **275**, 1511–1519
- Andrews, B. J., and Herskowitz, I. (1989) *Nature* **342**, 830–833
- Madden, K., Sheu, Y. J., Baetz, K., Andrews, B., and Snyder, M. (1997) *Science* **275**, 1781–1784
- Dodou, E., and Treisman, R. (1997) *Mol. Cell. Biol.* **17**, 1848–1859
- Jung, U. S., and Levin, D. E. (1999) *Mol. Microbiol.* **34**, 1049–1057
- Popolo, L., Gilardelli, D., Bonfante, P., and Vai, M. (1997) *J. Bacteriol.* **179**, 463–469
- Ketela, T., Green, R., and Bussey, H. (1999) *J. Bacteriol.* **181**, 3330–3340
- Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) *Mol. Biol. Cell* **11**, 4241–4257
- Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A. (2001) *Mol. Biol. Cell* **12**, 323–337
- Yoshimoto, H., Saltsman, K., Gasch, A. P., Li, H. X., Ogawa, N., Botstein, D., Brown, P. O., and Cyert, M. S. (2002) *J. Biol. Chem.* **277**, 31079–31088
- Hohmann, S. (2002) *Microbiol. Mol. Biol. Rev.* **66**, 300–372
- Jiang, B., Bussey, H., and Roemer, T. (2002) *Curr. Opin. Microbiol.* **5**, 446–448
- Woods, R. A., and Gietz, R. D. (2001) *Methods Mol. Biol.* **177**, 85–97
- Ram, A. F., Kapteyn, J. C., Montijn, R. C., Caro, L. H., Douwes, J. E., Baginsky, W., Mazur, P., van den Ende, H., and Klis, F. M. (1998) *J. Bacteriol.* **180**, 1418–1424
- Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, NY
- Martin, H., Dagkessamanskaia, A., Satchanska, G., Dallies, N., and Francois, J. (1999) *Microbiology* **145**, 249–258
- Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998) *Mol. Cell. Biol.* **18**, 1013–1022
- Innis, M. A., Gelfand, D. H., Sinsky, J. J., and White, T. J. (1990) *PCR Protocols: A Guide to Methods and Applications*, pp. 177–183, Academic Press, Inc., New York
- Rose, M., and Botstein, D. (1983) *Methods Enzymol.* **16**, 167–180
- Beier, M., and Hoheisel, J. D. (1999) *Nucleic Acids Res.* **27**, 1970–1977
- Hauser, N. C., Vingron, M., Scheideler, M., Krems, B., Hellmuth, K., Entian, K. D., and Hoheisel, J. D. (1998) *Yeast* **14**, 1209–1221
- Nguyen, C., Rocha, D., Granjeaud, S., Baldit, M., Bernard, K., Naquet, P., and Jordan, B. R. (1995) *Genomics* **29**, 207–216
- Siderius, M., Rots, E., and Mager, W. H. (1997) *Microbiology* **143**, 3241–3250
- Martin, H., Arroyo, J., Sanchez, M., Molina, M., and Nombela, C. (1993) *Mol. Gen. Genet.* **241**, 177–184
- Fellenberg, K., Hauser, N., Brors, B., Hoheisel, J., and Vingron, M. (2002) *Bioinformatics* **18**, 423–433
- Beissbarth, T., Fellenberg, K., Brors, B., Arribas-Prat, R., Boer, J., Hauser, N. C., Scheideler, M., Hoheisel, J. D., Schutz, G., Poustka, A., and Vingron, M. (2000) *Bioinformatics* **16**, 1014–1022
- Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14863–14868
- Sherlock, G. (2000) *Curr. Opin. Immunol.* **12**, 201–205
- Cherry, J. M., Adler, C., Ball, C., Chervitz, S. A., Dwight, S. S., Hester, E. T., Jia, Y., Juvik, G., Roe, T., Schroeder, M., Weng, S., and Botstein, D. (1998) *Nucleic Acids Res.* **26**, 73–79
- Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) *Nucleic Acids Res.* **23**, 4878–4884
- Van Helden, J., Andre, B., and Collado-Vides, J. (2000) *Yeast* **16**, 177–187
- Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I., and Schacherer, F. (2000) *Nucleic Acids Res.* **28**, 316–319
- Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W., el Sherbeini, M., Clemas, J. A., Mandala, S. M., Frommer, B. R., and Kurtz, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12907–12911
- Roemer, T., Paravicini, G., Payton, M. A., and Bussey, H. (1994) *J. Cell Biol.* **127**, 567–579
- Shahinian, S., and Bussey, H. (2000) *Mol. Microbiol.* **35**, 477–489
- Yip, C. L., Welch, S. K., Klebl, F., Gilbert, T., Seidel, P., Grant, F. J., O'Hara, P. J., and MacKay, V. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2723–2727
- Popolo, L., Vai, M., Gatti, E., Porello, S., Bonfante, P., Balestrini, R., and Alberghina, L. (1993) *J. Bacteriol.* **175**, 1879–1885
- Popolo, L., and Vai, M. (1999) *Biochim. Biophys. Acta* **1426**, 385–400
- Martin-Yken, H., Dagkessamanskaia, A., Talabi, D., and Francois, J. (2002) *Curr. Genet.* **41**, 323–332
- Ram, A. F., Wolters, A., Ten Hoopen, R., and Klis, F. M. (1994) *Yeast* **10**, 1019–1030
- Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M. (2001) *Nat. Genet.* **29**, 365–371
- Terashima, H., Yabuki, N., Arisawa, M., Hamada, K., and Kitada, K. (2000) *Mol. Gen. Genet.* **264**, 64–74
- Lagorce, A., Le Berre-Anton, V., Aguilar-Uscanga, B., Martin-Yken, H., Dagkessamanskaia, A., and Francois, J. (2002) *Eur. J. Biochem.* **269**, 1697–1707
- Mewes, H. W., Albermann, K., Heumann, K., Liebl, S., and Pfeiffer, F. (1997) *Nucleic Acids Res.* **25**, 28–30
- Alexandre, H., Ansanay-Galeote, V., Dequin, S., and Blondin, B. (2001) *FEBS Lett.* **498**, 98–103
- Walsh, R. B., Clifton, D., Horak, J., and Fraenkel, D. G. (1991) *Genetics* **128**, 521–527
- Okar, D. A., and Lange, A. J. (1999) *Biofactors* **10**, 1–14
- Muller, S., Zimmermann, F. K., and Boles, E. (1997) *Microbiology* **143**, 3055–3061
- Dihazi, H., Kessler, R., and Eschrich, K. (2001) *Biochemistry* **40**, 14669–14678
- Gancedo, J. M. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 334–361
- Waterland, R. A., Basu, A., Chance, B., and Poyton, R. O. (1991) *J. Biol. Chem.* **266**, 4180–4186
- Drgon, T., Sabova, L., Gavurnikova, G., and Kolarov, J. (1992) *FEBS Lett.* **304**, 277–280
- Takabatake, R., Siddique, A. B., Kouchi, H., Izui, K., and Hata, S. (2001) *J. Biochem. (Tokyo)* **129**, 827–833
- Devenish, R. J., Prescott, M., Roucou, X., and Nagley, P. (2000) *Biochim. Biophys. Acta* **1458**, 428–442
- Estruch, F. (2000) *FEMS Microbiol. Rev.* **24**, 469–486
- Mazur, P., Morin, N., Baginsky, W., el Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995) *Mol. Cell. Biol.* **15**, 5671–5681
- Lussier, M., Sdicu, A. M., Camirand, A., and Bussey, H. (1996) *J. Biol. Chem.* **271**, 11001–11008
- Lussier, M., White, A.-M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F. J., Kapteyn, J. C., Roemer, T. W., Vo, D. H., Bondoc, D. C., Hall, J., Zhong, W. W., Sdicu, A.-M., Davies, J., Klis, F. M., Robbins, P. W., and Bussey, H. (1997) *Genetics* **147**, 435–450
- Olsen, V., Cawley, N. X., Brandt, J., Egel-Mitani, M., and Loh, Y. P. (1999) *Biochem. J.* **339**, 407–411
- Kitagaki, H., Wu, H., Shimoi, H., and Ito, K. (2002) *Mol. Microbiol.* **46**, 1011–1022

73. Shimoi, H., Kitagaki, H., Ohmori, H., Iimura, Y., and Ito, K. (1998) *J. Bacteriol.* **180**, 3381–3387
74. Destruelle, M., Holzer, H., and Klionsky, D. J. (1994) *Mol. Cell. Biol.* **14**, 2740–2754
75. Puig, S., and Perez-Ortin, J. E. (2000) *Yeast* **16**, 139–148
76. Cappellaro, C., Mrsa, V., and Tanner, W. (1998) *J. Bacteriol.* **180**, 5030–5037
77. Goldman, R. C., Sullivan, P. A., Zakula, D., and Capobianco, J. O. (1995) *Eur. J. Biochem.* **227**, 372–378
78. Nebreda, A. R., Villa, T. G., Villanueva, J. R., and Del Rey, F. (1986) *Gene (Amst.)* **47**, 245–259
79. Konopka, J. B., DeMattei, C., and Davis, C. (1995) *Mol. Cell. Biol.* **15**, 723–730
80. Fellenberg, K., Hauser, N. C., Brors, B., Neutzner, A., Hoheisel, J. D., and Vingron, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10781–10786
81. Roberts, C. J., Nelson, B., Marton, M. J., Stoughton, R., Meyer, M. R., Bennett, H. A., He, Y. D., Dai, H., Walker, W. L., Hughes, T. R., Tyers, M., Boone, C., and Friend, S. H. (2000) *Science* **287**, 873–880
82. Uemura, H., Koshio, M., Inoue, Y., Lopez, M. C., and Baker, H. V. (1997) *Genetics* **147**, 521–532
83. Lenburg, M. E., and O'Shea, E. K. (1996) *Trends Biochem. Sci.* **21**, 383–387
84. Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) *EMBO J.* **15**, 2227–2235
85. Sorger, P. K. (1991) *Cell* **65**, 363–366
86. Dutton, J. R., Johns, S., and Miller, B. L. (1997) *EMBO J.* **16**, 5710–5721
87. Pan, X., and Heitman, J. (2000) *Mol. Cell. Biol.* **20**, 8364–8372
88. Gancedo, J. M. (2001) *FEMS Microbiol. Rev.* **25**, 107–123
89. Iyer, V. R., Horak, C. E., Scafe, C. S., Botstein, D., Snyder, M., and Brown, P. O. (2001) *Nature* **409**, 533–538
90. Bailey, T. L., and Elkan, C. (1994) *Proc. Sec. Int. Conf. Intell. Syst. Mol. Biol.* **3**, 28–36
91. Smits, G. J., Kapteyn, J. C., van den, E. H., and Klis, F. M. (1999) *Curr. Opin. Microbiol.* **2**, 348–352
92. Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W., and Cabib, E. (1995) *J. Biol. Chem.* **270**, 1170–1178
93. Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997) *J. Biol. Chem.* **272**, 17762–17775
94. Hahn, J. S., and Thiele, D. J. (2002) *J. Biol. Chem.* **277**, 21278–21284
95. Baetz, K., Moffat, J., Haynes, J., Chang, M., and Andrews, B. (2001) *Mol. Cell. Biol.* **21**, 6515–6528
96. Edlind, T., Smith, L., Henry, K., Katiyar, S., and Nickels, J. (2002) *Mol. Microbiol.* **46**, 257–268
97. Serran, R., Ruiz, A., Bernal, D., Chambers, J. R., and Ariño, J. (2002) *Mol. Microbiol.* **46**, 1319–1333