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Arnaud Lagorce, Nicole C. Hauser, Delphine Labourdette, Cristina Rodríguez, Hélène Martin-Yken, et al.. Genome-wide analysis of the response to cell wall mutations in the yeast Saccharomyces cerevisiae. Journal of Biological Chemistry, 2003, 278 (22), pp.20345-20357. 10.1074/jbc.M211604200 . hal-02681963

# HAL Id: hal-02681963 https://hal.inrae.fr/hal-02681963

Submitted on 1 Jun2020

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## 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

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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 PKC1regulated transcription factors involved in the activation genes related to cell wall biogenesis and G1/S transition. Moreover, this computational analysis also uncovered the 6-bp 5'-AGCCTC-3' CDRE (calcineurindependent response element) motif in 40% of the coregulated 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<sup>2+</sup>/calcineurindependent 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  $\beta$ -1,3- and  $\beta$ -1,6-glucan (50–60% of the cell wall dry mass), mannoproteins (40–50%), and chitin (2%).  $\beta$ -1,3-Glucan and chitin form a fibrillar network to which mannoproteins are anchored, mostly through  $\beta$ -1,6-glucan. Some cell wall proteins, like the PIR family, can be directly linked to  $\beta$ -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  $\beta$ -glucan, mannoproteins, and chitin is changed. For instance, lowering the amount of  $\beta$ -1,6glucan leads to a larger fraction of the cell wall proteins becoming linked directly to  $\beta$ -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 MAPK<sup>1</sup> 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

<sup>\*</sup> 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 Hoechst 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.

<sup>§</sup> Recipient of a predoctoral fellowship from the French Ministry of Research and Technology Education.

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

#### Transcriptome Analysis of Yeast Cell Wall Mutants

List of yeast strains used in this work								
Genotype	Source or reference							
MAT α MNN9 SUC2 mal mel gal2 CUP1	ATCC							
X2180-1A mnn9	ATCC							
MATa ura3-52	Ref. 52							
AR27 knr4::Kan <sup>r</sup>	Ref. 28							
AR27 cwh48 (kre6)	Ref. 52							
MAT $\alpha$ his3- $\Delta 200$ ura3-52 leu $2\Delta 1$ lys $2\Delta 202$ trp $1\Delta 63$	EUROSCARF							
FY834 cwh53 (fksl)	Ref. 52							
MATα ade2-1 ura3-1 leu2,3-112 trp1-1 his3-11, 15 can1-100	Ref. 18							
W303-1B gas1::LEU2	Ref. 18							
W303-1B rlm1::LEU2	Ref. 55							
$MATa$ $his3\Delta1$ $leu2\Delta0$ $met15\Delta0$ $ura3\Delta0$	EUROSCARF							
BY4741 crz1::Kan <sup>r</sup>	EUROSCARF							
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TABLE I List of yeast strains used in this work

expression of cell cycle-regulated genes at the G<sub>1</sub>/S phase (14, 15) and cell wall-related genes (16, 17), respectively. This illustrates that the maintenance of cell integrity is dependent on coordination between cell wall biogenesis and cell proliferation (9, 15). Cell wall perturbations including treatment of yeast cells with Calcofluor White,  $\beta$ -1,3-glucanase, or Congo red or mutations in cell wall-related genes cause an activation of the *PKC1-SLT2*-dependent pathway, suggesting an important role of this pathway in the mechanism to compensate for cell wall weakening (6, 12, 18, 19). However, the cell wall compensatory mechanism does not seem to rely exclusively on Pkc1p function, since a number of genes involved in cell wall biogenesis are also up-or down-regulated in response to a wide variety of environmental conditions (20, 21). Interestingly, this latter response is dependent to a large extent on the activity of Msn2p/Msn4p transcription factors. The calcium/calcineurin and the HOG1-MAPK signaling pathways are another two possible contributors to the regulation of cell wall construction; high calcium concentration activates expression of several genes implicated in cell wall synthesis and maintenance (22), and mutations in components of the HOG pathway ( $hog1\Delta$  and  $pbs2\Delta$ ) lead to defective cell wall phenotypes (2, 23).

To obtain a comprehensive view of the cellular responses to cell wall damage, we used DNA arrays to examine the transcription profiles of yeast strains that are defective in different functions required for cell wall biogenesis. Our rationale was that the comparison of gene expression profiles from different cell wall mutants would allow identification of a discrete cluster of co-regulated genes that is a hallmark of the cell wall compensatory mechanism. Moreover, unraveling this cluster might be fruitful from a drug discovery viewpoint toward identifying potential antifungal targets (24). Following this strategy, we isolated a group of 79 co-regulated genes that probably represent the stereotypical transcription response to cell wall damage and showed that the cell wall compensatory mechanism integrates the Slt2p-mediated cell integrity pathway together with the general stress response system and the calcium/calcineurin-dependent signaling pathway.

#### EXPERIMENTAL PROCEDURES

Strain Construction, Growth Conditions, and Phenotypic Analysis— The strains used in this work are listed in Table I. For yeast transformation, the method described in Ref. 25 was used. Strains bearing single gene deletion by the KanMX4 cassette were purchased from the EUROSCARF collection (available on the World Wide Web at www.uni-frankfurt.de/fb15/mikro/euroscarf/2000). Crossing, sporulation, and tetrad analysis were carried out as described by Rose *et al.* (27). For microarray analysis, yeast cells were cultured at 30 °C in a 1-liter shake flask on a rotary shaker set at 200 rpm and containing 0.2 liters of YEPD medium (10 g of yeast extract, 20 g of bactopeptone, and 20 g of glucose/liter). Cell samples for RNA extraction were taken early in the exponential phase of growth (*i.e.* in conditions where cellular growth is perfectly balanced). Cultures in the presence of Calcofluor White (50  $\mu$ g/ml) and CaCl<sub>2</sub> (0.2 M) were carried out as previously described in Refs. 6 and 22. Sensitivity assays on agar plates containing different drugs (*i.e.* Calcofluor White, Congo red, SDS, or geneticin (G418)) were performed according to Ref. 28.

Plasmids and PCR-directed Mutagenesis-The constructions  $pFKS2\Delta cdre-lacZ$  and  $pFKS2(CDRE)_6$ -lacZ reported in Table VI are derived from the pFKS2-lacZ plasmid, which bears a 706-bp promoter region of FKS2 fused in frame to the E. coli lacZ gene (29). To replace the AGCCTC by AGATCT (corresponding to a XbaI site), a recombinant PCR strategy was followed as described by Innis et al. (30), using 5'-ACGGCAAATCGCCAAAAGAGATGAT-3' and 5'-GCGGGCCTCTT-CGCTATTACGCCAG-3' as external primers as well as 5'-GAGATAA-TCTAGAGTTCGTTCTCTCCATTTCTTCCCTG-3' and 5'-AACGAATC-TAGATTATCTCATTTTATTCTACTCTTTG-3' as internal primers (the  $\overline{XbaI}$  restriction site is underlined) to yield the pFKS2 $\Delta cdre$ -LacZ gene fusion. To construct the FKS2- $(CDRE)_6$ -lacZ gene fusion, which contains five additional AGCCTC copies, the internal primers were 5'-G-AGATAACAATTGAGCCTCAGCCTCAGCCTCAGCCTCAGCCTCAGC-CTCGTTCGTTCTCTCCATTTCT-3' and 5'-AACGAACGAGGCTGAGG-CTGAGGCTGAGGCTGAGGCTGAGGCTCAATTGTTATCTCATTTTAT-TCTACTCTTTGCTATATA-3' (the MfeI restriction site is underlined; AGCCTC copies are in italics). PCR was performed with high fidelity Pfu polymerase (Stratagene). The constructs were verified by DNA sequencing (Genome Express S.A., Grenoble, France), prior to yeast transformations and  $\beta$ -galactosidase assays (31).

Surface Derivation of Polypropylene Membranes for DNA Arrays-All chemical reactions for surface derivation of the polypropylene membranes were carried out at 30 °C under gentle shaking as described in Ref. 32 with some modifications. Briefly, in the first step (acylation), polypropylene membranes (Pall, Dreieich, Germany) were incubated for 2 h in a mixture of 1.3 ml (16 mmol) acryloylchloride in 40 ml of anhydrous dichloroethane and 2.74 ml (16 mmol) of diisopropylethylamine. The membranes were washed thoroughly with dichloroethane  $(2 \times 40 \text{ ml})$  and then dimethylformamide (DMF)  $(1 \times 40 \text{ ml})$  and subsequently dried. In the second step, the acylated membranes were incubated over two nights in a solution of 3.6 ml (16 mmol) tetraethylene-pentamine in 40 ml of anhydrous, amine-free DMF. After two washes each with DMF and dichloroethane, the membranes were dried. Steps one and two were repeated, but instead of tetraethylene-pentamine in step two, 3.4 ml (16 mmol) of 1,4-bis-(3-aminopropoxy)butane were used. For control of reaction efficiency, aminated polypropylene strips were added to the reaction. After every reaction step, part of a control strip was removed, washed with DMF, and stained in a 0.05% (w/v) bromphenol blue solution in 2 ml of DMF for 2 min. After subsequent washes in pure DMF and ethanol, the color intensity was used to calculate the number of amino groups blocked during acylation or newly introduced with the amines.

Manufacturing DNA Arrays on Polypropylene Filters—The 6116 yeast genes were amplified as described in Ref. 33, using a commercially available set of primers (GenePair; Invitrogen). The amplification yielded 95% recovery of the initial amplicons. Prior to spotting, the polypropylene membranes were soaked in activation solution (28 mM *N*-ethylcarbodiimidehydrochloride in H<sub>2</sub>O, to which 44  $\mu$ l of *N*-methylimidazole per 100 ml of solution were added immediately prior to use) and left therein during the spotting process. After spotting, membranes were incubated for 2 h at 65 °C and dried overnight. The DNA arrays on polypropylene filters were reused at least 10 times without significant reduction of the signal intensity. The regeneration of arrays between experiments was carried out as described previously (33).

Single Strand cDNA Synthesis and Hybridization on DNA Arrays— Total RNA was extracted from exponentially growing cells as described

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  $\mu$ g of total RNA were mixed with 0.5  $\mu$ g of oligo(dT)<sub>15</sub> (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  $\mu l$  using SuperScript<sup>TM</sup>II (Invitrogen) in the presence of a 0.25 mM concentration each of dATP, dGTP, and dTTP, 1.66  $\mu$ M dCTP, and 30  $\mu$ Ci of [ $\alpha$ -<sup>33</sup>P]dCTP (Amersham Biosciences). After 30 min at 42 °C, another 1 µl of SuperScript<sup>TM</sup>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  $\mu$ l 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  $\mu$ l of 2 M HCl. After the addition of 5  $\mu$ l of 3 M sodium acetate, pH 5.3, 5  $\mu$ l of tRNA (10  $\mu$ g/ $\mu$ l), and 60  $\mu$ l of isopropyl alcohol, the cDNA was precipitated at -20 °C for 30 min, pelleted by centrifugation, and resuspended in 100  $\mu$ l of water. The incorporation of <sup>33</sup>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 denaturated 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 phosphofluoroimager (Amersham Biosciences). Experiments with each mutant strain and its isogenic wild type were repeated four times.

Northern Blot Analysis—Total RNA was separated in formaldehydeagarose gels (25  $\mu$ g/lane) as described by Siderius *et al.* (35) and transferred to Nylon-N<sup>+</sup> 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× SSC at room temperature and then washed twice for 10 min 0.1% SDS, 0.01× SSC at room temperature. Signal quantification was performed with the phosphofluoroimager, 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  $A_{595}$  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 antiphospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) 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  $\geq 2$  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  $\beta$ -1,3-glucan synthase catalytic subunit (45); KRE6, which encodes a protein required in the synthesis of  $\beta$ -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  $\beta$ -1,3-glucanosyl transferase activity (18, 49, 50); and KNR4, whose product connects the PKC1-SLT2 MAPK pathway with cell proliferation (51).<sup>2</sup> The DNA macroarrays, which bear 6116 yeast open reading frames (see, on the World Wide Web, www.dkfz.de/funct\_genome/YEASTfilters/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 upregulation 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

<sup>&</sup>lt;sup>2</sup> H. Martin-Yken, H. Martin-Brevia, A. Dagkessamanskaia, F. Basmaji, A. Lagorce, M. Molina, and J. Francois, submitted for publication.

		Northern-blot					Macroarray						
Strains Genes	PY834 (my) CWH53 (mu)	AR27 (84) HMR10 (6mm) CWR488 (6mm)	W303.1B (wy) WB24 (wy)	ADVIND (MT)	CUNH53 (Run)	HANTO (Kang)	CWH48 (kms)	WB24 (sur)	MIND (mar)				
YML123c (PHO84)		- 15 1.7	5.9	-10.7	-1.7	1	1.7	-5.4	-13.4				
YAR071w (PHO11)			-44	-2.7	1	1.8	1.6	-6.8	-3.3				
YLR160c (ASP3D)		1.3 96.4	- 14		1	1	181.9	2	1				
YLR158c ASP3C)	$\frac{1}{2}$	1 38			1	1	90.3	2.1	1				
VLR157c ASP3B)		1 47	- 14	- 1.6	1	1	106.8	2	1.6				
YLR155c ASP3A)		1 17.8	= 14	- 1.6	1	1	62.4	3.6	1.6				
(KL161c (MLP1)		2.7 49	1.8 1.8	=	10.9	4.7	8.9	1.8	15.8				
(KR091w (SRL3)	3.9	3.1 5.3		3.8	6.7	6.9	9.9	4.9	10.5				
(CWP1)		- 53 55	$-\frac{1}{1}$	$-\overline{\overline{2.1}}$	6.9	4.5	4.1	1	2.9				
(KL163w (PIR3)		. 2 23	5.6		1.9	1	2.3	ó.8	1				
YLR194C	6.3	2 45	- 11 .	- 125	4.8	2.8	6.9	5.9	13.7				
YNL 160w (YGP1)		7.9 9.8	$\overline{\pi}_{\overline{1}}$	- 3.5	3.4	5.5	13.5	1	5				
YER178w													

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  $fks1\Delta$  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 interpretation, 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 reduction of glycolytic flux in favor of a redirection of glucose 6-phosphate to cell wall  $\beta$ -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,  $\sim 10\%$  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  $\beta$ -glucan and mannan synthesis, like *FKS2*, which encodes the minor  $\beta$ -1,3-glucan synthase catalytic subunit (6, 29, 68); *KRE6* and *KRE11*, which are involved in the synthesis of  $\beta$ -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 ∆/wt ratio	Description of the gene product
YBR023c/CHS3	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.3	Chitin synthase 3
YBR076w/ECM8	$\Delta fks I$ , , $\Delta knr4$ , $\Delta kre6$ , $\Delta mnn9$	2.7	Involved in cell wall biogenesis and structure
YDR055w/PST1	Δfks1, Δkmr4, Δkre6, Δmnn9	4.5	Putative GPI-dependent cell wall protein
YDR077w/SED1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	4,5	Abundant cell wall glycoprotein, null mutant
YEL058w/AGM1	$\Delta fks1$ , $\Delta kmr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.2	exhibits increased sensitivity to zymolyase Phosphoacetylglucosamine mutase, chitin biosynthesis pathway
YER150w/SPI1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.7	Cell wall protein, stationary phase induced
YGR032w/FKS2	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	5.1	beta 1-3 glucan biosynthesis
YGR166w/KRE11	$\Delta fks1$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.4	beta 1-6 glucan biosynthesis
YGR189c/CRH1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	3.8	Cell wall organisation
YGR282C/BGL2	∆knr4, ∆kre6, ∆gas1, ∆mnn9	2.2	Cell wall endo-beta-1-3-glucanase
YHR030c/MPK1	$\Delta fks 1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas 1$ , $\Delta mnn9$	2.6	Ser/Thr kinase (cell wall integrity pathway)
YHR136c/SPL2	Δknr4, Δkre6	4.8	Cell wall protein
YJL165c/HAL5	Δgasl	3.5	Ser/ Thr protein kinase, involved in cell wall biogenesis and architecture
YJR106w/ECM27	$\Delta gas1, \Delta mnn9$	5.0	cell wall biogenesis and architecture
YKL096w/CWP1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta mnn9$	4.6	Cell wall mannoprotein
YKL104c/GFA1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.9	Glucosamine-fructose-6-phosphate-
YKL161c/MLP1	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	8.4	Putative ser/ thr kinase with similarity with
YKL163w/PIR3	Δfks1, Δkre6, Δgasl	3.7	Member of PIR family protein, cell wall structural protein
YKR061w/KTR2	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta mn9$	4.3	Mannosyltransferase
YKR076w/ECM4	$\Delta fks1$ , $\Delta kmr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mm9$	2.5	cell wall biogenesis and architecture
YLR121c/YPS3	$\Delta fks1$ , $\Delta kmr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	5.8	GPI-anchored aspartic protease 3
YLR155c/ASP3A	$\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	22.5	Nitrogen catabolite-regulated cell wall L- asparaginase II
YLR157c/ASP3B	$\Delta kre6, \Delta gasl, \Delta mnn9$	34.8	Nitrogen catabolite-regulated cell wall L- asparaginaseII
YLR158c/ASP3C	Δkre6, Δgasl	46.2	Nitrogen catabolite-regulated cell wall L- asparaginasell
ILRI60C/ASP3D	Δkreb, Δgasi	92.0	asparaginaseII
YLR194c	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	6.8	Unknown function and unknown phenotype
YLR300w/EXG1	$\Delta fks1$ , $\Delta kmr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mm9$	1.9	Exo beta 1-3-glucanase
YLR342w/FKS1	$\Delta gas1, \Delta mm9$	2.0	Beta 1-3 -D-glucan synthase
YMR238w/DFG5	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta gas1$ , $\Delta mnn9$	2.8	Protein involved cell polarity, cellular elongation and possibly in processing of GPI- dependent cell wall proteins
YMR305c/SCW10	Δkre6, ΔgasI, Δmnn9	3.3	Member of the glucanase gene family, soluble cell wall protein
YNL058c	$\Delta fks1$ , $\Delta knr4$ , $\Delta kre6$ , $\Delta mnn9$	2.0	Similarity to YIL117c
YNL160w/YGP1	Δfks1, Δkmr4, Δkre6, Δmnn9	6.8	Cell wall glycoprotein synthesized in response
YPR159w/KRE6	Δgas1, Δmm9	2.4	to nutrient limitation synthesis of beta 1-6 glucan





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  $\beta$ -glucanosyl/glucosyltransferase activity (*BGL2*, *CRH1*, and *SCW10* (76, 77)) and  $\beta$ -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  $\beta$ -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  $\beta$ -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 (Thr<sup>190</sup> and Tyr<sup>192</sup>) 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-

	Pror	noter		Gene	
- 700 bp			ATG		
Consensus	Software	Sequence	Frequency	Function	Organism
STRE	MatInspector	CCCCT	45/79	Stress	S. cerevisiae
STUA	MatInspector	NWWCGCGWNM	28/ 79	Cell cycle	A. nidulan
RLM1	DNA-pattern	NTAWWWTAG	39/79	Cell wall	S. cerevisiae
HSE	DNA-pattem	GAANNTCC	75/ 79	Heat stress	S. cerevisiae
GCR1	DNA-pattern	RGCTTCCWC	28/79	C-metabolism	S. cerevisiae
PHO4	DNA-pattern	NNNCACGTKNGN	17/79	P-metabolism	S. cerevisiae
CDRE	Oligo-analysis	AGCCTC	30/ 79	?	S. cerenisiae
K=G/T	M=A/C	N=A, T, G ou C	R=A	/G W=A	/ T

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 Oligoanalysis (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).

Consensus		w	М	A	G	С	С	T	C	Y	H
	Т	10	4	0	0	0	0	31	0	10	10
	G	5	6	0	31	0	0	0	0	5	5
	С	5	11	0	0	31	31	0	31	9	11
	Α	11	10	31	0	0	0	0	0	7	10

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  $Ca^{2+}$ /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  $\mu$ g/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 MSN2and 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  $rlm1\Delta$  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 Pl	HO4	STUA	STRE	HSE	
CDRE	16	10	5	13	18	24	
RLM1	11.12.17.12	12	10	14	18	28	
GCR1	STATES OF STATES		7	8	20	23	
PHO4		The second second	124810	6	8	15	
STUA	STOP TONE	ALL	000000	153.82	13	31	
STRE	Eless Alt (2)				Charles The State	34	
HSE	「「「「「「「」」」			前建制			
Motifs	CDRE/GCR1	CDRE/PHO4	CDRI	STUA	CDRE/STRE	CDRE/HS	Ē
RLM1	5	3	- ODIG	7	8	10	-
GCR1	Contraction of the	1		3	8	7	
PHO4	E MANY AND	ALL STREET	( ) ( ) ( ) ( ) ( ) ( ) ( ) ( ) ( ) ( )	3	3	2	
STUA	代にしてつきえん見な	Section Market	SO CERT	22 (S. 13	4	9	
STRE	1351.0 000000	「中心にないのやきをたちい」	the state		1 10 1 19 1 - 3 M	12	
	and a second to be		and the second second				
Motifs	RLM1/PHO4	RLM1/STUA	RLM	1/STRE	RLM1/HSE		
GCR1	3	1		7	6		
PHO4		4		5	1		
STUA	医生活 中外的			4	8		
STRE			NY WAY	12.23	9		
Motifs	GCR1/STUA	GCR1/STRE	GCF	1/HSE	٦		
PHO4	0	2		2			
STUA	CASE OF DELLA	4		7			
STRE				13			
Motifs	PHO4/STRE	PHO4/HSE	1				
STUA	3	2	1				
STRE		3					
Motife	STILA/HSE	7					
STRE	11	-					
OTICS	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 Ca<sup>2+</sup>/ 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

### Transcriptome Analysis of Yeast Cell Wall Mutants

#### 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

		DNA motifs in the noncoding up region <sup>a</sup> (-700 bp from AT	$\operatorname{Induction}^b$							
ORF name	Gene	Other motifs	CDRE motif	Our study	$\mathop{\mathrm{Calcium}}_{(22)^c}$	GAL- <i>PKC1</i> (81)	Dithiothreitol (20)	Heat shock (21)	fksl mutation (54)	$\frac{MKK1^{\rm S386P}}{(17)^d}$
YAL053w		STRE*3/PHO4*2/RLM1*2/GCR1/	CDRE	++	+++				++	
YBL064c		STRE/STUA/HSE*2	++			+++	+++			
YBL078c	AUT7	STRE/GCR1/RLM1	CDRE	++		++		++		
YBR023c	CHS3	STUA/KLM1/HSE*3	<u>тт</u>	++		++	± ±		+	Induced
VBR071w		STILA*9/RLM1/HSE	CDRE	++	+++	+++	++		+	
YBR076w	ECM8	STUA/HSE/GCR1	ODIE	++					1	
YBR222c	PCS60	STUA/RLM1/HSE*2	CDRE	++		++				
YCL040w	GLK1	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	INH1	STRE/GCR1*2/HSE*2	CDRE	++			+++	+++		
YDL234c	GYP7	STRE*2/GCR1/HSE/RLM1	CDRE	++	+++		+			
YDR055w	PST1	PHO4*2/RLM1*2/HSE		++		+++	+++	++	++	Induced
YDR077w	SED1	RLM1*3/HSE		++		+++	+++	++		Induced
YDR178w	SDH4	STRE/HSE*2	CDDE	++			++	++		
YEL058w	AGM1	STRE*2/RLM1*3/HSE	CDRE	++		++				
YER037w	CDI	STRE*3/HSE	CDDE	++			++	++		
YER150w	SPI1	STRE/RLMI/HSE*4	CDRE	++		++	+++	+++		
YEL 014m	NCB1 USD19	SIRE/GURI/HSE*D		++		++				
1FL014w VCI 027a	DNC1	STRE*4/REMI/HSE STPE*4/PLM1	CDPF	++		+++	+++	+++		
VCI 948m	PDF1	STRE 4/REMI STRE/DI M1/CCD1/HSF	CDRE	++		++	+++	+++		
VCP008e	STE9	STRE/REMI/GORI/HSE		++		+++	++ +++			
VGR032w	FKS9	STUA/PHO4/HSE/RI M1	CDRE	+++		+ +	+++	+++	++	Induced
VGR043c	FR32	STRE/HSE*9	CDRE	+++		+++	+++	++++	+ +	muuceu
VGR138c		GCB1/BLM1/HSE		++			+			
YGR149w		STRE/RLM1/HSE		++			+	++		
YGR153w		STUA*2/HSE	CDRE	++		+ +				
YGR189c	CRH1	STUA*3/GCR1/RLM1*2/HSE*4	CDRE	++	+++	+++	++		++	Induced
YGR244c	LSC2	STRE/RLM1/HSE		++			++	++		
YGR250c		HSE*2	CDRE	++			+	+++		
YHR030c	MPK1	HSE		+ +		+ + +	++	+	++	Induced
YHR087w		STRE*3/STUA/RLM1/HSE		++		+++	+ + +	+ + +		
YHR097c		STRE/GCR1/HSE	CDRE	++	+++	+ +	+++	+++		
YIL029c		RLM1/GCR1/HSE		++						
YIL107c	PFK26	STUA/STRE/PHO4/RLM1/HSE		++		++	++	++		
YIL117c		PHO4/HSE2/RLM1*2		++		+++	+ + +	++		Induced
YJL151c		STRE*2/PHO4/RLM1/HSE		+			+			
YJL155c	FBP26	STRE/GCR1/HSE		++		++	++	+		
YJL171c		STUA*2/RLM1/HSE	CDRE*2	++	+++	++	+			
YKL065c	YET1	STRE*2/HSE		++		++	+	+		
YKL096w	CWP1	HSE		++		+++		+ + +	++	Induced
YKL104c	GFA1	GCR1/RLM1/HSE		++		+++	+		+	
YKL159c	RCN1	STUA/PHO4/HSE*2		++	+++		+			
YKL161c	MLP1	STUA/GCR1/HSE*2	CDDD	+++		+++	++	+		Induced
YKR061w	KTR2	RLM1/HSE2	CDRE	++		+++	++		+	
YKR076w	ECM4	HSE	CDRE	++			+++	++		
YKR091W	SRL3	STRE/STUA/RLM1*2/HSE		+++		+++	+++	+++		
YLR031W		IDL <sup>*4</sup>		++		++				
VI P1916	VDC2	REMI/HSE'Z	CDPF	+++		++	+		± ±	
VLR136c	TIS1	STRE 2/STOR/IISE STRE/PHO4/HSE	CDRE	++	+++	+++	+++	+	+ +	
VLR178c	TSF1	STRE/THO4/HSE*2	ODILL	++			+++	+++		
YLR194c	1.011	STUA/RLM1*2/HSE2	CDRE	+++	+ + +	+++	+++	+	++	Induced
YLR300w	EXG1	STUA/PHO4/RLM1	02.00	+		+++				maaced
YLR303w	MET17	GCR1/PHO4/HSE		++	+++	++	+++	+		
YLR414c		GCR1/STUA/HSE	CDRE	++	+++	+++	++			
YLR438w	CAR2	STRE/HSE	CDRE	++				+++		
YML100w	TSL1	STRE*6/STUA/HSEGCR1		+ +			+ + +	+ + +		
YMR007w		STUA/RLM1/HSE		++	+++		+ + +			
YMR173w	DDR48	STRE*2/PHO4/HSE3		++			+++	+ +		
YMR184w		STRE		++		+ +	++			
YMR238w	DFG5	RLM1*3/HSE3		++	+++	+++	++			Induced
YMR315w		STRE*2/GCR1/RLM1*2/HSE	CDRE	++		++	+ + +	++		
YMR316w		GCR1/HSE		++		+++	+ + +	+		
YNL015w	PB12	STRE/STUA/PHO4/HSE2		++			+ + +	+++		
YNL036w	NCE3	PHO4*3/GCR1/HSE		++		++	+++	+++		
YNL058c		RLM1/HSE3	CDRE	++		++				Induced
YNL157w		STRE*2/STUA/GCR1/HSE2		+						
YNL160w	YGP1	STRE/HSE3	CDRE	+++			+++	+++		Induced
YNL208w		STRE/PHO4/HSEGCR1/RLM1		++	+++	++	+++	+		
YOL053c		STRE*4/HSE	ar	+++						
YOR220w		STRE*2/GCR1/HSE	CDRE*2	++	+++	++	+++	++		
YOR374w	ALD4	STUA/STRE*2/HSEGCR1		++			+	+++		
YPL088w	DOD1	PHO4/KLM1/GCR1/HSE		+++		+++	+++	+		
rPL221w	BOL1	STRE/HSE		++	+++	+++	+		+	
1PR0780 VPR1676	MET16	SIUA/REMI/HSE PHO//CCR1/HSE9		++	+++	+ -	+++	+		

<sup>a</sup> Asterisks indicate more than one copy in the 700-bp DNA noncoding sequence of the gene. <sup>b</sup> +, <2-fold induction; ++, 2-5-fold induction; +++, >5-fold induction. <sup>c</sup> List of genes from Ref.22 that were up-regulated after 30-min incubation in response to 0.2 M CaCl<sub>2</sub>.

<sup>d</sup> 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*( $\Delta cdre$ )-*lacZ* and *FKS2*(CDRE)<sub>c</sub>*lacZ* 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<sub>2</sub> (0.2 M final concentration from a 2 M stock solution) or Calcofluor White (CFW; 50  $\mu$ g/ml final concentration) was added, and cultures were sampled after 30 min (for CaCl<sub>2</sub>-treated cells) or 4 h (for Calcofluor White-treated cells) to measure  $\beta$ -galactosidase activity. The activity of  $\beta$ -galactosidase is expressed as nmol of *o*-nitrophenyl- $\beta$ -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	C t	Treat	ment	$\beta$ -Galactosidase activity				
Strain	Genotype	CFW	$CaCl_2$ FKS2-lacZ		$FKS2 \ (\Delta cdre) - lacZ$	$FKS2 (CDRE)_6$ - $lacZ$		
		µg/ml	тм					
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	$crz1\Delta$	0	0	1.70	0.90	1.22		
BYcrz1	$crz1\Delta$	50	0	11.6	5.11	13.5		
BYcrz1	$crz1\Delta$	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	$gas1\Delta$	0	0	305	75	3297		
JF1409	$rlm1\Delta$	0	0	0.9	0.8	2.1		
JF1409	$rlm1\Delta$	50	0	1.7	1.4	5.6		
JF1409	$rlm1\Delta$	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<sup>2+</sup>/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<sup>2+</sup>/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  $\beta$ -galactosidase in response to cell wall damage were evaluated. As shown in Table VI, the FKS2-lacZ gene fusion showed no significant increase in  $\beta$ -galactosidase activity after 30-min incubation with 200 mM calcium. This is consistent with the data of Yoshimito 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  $crz1\Delta$  strain lacking the transcription factor that binds CDRE. The addition of 50  $\mu$ 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 Calcofluor White experiment was carried out in a  $crz1\Delta$  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  $rlm1\Delta$  strain. As shown in Table VI, the loss of *RLM1* function led to a residual 2-fold increase in the levels of  $\beta$ -galactosidase from the different FKS2-lacZ constructs upon incubation with 50  $\mu$ g/ml Calcofluor White. More interestingly, the effect of high calcium concentration to activate the FKS2 (CDRE)<sub>6</sub> lacZ gene fusion in wild type cells was almost completely abolished in  $rlm1\Delta$ cells. These results illustrate that Ca<sup>+</sup>/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  $\beta$ -1,3- and  $\beta$ -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),  $\beta$ -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  $\beta$ -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 glucosyl/glucanolytransferases (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 nitrogencatabolite 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 wallrelated 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 Pdh1p 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  $G_1$  to S transition (15). This suggestion rests on the fact that Swi4p shares similarity within the APSES domains of StuAp, Sok2p, and Pdh1p (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 wallrelated genes (95). The second important result that illustrates the powerful input of computational analyses in functional genomic 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 Ca<sup>2+</sup>/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/calcineurindependent signaling pathways for optimal activation of this gene in response to cell wall drugs. Another illustration of cross-talks between signaling pathways was that *fks1* 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 wallrelated 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 PCRdirected 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.

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