The Global Transcriptional Response to Transient Cell Wall Damage in *Saccharomyces cerevisiae* and Its Regulation by the Cell Integrity Signaling Pathway*†[S]

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In the yeast *Saccharomyces cerevisiae*, environmental stress conditions that damage the cell wall lead to activation of the so-called “compensatory mechanism,” aimed at preserving cell integrity through remodeling of this extracellular matrix. Here we used DNA microarrays to investigate the molecular basis of this response to two agents that induce transient cell wall damage; namely Congo Red and Zymolyase. Treatment of the cells with these two agents elicited the up-regulation of 132 and 101 genes respectively, the main functional groups among them being involved in cell wall construction and metabolism. The main response does not occur until hours after exposure to the cell wall-perturbing agent. In some cases, this response was transient, but more sustained in others, especially in the case of the genes involved in cell wall remodeling. Clustering of these data together with those from the response to constitutive cell wall damage, revealed the existence of a cluster of co-regulated genes that was strongly induced under all conditions assayed. Those genes induced by cell wall damage showed an enrichment in DNA binding motifs for Rlm1p, Crz1p, SBF (Swi4p/Swi6p), Msn2p/Msn4p, Ste12p, and Tec1p transcription factors, suggesting a complex regulation of this response together with the possible involvement of several signaling pathways. With the exception of *PHO89* and *FKS2*, none of the genes induced by Congo Red was up-regulated in a *slt2* strain. Moreover, characterization of the transcriptional response to Congo Red in a *rlm1* mutant strain revealed that only a few genes (i.e. *PHO89, FKS2, YLR042C*, and *CHA1*) were induced at least partially independently of the transcription factor Rlm1p, the rest being totally dependent on this transcription factor for their activation. Our findings consistently demonstrate that the cell integrity signaling pathway regulates the cell wall damage compensatory response, mainly through transcriptional activation mediated by Rlm1p.

The cell wall is an external envelope that surrounds yeast cells. This structure is essential for maintaining cell morphology and to protect cells from the external environment by preserving their osmotic integrity. In the budding yeast *Saccharomyces cerevisiae*, the cell wall is composed primarily of β-1,3 glucan, β-1,6 glucan, chitin, and mannoproteins (1, 2, 3). These components are linked to each other as macromolecular complexes in which β-1,6 glucan acts as a cross-linker, being attached to the major components, β-1,3 glucan and mannoproteins, and occasionally to chitin (4, 5). β-1,3 glucan is covalently linked to chitin to make up the inner cell wall, which is the main element responsible for the mechanical strength of this structure. Mannoproteins form the outer cell wall layer. Two main classes of proteins are coupled covalently to cell wall polysaccharides: GPI-dependent cell wall proteins (GPI-CWPs), which are generally linked to β-1,3 glucan through a β-1,6 glucan chain, and PIR proteins (PIR-CWPs), which are directly linked to β-1,3 glucan (6, 7). For some of these cell wall mannoproteins, several functions have been characterized, including adhesion and cell wall construction (for a review see Ref. 3), although in many cases their precise function is unknown.

The cell wall cannot be a static structure since it needs to adapt to its surrounding growth conditions as the cell increases in size, and it also needs to be remodeled during morphogenetic processes such as mating, sporulation, or pseudohyphal growth (2, 8, 9). Moreover, in its attempt to survive the cell wall can change in composition and/or structure in response to environmental stress (3, 10). The existence of this cellular response, which has been called “compensatory mechanism,” clearly illustrates the dynamic nature of the cell wall. This cell wall salvage response involves: (i) a remarkable increase in the chitin content; (ii) changes in the association between cell wall polymers (while only 2% of CWPs are linked directly to chitin in a wild-type cell, this linkage is 20-fold more abundant in *gas1* cells) (11); (iii) an increase in the bulk of CWPs; and (iv) a transient redistribution of β-1,3 glucan synthase complex throughout the cell (12). All these mechanisms are involved in the repair of the cell wall under stress conditions.

Although we are now beginning to understand some aspects of the regulation of cell wall construction, exactly how all these processes are regulated is still largely unclear. In addition to the activation of the *SLT2*-MAPK pathway in response to several environmental stimuli, such as high temperature (13),

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplementary Data.

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1 The abbreviations used are: GPI-CWP, glycosylphosphatidylinositol-dependent cell wall proteins; MAP, mitogen-activated protein; MAPK, MAP kinase; ORF, open reading frame; CR, Congo Red.
contribution of these proteins and their corresponding signal-

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strains (BY4741 background) present the corresponding gene com-

KRE6, GAS1, KNR4, FKS1) with the expression profiles of wild-type cells (19). In addition to confirming the involvement of the SLT2-MAP kinase pathway, this genome-

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kinase (16). Additionally, mutant cells with a weakened cell wall, such as fks1Δ, gas1Δ, mnn9Δ, and others, show a constitutive activation of this pathway (18, 19). Several cell membrane proteins (Mid2p, Mlt1p, and Wsc1–4p) (16, 20, 21), should act as sensors for cell wall damage, activating the MAP kinase cascade (22). The phosphorylated Sltp2 finally activates several transcription factors, leading to changes in gene expression. Currently, two transcription factors have been identified as targets of Sltp2: the MADS-box transcription factor Rlm1p (23–25) and SBF (26). This latter is a heterodimeric complex of two proteins, Swi4p and Swi6p, that are involved in the activation of gene expression at the G1/S transition (27). Signaling through Rlm1p regulates the expression of many genes involved in cell wall biogenesis (28). Other works have also involved the Sho1p-Kss1p (29) and the HOG1-MAP kinase pathways (3, 30) in the control of the cell integrity. More recently, using DNA microarray technology we obtained a comprehensive view of the cellular response to constitutive cell wall damage by comparing the transcriptome of mutant cells deleted in several genes important for the construction of the cell wall (KRE6, GAS1, KNR4, FKS1) with the expression profiles of wild-type cells (19). In addition to confirming the involvement of the SLT2-MAP kinase pathway, this genome-wide analysis also led to the identification of the Calcineurin/Crz1p signaling pathway and the regulatory machinery from the general stress cellular response as regulators of the response to cell wall damage. Although the genomic characterization of the transcriptional response to cell wall mutations has allowed us to decipher relevant molecular aspects of the cell wall compensatory mechanism (19), important clues regarding the kinetics and regulation of the response were lacking. In order to obtain additional insight into these aspects, we used an alternative approach involving the characterization of the transcriptional yeast response to the presence of two drugs, Congo Red and Zymolyase, which are known to cause cell wall damage and activate the Sltp2-MAP kinase pathway (17). The global transcriptional response to transient cell wall damage demonstrates that the larger fraction of genes induced are devoted to cell wall remodeling in order to ensure cell integrity under cell wall damage-inducing growth conditions. The direct role of the cell integrity pathway in the response was analyzed using mutant strains in some elements of this pathway. The expression profiles of strains deleted in SLT2 and RLM1 disclosed the essential contribution of these proteins and their corresponding signaling pathway to the regulation of the response.

EXPERIMENTAL PROCEDURES

Strains—All experiments were performed with the S. cerevisiae BY4741 strain (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and mutant derivatives provided by Euroscarf (Frankfurt, Germany). Mutant strains (BY4741 background) present the corresponding gene completely deleted and replaced by the Genetica resistance-codifying KanMX4 module.

Culture Conditions—For routine cultures, S. cerevisiae was grown on YEPD (1% yeast extract, 2% peptone, 2% glucose). When necessary, Genetica (200 mg/liter) (Invitrogen) was added. Yeast cells were grown overnight at 24 °C to an optical density of 0.8–1 (OD600). The culture was refreshed to 0.2 OD and grown at 24 °C for 2 h 30 min. Next, the culture was divided into two parts. One part was allowed to continue growing under the same conditions (the non-treated culture) while the other one was supplemented with Congo Red to a final concentration of 30 μg/ml. The severity of the treatment was calibrated to preserve more than 90% of cell viability. The mechanism of action of this drug is not known, although its interference with proper cell wall assembly has been documented (31). At this time, cells (7 × 106) were collected by centrifugation (t = 0 h sample). Then, for the wild-type strain cultures were collected at centrifugation by 0.5, 2, 4, and 6 h of growth, frozen at −80 °C, and processed for RNA extraction (see below). For the rlm1Δ and slt2Δ strains, cells were collected at 4 h. For the Zymolyase experiments, the procedure was identical but the medium was supplemented with 5 units/ml of Zymolyase 10140F (ICN Biomedicals Inc, Aurora, OH), and the times studied were 0 and 2 h.

Preparation of Yeast Extracts and Western Blotting—Cells were grown, treated with Congo Red, and collected as described above. Lysis, collection of proteins, fractionation by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes, as well as the de-

HOG1
–

SLT2

kinase (Thr202/Tyr204) (antibody New England Biolabs, Beverly, MA) were performed as described by Martin et al. (32). Mouse anti-actin mon-

InSb1p

Nova Midi kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. RNA concentrations were determined by measuring absor-

RNA Isolation—Total RNA was isolated from exponentially growing cells (5 × 108) by the “mechanical disruption protocol” using the RNeasy Midi kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. RNA concentrations were determined by measuring absorbance at 260 nm. RNA purity and integrity were assessed using RNA Nano Labchips in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) following the manufacturer’s instructions.

DNA Synthesis and Chip Hybridization—DNA was synthesized from 25–30 μg of total RNA by reverse transcription, using the CyScri-

Calcineurin

Calcineurin/Crz1p signaling pathway and the regulatory machinery from the general stress cellular response as regulators of the response to cell wall damage. Although the genomic characterization of the transcriptional response to cell wall mutations has allowed us to decipher relevant molecular aspects of the cell wall compensatory mechanism (19), important clues regarding the kinetics and regulation of the response were lacking. In order to obtain additional insight into these aspects, we used an alternative approach involving the characterization of the transcriptional yeast response to the presence of two drugs, Congo Red and Zymolyase, which are known to cause cell wall damage and activate the Sltp2-MAP kinase pathway (17). The global transcriptional response to transient cell wall damage demonstrates that the larger fraction of genes induced are devoted to cell wall remodeling in order to ensure cell integrity under cell wall damage-inducing growth conditions. The direct role of the cell integrity pathway in the response was analyzed using mutant strains in some elements of this pathway. The expression profiles of strains deleted in SLT2 and RLM1 disclosed the essential contribution of these proteins and their corresponding signaling pathway to the regulation of the response.

Microarray Image Analysis—Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA) at a res-

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Response to Cell Wall Damage in Yeast

The microarray data described here follow the MIAME recommendations and has been deposited at the NCBI gene expression and hybridization array data repository (GEO, www.ncbi.nlm.nih.gov/geo/) with GEO accession numbers GSE959, GSE960, GSE961, GSE962, GSE963, GSE964, GSE965, and GSE966.

Promoter Analysis—For each gene induced at least 2-fold under the different conditions tested, 800-bp upstream from their start codon (ATG) were analyzed using the MEME algorithm (meme.sdsc.edu/meme/website/intro.html), the Regulatory Sequence Analysis Tools (rsat.ulb.ac.be/rsat) and Reduce (lognormal.bio.columbia.edu/reduce/), to identify possible common DNA motifs. Regulatory Sequence Analysis Tools were also used to search for specific sequence motifs.

Quantification of mRNAs using Real-time Quantitative RT-PCR—First-strand cDNAs were synthesized from 2.5 μg of total RNA, using the Reverse Transcription System (Promega) and following the recommendations of the manufacturer. As controls for genomic contamination, the same reactions were performed, but in the absence of reverse transcriptase. Real-time PCR was performed using an ABI 7700 instrument (Applied Biosystems) in a final volume of 25 μl containing 5 μl of a 100-fold dilution of the RT reaction and 12.5 μl of the 2× SYBRGreen Universal Master Mix (Applied Biosystems) together with the specific forward and reverse primers (Sigma), designed using the Primer Express Software 2.0 (Applied Biosystems). Real-time PCR conditions were selected according to the Universal conditions (default conditions) recommended by the manufacturer of the instrument. Each cDNA was assayed at least in duplicate PCR reactions. After amplification, a melting curve analysis was performed to verify the specificity of the reaction. Basic analysis was performed using the SDS 1.9.1 software (Applied Biosystems). For quantification, the abundance of each gene was determined relative to the standard transcript of ACT1 and the final data of relative gene expression between the two conditions tested on each microarray were calculated following the 2−ΔΔCT method, as described in (35). The following forward and reverse primers, respectively, were used: ACT1, 5′-ATACCGGGTTTTGGCTCCAT-3′ and 5′-CCATATCGACCAAGGATAGTCTTCT-3′; CRH1, 5′-ACTACCCAGTATACGGAAATACACA-3′ and 5′-TCACACCGTCTTGAAAATGTTGGAACA-3′; PIR3, 5′-CTCATATCTGGTCACCAAGACAGAT-3′ and 5′-CACCAACGCACTAGATAGCAG-3′; CWC4, 5′-TCACTACGCAAGGCTGCTGTTGGAAATTACC-3′ and 5′-TAAACCGCGGATAAGGTTTTGAGTAGA-3′; PRM5, 5′-AGACATAAGAAGACGGCCCCAAA-3′ and 5′-AGCATTTATCGTACCCATCATCTTCTTCT-3′; MLP1, 5′-TGTAATTCACAAAGATGCACAAACG-3′ and 5′-TCTTCTTCTCTACACATGTGT-3′; PIR809, 5′-ACTGGTCA-TGGTACCACTTGGT-3′ and 5′-TCATACACCAACAGCAATAG-3′; MLP1, 5′-AGCATTAAGAAGACGGCCCCAAA-3′ and 5′-AGCATTTATCGTACCCATCATCTTCTTCT-3′; CHA1, 5′-ATTCAAAAGTGGTTGAAGATTGTT-3′ and 5′-TGCTTCTCTCCCCACCACAT-3′; CHS3, 5′-TTGGGATACGAAGCTAGATGTT-3′ and 5′-TACGGGACCGAATACATCAGA-3′; FKS1, 5′-TGCTTCTCTCTGATACATCATATCATCAGA-3′; YPR1, 5′-GGACCTGATGTTTTTGATGACGCA-3′ and 5′-ACGGTGCGGAAAATATCCC-3′; FKS2, 5′-CGCTAAACCTCCTCCTCCT-3′ and 5′-TGACAGATGCATAGATCCTAC-3′.

Congo Red Sensitivity—Cells were grown overnight in YEPD and adjusted to an optical density at 600 nm (OD600) of 0.13 (±2×106 cells per ml). Five microliters of samples plus three serial 1:10 dilutions were spotted onto plates of YEPD supplemented with Congo Red at a final concentration of 100 μg/ml. Growth was monitored after 2 days at 28°C.

RESULTS

Global Analysis of the Transcriptional Response to Transient Cell Wall Damage Caused by Congo Red Treatment in Yeast—

The aim of this study was to characterize, in a comprehensive way, the response of the yeast Saccharomyces cerevisiae to the presence of transient cell wall damage and how this response is regulated. To achieve this, we exposed yeast cells, in the logarithmic growth phase, to 30 μg/ml of Congo Red (CR) and DNA microarrays were used to analyze the gene expression of 6306 open reading frames encoded by the yeast genome. We characterized the pattern of gene expression at time 0, 2, 4, and 6 h after exposure of the yeast to CR. The time-course experiments were performed by comparing the abundance of mRNA at each time in cells growing in the presence of CR relative to yeast cells growing in the absence of the drug (see “Experimental Procedures” for details). A complete data set of the genes induced in our study is shown in Table I. The analysis revealed that 132 genes were induced at least 1.8-fold at one or more of the time points tested (67 genes after 2 h, 58 genes after 4 h, and 75 genes after 6 h).

We then distributed the significant responsive genes into functional categories according to YPD/SGD classification. As shown in Table I, the main functional groups of up-regulated genes belong to those involved in cell wall organization and biogenesis, metabolism and the generation of energy, together with unknown genes. From the large group of 34 cell wall-related genes, four different sets can be highlighted. The first subgroup comprises 15 genes out of the 60–70 genes encoding GPI-cell wall proteins (GPI-CWPs) so far identified in the yeast genome (34). Of special relevance are proteins such Cwp1p, which has been implicated in cell wall strengthening (35); Pst1p, which is secreted from protoplasts under conditions of cell wall regeneration (36); and Crh1p and Crh2p, two putative transglycosidases involved in the cross-linking between yeast cell wall polymers (37, 38). A second set in this group includes PIR1, PIR2/HSP150, PIR3, and PIR4/CIS3, encoding for PIR-CWPs. The products encoded by these genes are directly linked to β-1,3 glucan (6). Deletion of all four genes results in swollen cells that are sensitive to Calcofluor White and Congo Red (6) while overexpression of PIR2 confers resistance to the antifungal protein osmotin (39). A third subgroup encompasses four genes involved in chitin synthesis: namely, GFA1, encoding the glutamine-fructose-6-phosphate amidotransferase, an enzyme involved in the first steps of the chitin synthesis pathway (40); CHS3, coding for the enzyme responsible for the synthesis of the bulk of chitin, both at the lateral cell wall and at the ring between mother and daughter cells (41); CHS1, which contributes to repairing mechanisms during cytokinesis (42); and PCM1, which is involved in the biosynthesis of N-acetylglucosamine (43). The presence of these genes is consistent with the finding that cell wall damage causes an increase in chitin deposition (44). The fourth set of cell wall related genes up-regulated under our conditions contains those involved in the biosynthesis and degradation of β-1,3 and β-1,6 glucan. This group includes FKS1, FKS2, KRE6, KRE11, and genes encoding exoglucanase (EXG1 and EXG2) (45) and endoglucanase/gluconosyl transferase activities (BGL2) (46). Finally, KTR2, encoding for a mannosyltransferase of the KRE2 family (47), and PSA1, coding for the mannose-1-phosphate guanylyltransferase (48), both required for the biosynthesis of mannoproteins, were also up-regulated.

The induction of the functional cluster of genes related to the metabolism of carbohydrates, amino acids, and sphingolipids was not unexpected. In particular, active remodeling of the cell wall would require a considerable degree of carbohydrate mobilization. Prolonged treatment with CR (6 h) also leads to the activation of genes involved in phosphate metabolism.

Interestingly, we found a cluster of genes related to the SLT2-MAP kinase pathway that were clearly induced at 2–4 h after addition of the drug (Table I), the gene most strongly induced within this cluster being MLP1 (YKL161C). The role of MLP1 in the cell integrity pathway has not been yet elucidated, but it is known to encode a protein kinase-like homologous to Sit2p that interacts with the Rlm1p transcription factor (24).

In contrast to the transcriptional induced response, 111 genes were repressed under the CR conditions studied, most of them (93 genes) being repressed after prolonged treatments (6 h). One of these genes was SWI6 that encode for one of the transcription factors of the SBF complex. The data concerning expression for the repressed set of genes are shown in Table II,
| ORF   | Gene  | CR 2h | CR 4h | CR 6h | Zym 2h | Function                        |
|-------|-------|-------|-------|-------|-------|--------------------------------|
|       |       |       |       |       |       | Cell wall organization and biogenesis |
| YLR042C | CWP1  | 2.0   | 2.6   | 2.6   | 14.0  | GPI-CWP                        |
| YKL096W | CRH1  | 2.9   | 2.6   | 2.4   | 2.9   | GPI-CWP                        |
| YER150W | SP1   | 1.8   | 2.1   | 3.1   | 2.6   | GPI-CWP; endopeptidase          |
| YNL192W | CHS1  | 2.7   | 2.0   | 3.6   |       | Chitin biosynthesis            |
|        |       |       |       |       |       | Cell septation (CW)            |
|        |       |       |       |       |       | Cell polarity                  |
| YGR282C | TIR2  | 1.9   | 1.9   | 1.9   | 1.9   | β-1,3 glucan synthase          |
| YRP159W | KRE11 | 2.0   | 2.1   | 2.0   | 2.1   | 1,6 β-glucan biosynthesis      |
| YBL043W | ECM13 | 2.0   | 2.0   | 2.0   | 2.0   | Putative CW-related protein     |
| YOR210C | VRP1  | 1.9   | 1.8   | 1.9   | 1.8   | Putative CW-related protein     |
| YMR374W | DDR2  | 3.3   | 3.5   | 3.5   | 3.5   | MAP kinase (cytokinesis)       |
| YDL162W | CWP3  | 2.4   | 2.6   | 2.6   | 2.6   | Putative regulation of cell cycle |
| YGL157W | PNC1  | 2.1   | 2.1   | 2.1   | 2.1   | Sensor of CW integrity pathway  |
| YGL008C | CAK1  | 2.4   | 2.4   | 2.4   | 2.4   | Cdk-activating kinase           |
| YDL022W | GPD1  | 9.6   | 7.7   | 10.3  | 7.7   | Putative aryl dehydrogenase     |
| YHR209W | FBP26 | 3.1   | 2.3   | 2.8   | 2.5   | Glycerol-3-P dehydrogenase      |
| YED121C | ARO10 | 1.9   | 2.0   | 2.0   | 2.0   | Putative methyltransferase      |
| YDL060W | ALD4  | 2.0   | 2.0   | 2.0   | 2.0   | Putative purine metabolism      |
| YOR374W | ALD4  | 2.0   | 2.0   | 2.0   | 2.0   | Putative purine metabolism      |
| ORF     | Gene   | CR  | CR  | Zym                                    | Function                                      |
|---------|--------|-----|-----|----------------------------------------|-----------------------------------------------|
| YER062C | HOR2   | 2.8 |     | m-glycerol-P phosphatase.              |                                               |
| YCL064C | CHA1   | 3.7 |     | Serine and threonine catabolism        |                                               |
| YHR104W | GRE3   | 2.4 |     | Aldoketo reductase                     |                                               |
| YIR038C | GGT1   | 2.3 |     | Glutathione transferase                |                                               |
| YLR069C | PRB1   | 2.1 | 1.8 | Protein degradation                    |                                               |
| YCR036W | RRK1   | 1.9 |     | Carbohydrate metabolism               |                                               |
| YHR201C | PPX1   | 1.9 | 2.1 | Exopolyphosphatase activity            |                                               |
| YPL231W | FAS2   | 1.9 |     | Fatty acid biosynthesis                |                                               |
| YNR091C | CTT1   | 1.8 | 1.8 | TCA cycle; Citrate synthase            |                                               |
| YPR121W | THI22  | 1.8 |     | Thiamin biosynthesis                   |                                               |
| YKL053W | RHR2   | 1.9 |     | Glycerol metabolism                    |                                               |
| YKR001C | MET14  | 1.8 |     | Sulfate assimilation                   |                                               |
| YFL004C | PDC1   | 2.0 |     | Energy; Putative oxidoreductase        |                                               |
| YDL052C | SLC1   | 1.9 |     | Phospholipid biosynthesis              |                                               |
| YGL157C | COX4   | 1.9 |     | Cytochrome c oxidase                   |                                               |
| YOL086C | ADH1   | 1.8 |     | Energy; Alcohol dehydrogenase          |                                               |
| YPL257C | ALG5   | 1.9 |     | Dolichyl-P β-glucosyltransferase       |                                               |
| YER055C | HIS1   | 2.0 |     | Histidine biosynthesis                 |                                               |
| YLR372W | SUR4   | 2.3 |     | Sphingolipid biosynthesis              |                                               |
| YKL053C | YSR3   | 1.9 |     | Sphingolipid metabolism                |                                               |
| YLR359W | ADE13  | 2.0 |     | Purine metabolism                      |                                               |
| YML123C | PHO84  | 4.7 |     | Phosphate metabolism, transporter      |                                               |
| YBR093C | PHO5   | 3.0 |     | Phosphate metabolism (hydrolyase)      |                                               |
| YBR092C | PHO3   | 1.9 |     | Thimiane transport                     |                                               |
| YHR215W | PHO12  | 0.4 | 2.3 | Phosphate metabolism (hydrolase)       |                                               |
| YLR140W | VPI1   | 1.9 |     | Protein aminosacid acetylation         |                                               |
| YKR063W | OSH6   | 3.7 |     | Sterol homeostasis                     |                                               |
| YDR074W | TPS2   | 1.8 |     | Glucose and fructose metabolism        |                                               |
| YOL151W | GNE2   | 2.2 |     | Methylyglycol catabolism               |                                               |
| YPL057C | SUR1   | 2.8 | 2.3 | Sphingolipid biosynthesis              |                                               |
| YGR008C | STF2   | 2.3 |     | ATPase stabilizing factor              |                                               |
| YPR062C | YLM1   | 2.8 | 2.1 | Transcription factor (integrity path.) |                                               |
| YGR097W | ASK10  | 1.8 | 1.8 | Two-component signal transduction      |                                               |
| YKR062W | TFA2   | 1.8 |     | RNA pol II transcription factor TFIIH  |                                               |
| Transcription | | | | | |
| YPL089C | RLM1   | 2.8 |     | Putative transport related             |                                               |
| YGR097W | ASK10  | 1.8 | 1.8 | Putative transport related             |                                               |
| Transport | |     | | | |
| YLR036W | PEPI1  | 2.8 | 2.9 | Putative transport related             |                                               |
| YJR042W | NUP85  | 2.3 |     | Putative transport related             |                                               |
| YHR094C | HXT1   | 1.9 |     | Putative transport related             |                                               |
| YDR147C | ITR1   | 2.2 |     | Putative transport related             |                                               |
| YOR306C | MCH5   | 3.7 | 2.2 | Putative transport related             |                                               |
| YJR075C | MIR1   | 2.0 |     | Putative transport related             |                                               |
| YDL145C | COF1   | 1.9 | 1.9 | Putative transport related             |                                               |
| YLR223W | PDE5   | 2.2 |     | Putative transport related             |                                               |
| YBR296C | PHO89  | 3.6 |     | Putative transport related             |                                               |
| YOL122C | SFI1   | 2.6 | 1.8 | Putative transport related             |                                               |
| YKR062C | TFA2   | 1.8 |     | Putative transport related             |                                               |
| Cell cycle | |     | | | |
| YKL018W | SLD2   | 2.6 | 2.3 | DNA replication                        |                                               |
| YLR178C | TFS1   | 1.8 |     | DNA replication                        |                                               |
| YMR199W | CLN1   | 1.8 |     | DNA replication                        |                                               |
| YIL123W | SIM1   | 1.8 |     | DNA replication                        |                                               |
| Protein synthesis | |     | | | |
| YNL301C | RP28B  | 3.3 |     | Constituent of ribosome                |                                               |
| YOR312C | RPL18A | 2.0 |     | Constituent of ribosome                |                                               |
| YLR075W | GRC5   | 1.8 |     | Constituent of ribosome                |                                               |
| YFR081C-A | RPL5B | 2.0 |     | Constituent of ribosome                |                                               |
| YNL178W | RPS3   | 2.2 |     | Constituent of ribosome                |                                               |
| YDR148W | RPL15A | 2.3 |     | Constituent of ribosome                |                                               |
| YNL067W | RPL9B  | 1.8 |     | Constituent of ribosome                |                                               |
| YGL031C | RPL30A | 2.1 |     | Constituent of ribosome                |                                               |
| YBR031W | RPL4A  | 1.9 |     | Constituent of ribosome                |                                               |
| YOR313W | TCM1   | 2.2 |     | Constituent of ribosome                |                                               |
| YNL053C | RPS16B | 2.2 |     | Constituent of ribosome                |                                               |
| YIL133C | RPL16A | 2.2 |     | Constituent of ribosome                |                                               |
| mRNA metabolism | |     | | | |
| YER032W | FIR1   | 2.4 |     | mRNA processing.                      |                                               |
| YNI008C | SLU7   | 2.4 |     | mRNA processing.                      |                                               |
| YOR204W | DED1   | 2.4 |     | mRNA processing. Helicase              |                                               |
| YLL013C | PUF3   | 2.4 |     | mRNA processing. Helicase              |                                               |
| Other functions | |     | | | |
| YKR061C | VPS1   | 3.2 |     | Vascular sorting (GTPase)              |                                               |
| YJL118C | NCA3   | 2.6 |     | Mitochondriogenesis                    |                                               |
| YLR27C | ORP19  | 2.6 |     | Organelle biogenesis                   |                                               |
| YBL078C | AUT7   | 2.6 |     | Autophagy. Vesicle transport           |                                               |
| YML130C | ERO1   | 2.2 |     | ER disulfide bond formation            |                                               |
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the main functional categories included in this response being devoted to metabolism (28%), RNA processing (10%), together with unknown genes (21.6%).

In order to validate the expression profiles obtained by microarray analysis, real-time quantitative RT-PCR was performed. For this analysis, a subset of genes including different levels of induction was selected. The same RNAs used in the microarray experiments to analyze the expression profile after 4 h of CR treatment were used for the Q-RT-PCR assays. As shown in Table III, in general we found a very good correlation between both data sets, supporting the validity of the microarray experiments. Additional detailed information of the complete data sets of microarray experiments is available on the World Wide Web at www.ucm.es/info/mfar/.

Kinetics of the Cellular Response to Cell Wall Damage—In contrast to many other environmental stresses that activate rapid cellular responses, the response to cell wall damage does not occur until hours after exposure to the cell wall-perturbing agent. The global transcriptional response 30 min after the addition of CR revealed only a small set of genes that were significantly activated, including YLR194C, YIL117C (PRM5), YDR055W (PST1), and YJL171C, all of them being highly up-regulated at longer times. Detailed analysis of the CR temporal expression pattern (Table I) revealed that the global response to cell wall stress was transient in some cases, but more sustained in others. About 50% of the up-regulated genes were induced at 2 h after the start of drug treatment, 34% of them, maintaining a sustained response until 6 h. In contrast, 34% were only induced at 2 h, and 31% at 2 to 4 h. In addition, one group of genes (12%) was induced only at 4 h and, finally, a set of about 38% of differentially expressed genes was induced late in the response (6 h).

Interestingly, the largest set of genes with a sustained response along the time of the kinetic study belonged to the family of genes involved in cell wall construction, underscoring the importance of these genes in the preservation of cell integrity. Within this functional family, we found four different temporal patterns: (i) genes with a sustained increase in induction throughout the kinetics (CWP1, PIR3, YPS3, FKS1, PIR1, and CIS3) (Fig. 1A); (ii) genes that peaked at 2 h and then fell, in some cases being maintained over 2-fold after 6 h (YLR194C, GFA1, PST1, SED1, YJL171C, CRH1, CHS1, YLR040C, BGL2, KRE6, KRE11, HSP150, and YPS1) (Fig. 1B); (iii) genes that peaked at 4 h (EXG2, FKS2, CHS3) (Fig. 1C); and (iv) genes that were induced late in the response (TOS6, TIR2, SVS1, EXG1, SUN4, DSE2, CRH2, PDA1) (Fig. 1D). The levels of induction in this latter group were in general low.

Global Analysis of the Transcriptional Yeast Response to Zymolyase Treatment—To test whether other types of cell wall damage might lead to a similar transcriptional response, we next analyzed the expression profile of yeast cells growing in the presence of 5 units/ml of Zymolyase100T for 2 h. Zymolyase treatment affects cell wall integrity owing to the presence of β-1,3 glucanase and mannanase activities in its composition. The response to this drug included the up-regulation of 101 genes (Table I) and the repression of about 175 genes; the

### Table I—continued

| ORF       | Gene     | CR  | CR  | CR  | Zym |
|-----------|----------|-----|-----|-----|-----|
| YDR068C   |          |     |     |     |     |
| YMR173W-A |          |     |     |     |     |
| YNL294C   |          |     |     |     |     |
| YOR161C   |          |     |     |     |     |
| YPR211W   |          |     |     |     |     |
| YKL065C   |          |     |     |     |     |
| YDR262W   |          |     |     |     |     |
| YMR173W-A |          |     |     |     |     |
| YNL294C   |          |     |     |     |     |
| YOR161C   |          |     |     |     |     |
| YPR211W   |          |     |     |     |     |
| YKL065C   |          |     |     |     |     |
| YDR262W   |          |     |     |     |     |
| YJL161W   |          |     |     |     |     |
| YRL065C   |          |     |     |     |     |
| YMR184W   |          |     |     |     |     |
| YMR295C   |          |     |     |     |     |
| YOR247W   |          |     |     |     |     |
| YKL065C   |          |     |     |     |     |
| YDR262W   |          |     |     |     |     |
| YJL161W   |          |     |     |     |     |
| YRL065C   |          |     |     |     |     |
| YMR184W   |          |     |     |     |     |
| YMR295C   |          |     |     |     |     |
| YOR247W   |          |     |     |     |     |
| YKL065C   |          |     |     |     |     |
| YDR262W   |          |     |     |     |     |
| YJL161W   |          |     |     |     |     |
| YRL065C   |          |     |     |     |     |

The response to this drug included the up-regulation of 101 genes (Table I) and the repression of about 175 genes; the
induced by Zymolyase; namely, SBE22, a gene involved in the transport of Chs3p to the membrane (49), SPI1 encoding a GPI-CWP, ECM13 (50); and PAU6 coding for a PIF-CWP. In common with both drug treatments was the up-regulation of genes involved in the Slt2p-signaling pathway, indicating a major role of this mechanism under these conditions. Some of the significant differences between both stimuli were found in the groups of genes related to metabolism and in genes of unknown function (Table I).

Promoter Analysis of the Up-regulated Genes—We reasoned that the identification of multiple transcription factor binding sites in the promoters of genes commonly induced under conditions eliciting cell wall damage should give an idea of the inputs coming from different regulatory signaling cascades involved in the regulation of this response. Using the RSAT program (51), we analyzed DNA binding motifs in the 800 bp non-coding upstream sequences of genes that were up-regulated at least 2-fold by CR at all the time points studied, and by Zymolyase. The algorithm used by this program localizes regulatory motifs that appear more frequently than would be expected if they had a random distribution. We also searched for DNA sequences that matched DNA motifs annotated in the databases (52). The most important results from this analysis can be seen in Table IV, showing the percentages of induced genes containing the corresponding DNA consensus motifs compared with their random appearance in the genome. Under all the conditions studied, we found the highest enrichment in genes carrying motifs for Rlm1p, suggesting a major role for the cell integrity pathway mediated by the MAP kinase Slt2p in the regulation of the cell wall compensatory transcriptional response. Thus, as shown in Fig. 2, the levels of the active dually phosphorylated form (Thr^130–Tyr^132) of Slt2p were clearly increased in response to treatment with Congo Red at the different times. Additionally, the significant enrichment in genes bearing motifs for the transcription factors Crz1p (53),

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TABLE II

Classification into functional families of ORFs whose transcripts are down-regulated at least 2-fold in any of the different Congo Red treatments assayed

| Cell Cycle | MUM2 (6h), WWM1 (6h), NAP1 (6h), RRD2 (6h), TFS1 (6h), YIL041W (6h), YOR023C (6h) |
| Cell Wall | QBR1 (6h), YIL169C (4h) |
| Mating | TPS1, FUS1 (6h), FIG2 (4h) |
| Metabolism | IMD1 (4h, 6h), SFA1 (6h), HSP78 (6h), CYC7 (6h), SER3 (4h), UBP5 (6h), FPL2 (6h), GSY1 (4h, 6h), ASN2 (6h), SOL4 (6h), FMR12 (2h), PFK26 (6h), GAT2 (6h), CDD1 (6h), GLO1 (6h), YML1 (6h), SGT2 (6h), BPT4 (6h), PEX19 (6h), EUG1 (6h), YBR056W (6h), YDR236C (6h), YHR112C (6h), YLR098C (4h), YMR323W (2h), YPR127W (6h), YHR080C (6h) |
| Morphogenesis | STU1 (6h), HSP42 (6h), TWF1 (6h), CTK1 (2h), KEL3 (6h), BFR1 (6h) |
| Nuclear | EBS1 (6h), APC5 (6h) |
| Protein Synthesis | YGR201C (6h) |
| Transcription factors | SWI6 (6h) |
| Stress | HSP26 (2h), YSA1 (6h), JCS2 (6h), KTI12 (6h), YHR029C (6h) |
| Transcription and/or RNA processing | MFCp (6h), KRR1 (6h), BDP2 (6h), NPL3 (6h), SIK1 (6h), CWC24 (6h), NPI46 (6h), GAT2 (6h), HSP42 (6h), RPC34 (6h), DBP10 (6h) |
| Transport | PHO3 (2h), OPT1 (4h), GAPI1 (4h), ATG19 (6h), COT1 (6h), GDRI19 (6h), FIT2 (6h), OSH1 (6h) |
| Others | TEC1 (6h), KAR4 (6h), PEX3 (6h), RAD23 (6h), TOM71 (6h), LAP4 (4h, 6h), YAF9 (6h), HCH1 (6h), YER076C (6h), YIL080W (6h) |
| Unknown | YSW1 (6h), GIR2 (6h), TC1 (2h), ZRS8 (6h), TOS5 (6h), ICS2 (6h), NCE102 (2h), YBR063C (6h), YDR367W (2h), YDR533C (6h), YGR127W (6h), YHR139C-A (2h), YIL082W-A (4h, 6h), YJL135W (2h), YIL212C (6h), YKL030W (2h), YKL083W (6h), YLR217W (6h), YNL120C (6h), YNR024W (6h), YOR052C (6h), YOR331C (6h), YPL245W (6h), YPR045C (6h) |

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TABLE III

Confirmation by real-time quantitative RT-PCR of some selected genes

| ORF  | Gene name | CR+/CR- Arrays | Q-RT-PCR fold |
|------|-----------|----------------|---------------|
| YBR202C | CHS3 | 2.1 | 2.0 |
| YBR296C | PHO89 | 3.6 | 4.7 |
| YCL064C | CHA1 | 3.7 | 1.9 |
| YDR261C | EXG2 | 1.9 | 1.9 |
| YDR451C | YPH1 | 1.1 | 1.1 |
| YGR032W | FKS2 | 3.2 | 6.8 |
| YGR189 | CRH1 | 2.6 | 3.0 |
| YIL117C | PRM5 | 3.8 | 4.7 |
| YKL161C | MPL1 | 9.4 | 9.8 |
| YKL163W | FRR3 | 5.0 | 4.0 |
| YLR042C | FKS1 | 2.6 | 2.7 |
| YLR342W | FKS1 | 1.8 | 1.5 |
| YLR390W-A | CCW14 | 2.0 | 2.5 |
| YNL289W | PCL1 | 1.1 | 1.2 |

The complete data set is available on the World Wide Web at www.ucm.es/info/mfar/. In general, comparative analysis of the genes induced by CR and Zymolyase revealed a similar response pattern in terms of the number of induced genes within the different functional clusters (Cell Wall: CR-34/Zym-25; Metabolism: 29/20; Signal Transduction: 7/5; Transcription: 2/2; Morphogenesis: 6/2; Stress: 4/6; Transport: 8/5; Protein synthesis: 12/0; Cell cycle: 3/2; Mating: 2/1; RNA metabolism: 2/2; Others: 3/7; unknown: 22/24). A more detailed analysis indicated the existence of certain specific CR and Zymolyase effects on gene expression. Within the core group of cell wall-related genes induced by CR and not by Zymolyase we found CHS3, FKS2, PSA1, TOS6, DSE2, CRH2, SUN4, FPR1, FKS1, YPS1, KRE6, KTR2, and EXG2, most of them induced late (4–6 h) in the presence of CR. Additionally, 3 genes were specifically induced by Zymolyase; namely, SBE22, a gene involved in the transport of Chs3p to the membrane (49), SPI1 encoding a GPI-CWP, ECM13 (50); and PAU6 coding for a PIF-CWP. In common with both drug treatments was the up-regulation of genes involved in the Slt2p-signaling pathway, indicating a major role of this mechanism under these conditions. Some of the significant differences between both stimuli were found in the groups of genes related to metabolism and in genes of unknown function (Table I).
SBF (Swi4p/Swi6p) (26), Msn2p/Msn4p (54), Ste12p (55), and Tec1p (56) (Table IV) suggests a more complex regulation of this response, with the possible participation of other signaling pathways. A clear increase occurs in genes carrying binding sites for Tec1p in their promoters in the late response to CR (after 6 h of Congo Red treatment), concomitant with a decrease in the proportion of genes carrying Rlm1p binding sites; this suggests that the Kss1p-mediated pseudohyphal signaling pathway could have a regulatory role at this time.

Comparison of the Transient Cell Wall Damage Response with Other Transcriptomic Analyses—The data concerning the response to transient cell wall damage, together with previous data on the transcriptional response to constitutive cell wall damage derived from mutations in cell wall-related genes (19), were organized by hierarchical clustering (see “Experimental Procedures”). The clustering algorithm arranges the genes according to their similarity in expression under the different conditions, and hence genes with similar expression patterns are clustered together (57). In the present work, this clustering identified two significant groups of co-regulated genes, containing 91 (cluster 1) and 42 genes (cluster 2) respectively (Fig. 3). Within cluster 1, two different sub-clusters were visualized: subcluster 1a contained genes induced under transient conditions and was mainly enriched in cell wall-related genes and genes of unknown function. In contrast, subcluster 1b mainly contained genes involved in cellular metabolism, which were preferentially up-regulated in cell wall mutants and by Zymolyase treatment. Finally, with the exception of the first 6 genes those included in cluster 2 were common to all cell wall damage conditions investigated, both constitutive, due to cell

### TABLE IV

Promoter analysis of induced genes (ratio > 2)

|                | Congo Red | Congo Red | Congo Red | Zymolyase | Random | Promoter binding sites |
|----------------|-----------|-----------|-----------|------------|--------|------------------------|
|                | 2 h       | 4 h       | 6 h       | 2 h        |        |                        |
| 81.8           | 84.6      | 56.9      | 70.7      | 37.7       | RLM1   | CT(A/T)TA             |
| 6.8            | 17.9      | 21.6      | 10.9      | 7.8        | SCB1 (SBF)| CACGAAA      |
| 9.1            | 10.3      | 15.7      | 4.3       | 3.3        | SCB2 (SBF)| CGCGAAA      |
| 45.5           | 53.8      | 43.1      | 53.2      | 34         | STRE (Msn2/Msn4)| CCCCT      |
| 20.5           | 23.1      | 15.7      | 18.5      | 14.3       | STE12  | TGAACA                  |
| 20.5           | 20.5      | 31.4      | 21.7      | 16.9       | TEC1   | CATTCC                  |
| 68.2           | 76.9      | 70.6      | 65.2      | 46.8       | CDRE (Cza1) | (A/C)GCCNC     |
| 6.8            | 12.8      | 13.7      | 7.6       | 5.5        | PHO4   | CACGTG                  |
| 9.1            | 10.3      | 19.6      | 8.7       | 12.4       | SWI5   | ACCAGC                  |

![Fig. 1. Temporal expression patterns of cell wall-related genes during Congo red treatment.](http://www.jbc.org/)

The percentages of genes with at least one promoter binding site for the transcription factor indicated are included together with its random appearance in the yeast genome.
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Fig. 2. Activation of Pkc1-Slt2 pathway in cells growing in the presence of CR determined by the levels of dually phospho-rylated form of Slt2p. Protein extracts were prepared from the same samples of BY4741 yeast cells growing in the presence of 30 μg of Congo Red previously used in the microarray experiments. Top panel, immuno- blot assays of cell extracts with anti-phospho-p44/42 MAPK antibod- ies. Bottom panel, anti-actin immunoblot assay of the same sample with a mouse anti-actin monoclonal antibody.

wall defects, and transient -caused by CR and Zymolyase treat- ments. Within cluster 2, the twenty genes included in subcluster 2b were strongly induced under all cell wall damage condi- tions and can therefore be considered the “core” of the transcriptional compensatory response to cell wall damage. It is likely that this group of genes would be essential for adap- tation of the yeast to cell wall stress.

We next compared our expression profiles with other previ- ously published DNA microarray datasets relevant to our ex- periments. Around 35–40% of the genes up-regulated under our conditions were induced in cells treated with dithiothreitol (58), a drug that may affect the cell wall by altering cell wall disulfide linkages, or caspofungin (59), a drug that inhibits the activity of β-1,3 glucan synthase (see Supplemental Data in Table V at www.ucm.es/info/mfar/).

The expression profiles of cells harboring activated allelles of Pkc1 (Pkc1-R598A) and Rho1 (Rho1-Q68H) (55) as well as MKK1 (28), components of the MAP kinase Slt2p cell integrity pathway, share many genes (50% of the global response; 75% within the cell wall-related cluster) in common to our profiles (see supplemental data in Table V on the web site for details), indicating a major regulatory role for this pathway in the response. Interestingly, 58 and 45% of the cell wall genes induced under our experimental conditions were also induced in cells overexpressing the transcription factor Tec1p (56), and also after long treatments with pheromones (55), respectively, suggesting additional levels of regulation for genes involved in the transcriptional response to cell wall damage through the pseudohyphal growth and mating MAP kinase pathways. In contrast, although analysis of the promoters of the co-regulated genes suggests a role for the Calcineurin-dependent pathway in the response to cell wall damage, only 25 genes out of those activated in the presence of CR and Zymolyase were found to be up-regulated by the Ca2+/calcineurin-dependent pathway (53).

Transcriptional Response to Cell Wall Damage in slt2 and rlm1 Mutant Strains—Our current findings strongly support the notion that the regulation of the transcriptional response to cell wall damage mainly depends on the Slt2p-cell integrity pathway. We therefore addressed the involvement of the Slt2p pathway on the regulation of this response by characterizing the transcriptional profiles of slt2 and rlm1 deletant strains grown for 4 h in the presence of CR. Interestingly, we found that the presence of Slt2 is essential for the transcriptional activation induced by CR, except for two genes, PHO89 (2.8-fold) and FKS2 (2-fold). PHO89 encodes a phosphate/Na+ co-transporter involved in phosphate uptake under high-phosphate growth conditions (60), while FKS2 codes for the alternative subunit of β-1,3 glucan synthase. With respect to the transcriptional profile of the rlm1 strain, surprisingly we found that almost the whole transcriptional response to Congo Red depends on Rlm1p, except for PHO89, FKS2, YLR042C (encoding a GPI-CWP), and CHA1 (encoding for serine-threonine ammonia lyase activity (61)). These results were further verified by Q-RT-PCR. PHO89 was induced in the rlm1 mutant to levels similar to those of the wild-type strain (WT-3.6-fold/rlm1-1.3 fold), while the others showed a partial dependence on Rlm1p for their up-regulation (YLR042C (WT-2.8-fold/rlm1-1.8-fold), FKS2 (WT-3.2-fold/rlm1-1.8-fold) and CHA1 (WT-3.6-fold/rlm1-1.8-fold)). A complete data lists on the experiments described in this section is available on line at www.ucm.es/info/mfar/.

Phenotypic Analysis of Mutant Strains in Genes Involved in the Compensatory Mechanism—Next, we wished to further characterize how the response to cell wall damage is linked to the cell wall remodeling by measuring the sensitivity to Congo Red of BY4741-derived strains with deletions in the genes induced in response to CR that belong to the functional clusters of cell wall construction and signaling. The results are shown in Fig. 4. Strains deleted in the genes encoding regulatory proteins displayed the most severe phenotypes. Mutant strains deleted in SLT2, PTP2, or SWI6 were unable to grow in the presence of 100 μg/ml of Congo Red, while those deleted in MSG5, CRZ21, and SWI6 were sensitive to the drug. Regarding the genes involved in cell wall construction, the most severe phenotype was due to the absence of FKS1, encoding β-1,3 glucan synthase. Deletion of CRH1 (encoding a putative GPI-CWP trans- glycosidase) or EXG1 (coding for an exo-glucanase) rendered these strains sensitive to CR, while those deleted in PIR2 or BGL2 were slightly sensitive. The rest of the strains were insensitive to the presence of the drug.

DISCUSSION

Stressful conditions that cause damage to the cell wall, a structure that is essential for cell survival, leads to cellular responses that guarantee the osmotic integrity of the cell and therefore cell survival through the remodeling of this extracel- lular matrix (10). In a previous work, we identified a specific group of 80 genes that are up-regulated in response to persist- ent cell wall damage caused by mutations in cell wall-related genes, and we showed that at least three signaling pathways, including the general stress response mediated by Mns2p/ Msn4p, the Ca2+/calcineurin and the Pkc1p-Slt2p pathways, were involved in the cell wall compensatory response. Here we have expanded our understanding of the cell wall compensa- tory mechanism by investigating the transcriptomic response to transient cell wall damage caused by CR and Zymolyase, two drugs that specifically alter the integrity of the cell wall, and we show that this response is regulated by the Slt2p-MAP kinase signaling pathway. The kinetics of the “compensatory response,” described here for the first time, indicates the exist- ence of a transient response for one cluster of genes and a sustained response for another set of genes. The genes that exhibit transient increases must be involved in the transition to the new environment; for instance, the genes included in the “signal transduction” cluster, which show a transient response peaking at 2–4 h. Genes whose expression changes to a new level and remains altered probably encode proteins with a persisting role under the new conditions. Interestingly, the largest set of genes with a sustained response is included in the family of genes involved in cell wall construction and remodeling.

The transcriptional profile of the response to cell wall dam- age clearly reflects the necessities of remodeling of the cell wall under these circumstances. The alterations in the cell wall caused by CR could be compensated by changes in the balance among the different cell wall components or by changes in the type of association between them. It is very likely that the group of cell wall-related genes up-regulated under the condi-
FIG. 3. SOTA-Clustering of yeast gene expression profiles to cell wall damage. Clustering analysis of differentially expressed genes after Congo Red and Zymolyase treatments and in some cell wall mutants (fks1, gas1, knr4, kre6, and mnn9) (19), represented in each column, is shown. Each row represents the expression ratios for a particular gene under the conditions listed. Clustering was obtained by the Sotarray Server from GEPAS (gepas.bioinfo.cnio.es/tools.html). Red represents expression ratios higher than 1, and green indicates ratios lower than 1. The degree of color saturation represents the amount of the expression ratio, as indicated by the scale bar. White denotes missing values.
tions studied here would play a direct role in compensating for transient cell wall damage. Although the function of many of these proteins remains unknown, they must play a role in cell wall strengthening and remodeling. An interesting issue is that despite the high transcriptional activation of some of these genes encoding CWPs their deletion mostly leads to mild phenotypes of sensitivity to CR. It is probable that the simultaneous deletion of several genes working in a coordinated fashion would be required for the complete impairment of cell wall remodeling that takes place under cell wall damage conditions. Alternatively, this may be explained, at least in part, by the existence of redundant functions for some of these CWPs. For example, the simultaneous deletion of \textit{CRH1} and its homologous \textit{CRH2} renders this strain unable to grow in CR (37). On the other hand, double disruptants of \textit{dfg5} (up-regulated under our conditions) and \textit{dcw1}, coding for homologous mannosidases, are synthetically lethal (62).

Does the yeast cell respond to different cell wall defects in a specific manner? Although there are differences in the transcriptional profiles induced by different stimuli leading to cell wall damage, the main core of the response is similar. Comparison of the transcriptional profiles under conditions of constitutive damage resulting from mutations at different steps of cell wall construction (\textit{gas1}/H9004, \textit{knr4}/H9004, \textit{fks1}/H9004, \textit{kre6}/H9004, \textit{mnn9}/H9004) (19) with the transient damage caused by Congo Red and Zymolyase indicates that, besides some differences between them, the genes included in the functional groups involved in cell wall remodeling (cell wall biogenesis, morphogenesis, signal transduction, and stress) under transient cell wall damage conditions are also up-regulated in at least one of the mutants previously characterized. It should be noticed that the transcriptomic profile of the \textit{gas1} mutant was the most similar to those identified in response to CR, reflecting the fact that this mutant exhibits the most pronounced cell wall defects (63). The hierarchical clustering of the transcriptional profiles of transient and constitutive cell wall damage permitted us to define a cluster of 20 genes that are strongly induced under all the conditions assayed, and would represent the main transcriptional fingerprint for cell wall stress. This cluster includes genes involved in cell wall biogenesis (\textit{CWPI}, \textit{SED1}, \textit{PIR3},

**Fig. 4. Analysis of sensitivity to Congo Red.** Several yeast strains deleted in genes up-regulated in the microarray experiments were tested for their ability to grow on solid medium in the presence of 100 \(\mu\)g/ml of Congo Red (see “Experimental Procedures” for details). Data in each column correspond to different experiments and therefore wild-type BY4741 and \textit{sli2} (sensitive to CR) strains were included for comparison. Strains sensitive to the drug are underlined.
CRH1, KTR2, GFA1, PST1, and YLR194C), signaling (SLT2, MLF1), metabolism (YPL088W, YP54, FBP26), stress (HSP12, PRM5), and genes with unknown function (SRL3, YL023C, YLR414C, YHR097C, YAL053W). The inclusion of this latter group in the cluster is very interesting since they can now be annotated as being involved in the compensatory response to cell wall damage.

Characterization of the expression profiles of cells deleted in SLT2 growing in the presence of CR (described here) indicates that the compensatory response activated in the presence of transient cell wall damage is totally regulated through the MAP kinase Slt2p, except for PHO89 and (although to a lesser extent) for FKS2. Interestingly, up-regulation of both genes in the presence of CR is dependent on the Crz1p transcription factor, indicating that these genes are regulated through the Calcineurin pathway. Moreover, our data indicate that nearly all the response is mediated through Rlm1p, the MADS-box transcription factor acting downstream from the MAP kinase Slt2p, with the exception of PHO89, FKS2, YLR042C, and CHA1, which should be regulated by other different transcription factors. Previous reports have shown that the induction of gene expression under artificial conditions and by an increase in temperature is dependent on Rlm1p, with the exception of the FKS2 gene (28). Our results further underscore the importance of Rlm1p-dependent regulation in the response to temperature shift to the transcriptional response to cell wall damage. Furthermore, our experiments also indicate that in the absence of Rlm1p there is no induction of an alternative transcriptional response. This result is surprising looking at the phenotype of a strain deleted in RLM1. In contrast to a mutant in SLT2, which is unable to grow under wall cell growth conditions, deletion of RLM1 does not lead to defects in cell integrity; and even rlm1 strains are resistant to treatments with Zymolyase or Calcofluor White (24). These phenotypes suggest that Rlm1p is not the only transcriptional targets of Slt2p. However, our findings indicate that Rlm1p mediate the majority of the effects of Slt2p on transcriptional activation under conditions of cell wall damage and suggest that the possible functional link between the cell integrity pathway and the transcription factors of other signaling pathways must be regulated specifically by Tec1p under conditions of cell wall damage and suggest that the real participation of other signaling pathways, additionally to the cell integrity pathway, in the regulation of the compensatory response to cell damage must await further investigation.

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