

# The 'yeast cell wall chip' – a tool to analyse the regulation of cell wall biogenesis in *Saccharomyces cerevisiae*

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Within the field of *Saccharomyces cerevisiae* functional genomics, DNA microarrays have become a very useful tool to study genome-wide gene-expression changes under diverse experimental conditions. Here, the design and production of a gene microarray, called the 'yeast cell wall chip', specifically tailored to investigate cell wall functions, is described. This array has been validated and shown to be useful to address gene involvement in the regulation of the response to cell wall damage in yeast. The advantages of this tailored gene microarray, which contains 390 genes, in terms of reproducibility, accuracy, versatility and ease of use are reported. Importantly, the microarray design permits the performance of a double hybridization process (two experiments) on the same slide. Cell wall stress leads to the transcriptional activation of a set of genes involved in cell wall remodelling. This response has been shown to be strongly controlled by the MAP kinase (MAPK) Sit2p, but other signalling pathways have also been suggested to be involved in this process. Here, using the tailored microarray, the role of the HOG1 pathway in the regulation of the transcriptional compensatory response to cell wall damage was evaluated by comparing the transcriptional profiles of a *hog1* mutant and a wild-type strain in the presence of Congo red. Two genes, *YFL014W* (*HSP12*) and *YLR414C*, were found to be dependent on the Hog1p MAPK for their induction, indicating that an additional level of regulation of cell wall functions is mediated by this MAPK.

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## INTRODUCTION

Over the past few years, DNA microarray technology has aroused increasing interest. Several applications have been developed and optimized, such as the study of gene expression and the employment of the analysis of DNA sequence variation in genotyping and comparative genome hybridization (CGH) (for a complete review of microarray technology see the *Nature Genetics* supplement of December 2002). The main application of microarrays has been transcriptional analyses: these currently being the method of choice for the large-scale analysis of gene expression. Very recently, within the microarray field, a new experimental approach has emerged, aimed at the design of expression

experiments involving a reduced and selected group of target genes (Evans *et al.*, 2003; Guimond *et al.*, 2003; Hayashi, 2004; Lorenz *et al.*, 2003; Rae *et al.*, 2004; Reinhold *et al.*, 2003). This kind of microarray has received the name of custom or tailored arrays, or 'minichips'. Microarrays containing a reduced number of features related to a common biological role permit microarray experiments that involve lower cost, better interpretation of the results, and a simple way of decreasing the complexity of the analysis process compared with time-consuming traditional genome-wide microarrays. The main advantage of these microarrays, apart from their low cost and affordability for academic research groups, is the possibility of focusing on the expression patterns of a group of genes closely related to the research field of interest.

The yeast cell wall is an essential structure for cell survival. Globally, the cell wall consists of mannosylated proteins and three kinds of polysaccharide chain (for reviews, see Cid *et al.*, 1995; Klis *et al.*, 2002). This apparently rigid structure

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Abbreviations: CR, Congo red; MAPK, MAP kinase; Q-RT-PCR, quantitative RT-PCR; SD, standard deviation.

maintains a high degree of flexibility for adaptation to different developmental programs, such as budding, mating and sporulation (Cid *et al.*, 1995; Duran & Nombela, 2004; Molina *et al.*, 2000; Smits *et al.*, 1999). Treatment with cell-wall-perturbing agents, such as Congo red (CR) and Calcofluor white, or with Zymolyase, which degrades the  $\beta$ -1,3 glucan network, leads to a cellular response, in an attempt by the cell to survive, that has been called the 'compensatory mechanism'. This response clearly illustrates the dynamic nature of the cell wall, and it is characterized by an increase of chitin content, an overproduction of many mannoproteins, changes in the association between cell wall polymers, as well as a transient redistribution of  $\beta$ -1,3 glucan synthase (see Popolo *et al.*, 2001; Smits *et al.*, 2001, and references therein). Most of these changes are the consequence of a global transcriptional response that we and other groups have recently characterized both in mutants affected in different steps of the cell wall construction process (Lagorce *et al.*, 2003) and in wild-type yeast cells growing under different transient cell-wall-damage conditions (Agarwal *et al.*, 2003; Boorsma *et al.*, 2004; García *et al.*, 2004).

The regulation of this compensatory response is mainly controlled by the MAP kinase (MAPK) Slt2p through the cell integrity pathway. This pathway is activated in response to several environmental stimuli, including cell-wall-damage conditions (De Groot *et al.*, 2001; De Nobel *et al.*, 2000; García *et al.*, 2004; Gustin *et al.*, 1998; Ketela *et al.*, 1999). As a consequence of this activation, a transcriptional program is turned on through the Rlm1p and Swi4p transcription factors, leading to a remodelling of the cell wall (Baetz *et al.*, 2001; García *et al.*, 2004; Igual *et al.*, 1996; Jung & Levin, 1999). Other authors have also suggested that the Sho1p-Kss1p (Lee & Elion, 1999) and HOG1-MAPkinase pathways (García-Rodríguez *et al.*, 2000; Kapteyn *et al.*, 2001; Klis *et al.*, 2002) could be involved in the control of cell integrity. Recent genomic approaches have revealed that, in addition to the above-mentioned pathways, the calcineurin/Crz1p signalling pathway and the regulatory machinery from the general cellular stress response could also be regulators of the response to cell wall damage (García *et al.*, 2004; Lagorce *et al.*, 2003). However, additional work is still necessary to fully evaluate the role of these pathways in the regulation of this response and to characterize in detail the possible cross-talk between them.

In the present work, we report a custom array (the 'yeast cell wall chip') for the study of transcriptional variations related to yeast cell wall homeostasis that has a significant number of technical advantages. Based on previous information obtained in our laboratory (García *et al.*, 2004), we have been able to validate this tool for the study of the transcriptional profile of wild-type yeast cells subjected to treatment that induces cell wall stress. The use of this array has also enabled us to obtain novel insights regarding the regulation of the transcriptional compensatory response to cell wall damage of yeast cells lacking the Hog1p MAPK by

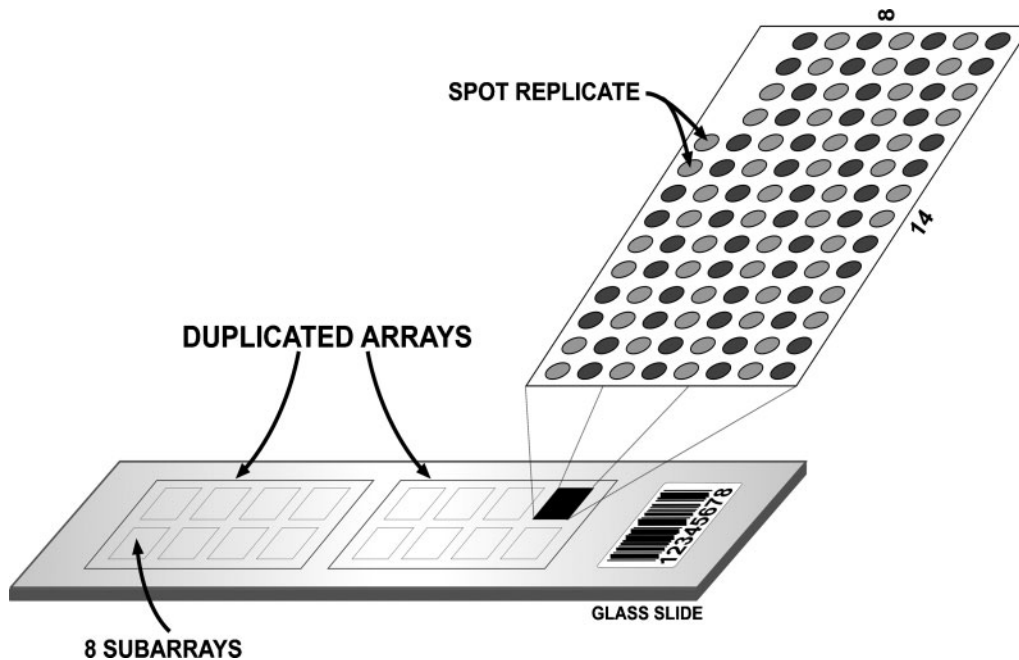
characterizing the transcriptional profile in response to Congo red.

## METHODS

**Generation of PCR products.** The probes selected for microarray construction (390) were obtained by PCR using different strategies. The principal strategy (1) was amplification of each complete ORF inserted into a plasmid of the Research Genetics Exclone collection. Each clone contains a different and characterized full-length yeast ORF (Martzen *et al.*, 1999). The inserts of the selected plasmids (ORFs) were amplified in a first PCR round using a pair of common plasmid primers, eYGUF (5'-ATTCGATGATGAAG-ATACC-3') and eYGUR (5'-ACGATTCATAGATCTCTGC-3'). In a second PCR round, the product of the first PCR was amplified with a new pair of inner primers, YGUF (5'-CGAATTCAG-CTGACCACCATG-3') and YGUR (5'-GATCCCCGGGAATTGCCATG-3') (Alberola *et al.*, 2004). A second strategy (2), for ORFs with a length greater than 3 kb, was for an internal oligonucleotide (close to 1000 bp upstream from the ORF stop codon) compatible with eYGUR and containing a 5' YGUF tail, in order to allow a second round of amplification, to be designed using Pride software (Haas *et al.*, 1998). A third strategy (3), in the case of the absence of amplification (negatives) or insufficient product quality from the first two approaches, was to carry out PCR using genomic DNA as template by means of the commercial GenePairs (Research Genetics) technique, again involving a double PCR round to obtain enough PCR product. Finally (4), when all the above strategies failed to amplify a selected ORF, two primers were designed, comprising around 900 bp of the 3' end to amplify the specific ORF region directly from genomic DNA. As negative hybridization controls, we chose and then PCR-amplified the *Escherichia coli*  $\beta$ -lactamase gene from plasmid pBR322 (primers: forward 5'-AGTATCAACAT-TTCCGTGTCG-3' and reverse 5'-CTAAAGTATATATGAGTAA-CTTG-3') and the phosphatidylinositol phosphatase (*sopB*) from *Salmonella typhimurium* (primers: forward 5'-CCAAGCTTGGAT-GCAAATACAGAGCTTC-3' and reverse 5'-CGGGATCTCAAGAT-GTGATTAATGAAG-3').

PCR conditions were modified according to the particular  $T_m$  of the primers used and the length of the amplified region. Biotools DNA polymerase (Biotools) was used for PCR reactions. PCR products were purified before printing with the Multiscreen PCR 96-well Filtration System (Millipore) and inspected for quality and quantity by gel electrophoresis, following gel analysis in a Molecular Imager ProPlus FX using the Quantity One software (Bio-Rad). All PCR products were verified by direct sequencing from at least one side after PCR purification on an automated DNA sequencer 3730 (Applied Biosystems).

**Microarray design and printing.** Purified PCR products were spotted onto Ultra-GAPS coated slides (Corning), using a 45% (v/v) DMSO solution as spotting buffer. For this purpose, we used the MicroGrid II arrayer from BioRobotics. Printing was accomplished with an eight-pin head in a 4 × 2 pin configuration, each probe being printed in duplicate consecutively, yielding a total of 864 available positions, corresponding to 54 source visits per pin. DNA binding to the surface was done by UV cross-linking following the slide manufacturer's instructions. In the microarray layout (see Fig. 1) there were 778 spots corresponding to *S. cerevisiae* ORFs, 38 spots were only printed with spotting buffer and were required for the evaluation of basal slide background, 32 spots contained the controls for hybridization specificity (*E. coli* and *S. typhimurium* DNA), and finally 16 spots (two printed for each pin) contained the housekeeping gene *ACT1*. Additionally, the complete grid was printed in duplicate within each slide (separated by 2–3 mm), giving



**Fig. 1.** Layout of the *Saccharomyces cerevisiae* yeast cell wall chip.

rise to two independent slide surfaces available for hybridization. Quality control of the printing of each slide batch included staining with Syto61 (Molecular Probes) and hybridization with Cy3-labelled random 9-mer oligonucleotides (Qiagen).

**Strains and culture conditions.** All experiments were performed with *S. cerevisiae* strain BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and mutant derivatives provided by Euroscarf. Mutant strains (BY4741 background) had the corresponding gene completely deleted and replaced by the geneticin-resistance-coding KanMX4 module. For routine cultures, *S. cerevisiae* was grown on YEPD (1% yeast extract, 2% peptone, 2% glucose). When necessary, 200 mg geneticin l<sup>-1</sup> (Gibco) was added. Yeast cells were grown overnight at 24 °C to OD<sub>600</sub> 0.8–1. The culture was refreshed to OD<sub>600</sub> 0.2 and grown at 24 °C for 2 h 30 min. Then, the culture was divided into two parts. One part was allowed to continue growing under the same conditions (the untreated culture), while the other was supplemented with Congo red to a final concentration of 30 μg ml<sup>-1</sup>. After 4 h of growth, cells (~5 × 10<sup>8</sup>) were collected by centrifugation, frozen at -80 °C, and processed for RNA extraction.

#### **RNA isolation, cDNA synthesis, and microarray hybridization.**

All these processes were basically carried out following the protocols described previously by García *et al.* (2004), with minor modifications. Briefly, cDNA was synthesized from 15–20 μg total RNA (isolated using the RNeasy MIDI kit, Qiagen) by reverse transcription using the CyScribe Post-Labeling Kit, incorporating Cy3-dUTP or Cy5-dUTP (Amersham Biosciences) into the cDNA corresponding to each sample to be compared. Both labelled cDNA populations were combined, dried in a vacuum trap, and used as a hybridization probe after resuspension in 15 μl hybridization solution [50% formamide, 6 × SSC, 0.5% SDS, 5 × Denhardt's, 20 μg poly(A) (P-9403, Sigma) and 100 μg salmon sperm (Gibco-BRL) ml<sup>-1</sup>]. This volume was found to be optimum for the hybridization surface of the tailored microarray described above when hybridization was done under 22 × 22 mm glass microscopy cover slips (Sigma). The amount of cDNA as well as the incorporation of Cy3 and Cy5 dyes into cDNA targets was quantified on an Ultrospec 3300 Pro UV/

visible spectrophotometer (Amersham Biosciences) by measuring the absorbance of each sample at 260, 550 and 660 nm, respectively. For each condition tested, comparing the treated and untreated samples, the total RNA from at least two different cultures was analysed, and in addition for each RNA sample, at least two different hybridizations were performed, including fluorochrome swapping in order to minimize transcriptional changes due to technical variability. Therefore, a minimum of four DNA microarrays was analysed for each experimental condition.

**Microarray image analysis.** Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments) at a resolution of 5 μm (PMT values 550–700, laser power 100%). GenePix Pro 4.0 analysis software (Axon Instruments) was used to locate spots in the microarray with the appropriate grid and to obtain the two Cy3/Cy5 image TIFF files. All images were further processed using GenePix 4.0 software, according to the manufacturer's instructions.

**Data processing and statistical methods.** Data processing was performed following the protocol described by García *et al.* (2004). Owing to the small number of features in the yeast cell wall chip with respect to traditional genome-wide microarrays, we had to set up the most convenient normalization method to compensate for treatment (condition assayed)-independent ratio variations. This process was accomplished by evaluating the three most widely used normalization methods (for a review, see Quackenbush, 2002): i) total intensity normalization, where a normalization factor is obtained from the two channels used (this method assumes that only a few transcripts are regulated by the treatment under investigation); ii) the internal reference method, where the normalization factor is calculated using the data corresponding to a gene with no transcriptional change in the conditions tested (this is the case of the actin gene in this work); and iii) intensity-dependent normalization (Lowess method) to account for systematic biases dependent upon spot intensity. Under our experimental conditions, the second method produced the best results in control experiments, and was therefore chosen for application to all datasets. Significance analysis

of the results was conducted using Student's *t* test (GeneSight 4.0, BioDiscovery). Genes with *P* values of less than 0.05 were considered to be significantly differentially expressed.

The Microarray data described here follow the MIAME recommendations and have been deposited at the NCBI gene expression and hybridization array data repository (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) with GEO accession numbers GSE2105, GSE2106, GSE2107 and GSE2108.

**Quantification of mRNAs using real-time quantitative RT-PCR (Q-RT-PCR).** Real-time Q-RT-PCR assays were performed as described by García *et al.* (2004) using an ABI 7700 instrument (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  $2^{-\Delta\Delta C_t}$  method, as described in Livak & Schmittgen (2001). The following forward and reverse primers, respectively, were used: *ACT1*, 5'-ATCACCGCTTTGGCTCCAT-3' and 5'-CCAATCCAGACGGAGTACTTTCTT-3'; *YFL014W*, 5'-GTCCAGACTCTGCCGAAA-3' and 5'-GCCAAAGATTCACCTTGACCTT-3'; *YLR414C*, 5'-TTGGTGCC-TTTTTTTCATTTTTC-3' and 5'-GCCCAAACACGAACCTATACCTGA-3'; *YKR061W*, 5'-TCGATTGCTGCCTCACTTCTT-3' and 5'-CAGGTAGTATGCAGATGGACACG-3'.

## RESULTS AND DISCUSSION

### Design and development of a *S. cerevisiae* cell wall custom microarray: the yeast cell wall chip

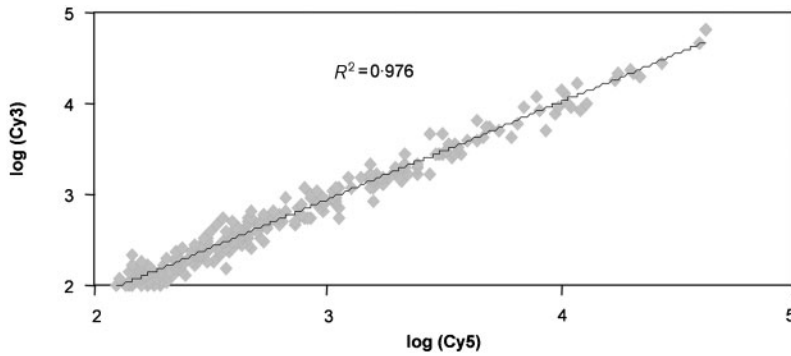
Our main goal consisted of the design and generation of a custom microarray focused on the process of cell wall construction and biogenesis in *S. cerevisiae* that would be useful to gain a deeper understanding of the fundamental processes required for the generation of this complex cell structure. For this purpose, we selected a set of genes that had previously been found to be directly or indirectly related to cell wall construction. To achieve this objective, we took advantage of the SGD ([www.yeastgenome.org](http://www.yeastgenome.org)) and YPD (Proteome BioKnowledge Library) databases to identify and select cell-wall-related genes. Eventually, 390 ORFs were chosen, including, in addition to cell-wall-related ORFs, many of the components of the *S. cerevisiae* signal transduction pathways (Gustin *et al.*, 1998; Schwartz & Madhani, 2004), several stress-induced genes, and the standard housekeeping gene *ACT1*. Interestingly, the cluster of genes recently characterized as the fingerprint of the transcriptional compensatory response to cell wall damage (García *et al.*, 2004) was included in the cell wall chip. The variety of gene functional categories in this microarray is in agreement with the idea of cell wall biogenesis as an integrated process in a global network of cellular functions (Firon *et al.*, 2004). Detailed information about these genes is listed on our web page ([www.ucm.es/info/mfar/U4/Data/cwgeneinfo.xls](http://www.ucm.es/info/mfar/U4/Data/cwgeneinfo.xls)). Additionally, two genes corresponding to the  $\beta$ -lactamase (*bla*) and phosphatidylinositol phosphatase (*sopB*) of *E. coli* and *Sal. typhimurium*, respectively, were included at several positions within the chip as controls for organism specificity. In this study, a significantly novel strategy was employed in the design of the microarray

layout, by which the complete microarray grid was printed in duplicate on each slide, thereby allowing two independent and simultaneous hybridizations on the same surface (Fig. 1). Several spots containing only printing buffer were spread along the grid to monitor the background (noise) of the system. At the same time, employing a strategy generally accepted for reducing the effects of noise, each independent grid had all the features printed consecutively in duplicate (left to right). The layout of the yeast cell wall chip formed by eight subarrays of fourteen columns and eight rows is shown in Fig. 1. The design of this tailored microarray is open, and allows the easy introduction of different or additional ORFs to adapt the microarray structure to particular experimental requirements.

### Evaluation of the double hybridization procedure and validation of the yeast cell wall chip

One of the most important prerequisites in microarray technology is to achieve systems with acceptable reproducibility. For the evaluation of this aspect with the yeast cell wall chip, several control steps were established. First, 'self to self' or 'yellow' experiments were carried out, in which the same RNA sample (from a wild-type yeast strain) was labelled independently with the two fluorochromes (Cy3 and Cy5). As shown in Fig. 2, none of the features present in the array showed significant variations higher than twofold, indicating a good data correlation. Second, we investigated the level of intra-array variability within each complete array (grid). For this and further validation assays, we chose the treatment of yeast cells with the dye Congo red for 4 h as the reference experimental condition. The mechanism of action of this drug is not known, although its interference with proper cell wall assembly is well documented (Kopecka & Gabriel, 1992). The effect on gene expression brought about by this treatment in wild-type yeast cells has recently been characterized in great detail by our group using microarrays in a genome-wide format (García *et al.*, 2004). The analysis of the ratios of duplicated spots within the yeast cell wall chip revealed a low degree of data dispersion in terms of deviation from the normal distribution of the data population. This can be deduced from the narrow boxes obtained when the comparisons are represented using box-and-whiskers plots (Fig. 3). From a quantitative point of view, only about 5% of the duplicates, on average, differed in ratio by more than two standard deviations (SD) from the mean of the normal distribution.

Third, we tested the level of inter-array variability for each feature in our platform. To this end, we first carried out four independent hybridizations on two different slides (our design allowed us to carry out two simultaneous hybridizations on the same slide). Fluorochrome swapping was also performed in the simultaneous hybridizations to evaluate the extent to which the variability of the data was dependent on the labelling process. Comparisons were made by generating a normal distribution in which the ratio of each feature from one hybridization was multiplied by the inverse

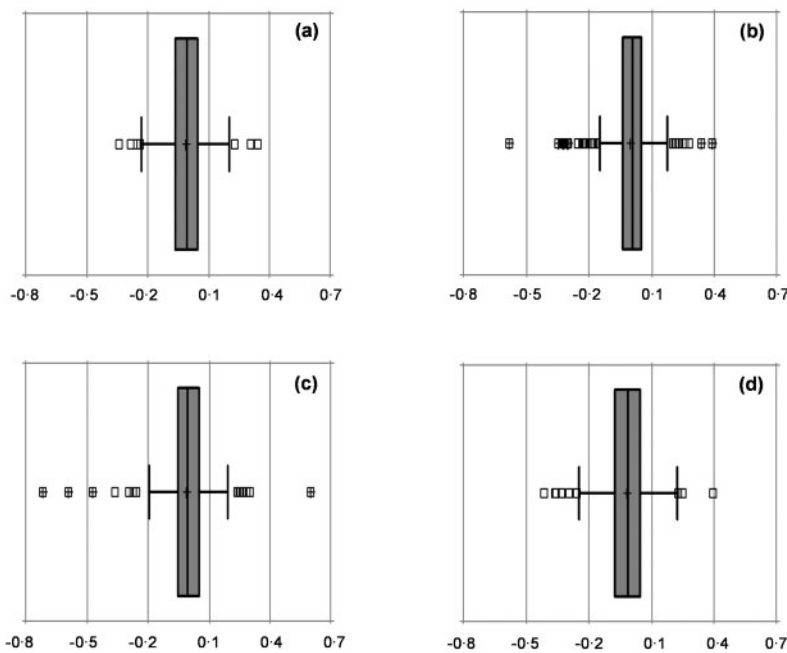


**Fig. 2.** Representation of ratio accuracy and signal specificity. Scatter plot of normalized fluorescence intensity values from a yeast cell wall chip hybridized with the same cDNA sample, obtained from total RNA extracted from the wild-type strain grown under normal conditions, labelled with Cy3 or Cy5 (self-to-self hybridization).

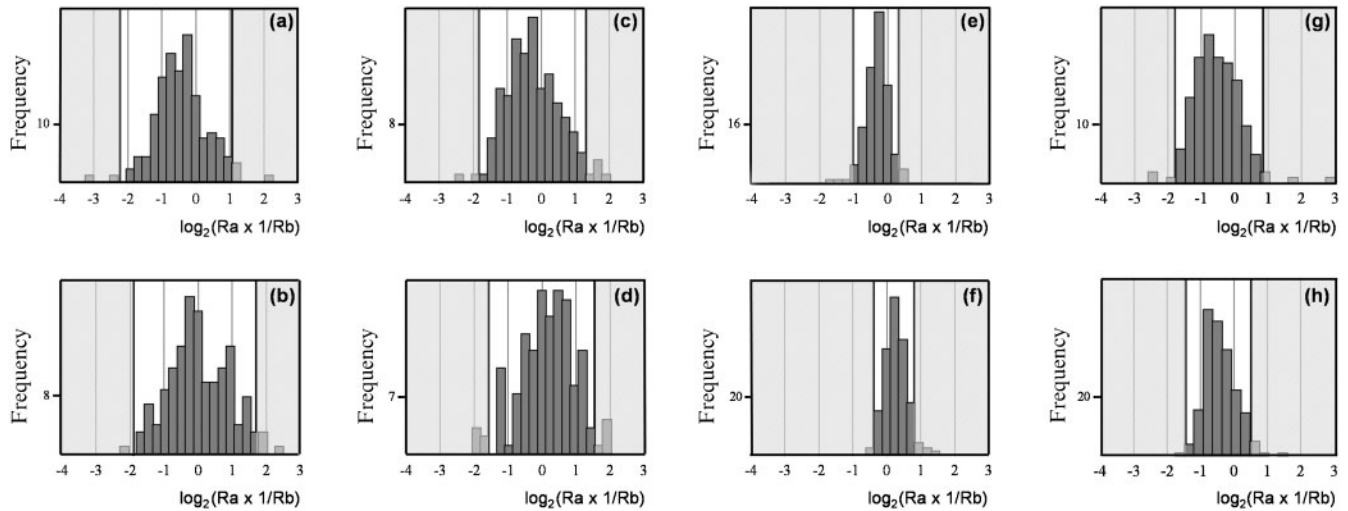
of the ratio of the second one. As shown in Fig. 4, the level of ratio variations was very similar in the experiments carried out on the same slide (Fig. 4a, b) or on different ones (Fig. 4c, d). In fact, in both cases, less than 5% of the genes showed a deviation of more than 2SD. However, when we compared experiments using the same labelling combination (Cy3 or Cy5 for CR-treated or -untreated samples) on different slides, data dispersion was notably lower (Fig. 4e, f), with a similar percentage of genes outside 2SD. All these determinations suggest that: 1) simultaneous hybridization on one slide does not significantly improve the reproducibility of the data obtained by hybridization on independent slides, although the option of performing two assays on the same surface is a significant advantage with respect to traditional platforms in view of the lower cost per experiment and the time saving achieved by running two tests simultaneously; and 2) the highest level of variability is introduced by differential labelling with the Cy3 or Cy5 fluorophores. This was further confirmed by evaluating the

effect on the ratio values of labelling different RNA samples with the same fluorochrome combination, carrying out hybridizations on different slides. As shown in Fig. 4(g, h), in this case data dispersion was intermediate, but lower than in the two former combinations in which fluorochrome swapping was considered. This is why it is essential to include technical replicates incorporating fluorochrome swapping in the DNA microarray experimental design.

Once the yeast cell wall chip had been seen to work suitably from the technical point of view, we were prompted to validate the data generated with this microarray by using the data we had previously obtained with whole-genome microarrays, in which the upregulation of a set of 43 genes induced at least twofold after 4 h of CR treatment was characterized (García *et al.*, 2004). Eventually, 42 out of these 43 upregulated genes were present in the yeast cell wall chip, and thus they constituted the main basis for comparison between the two platforms (genome-wide



**Fig. 3.** Determination of intra-array variability. The reproducibility of replicates (two spots per ORF) was analysed by creating a normal distribution of  $\log_2(Ra \times 1/Rb)$ , where  $Ra$  and  $Rb$  are the ratios of replicated spots after background subtraction and normalization. Replicate reproducibility is represented in box-and-whiskers plots corresponding to four independent hybridizations (comparison of wild-type strain grown for 4 h in the presence or absence of Congo red). The width of the central boxes indicates the level of intrachip ratio dispersion. Values of  $\log_2(Ra \times 1/Rb)$  are indicated in each plot.



**Fig. 4.** Histogram representation of inter-array variability. Each histogram is constructed from the comparison of the ratios of two different hybridizations creating a normal distribution  $\log_2(Ra \times 1/Rb)$ , where Ra and Rb are the ratios for the same spots from two different experiments after background subtraction and normalization. Two histograms (corresponding to repeated experiments) per condition are shown. In all cases, competitive hybridizations were carried out in which the transcriptional profile of a yeast wild-type strain grown for 4 h in the presence or absence of Congo red (CR) was tested. (a, b) The experiments compared in each case were done with the same RNA sample using fluorochrome swapping (in one hybridization cDNA from CR-treated cells labelled with Cy3 and in the other with Cy5) and carried out on the same slide. (c, d) As in (a, b), but using different slides for the hybridizations to be compared. (e, f) Experiments for comparative purposes done on different slides with the same RNA sample and labelling distribution. (g, h) Experiments on different slides using different RNA samples, but with the same labelling distribution (for example Cy5 or Cy3 labelling was always associated with CR-treated cells). Shaded sections delimit features more than 2SD from the mean of the normal distribution.

versus tailored). In order to decrease the influence of technical variability and to obtain data with high statistical significance, we carried out 12 competitive hybridizations distributed in double experiments per slide as well as fluorochrome swapping, and also tested the use of different biological samples (see Methods). From a general view of the results generated with our tailored microarray, it was clear that it gave rise to the expected expression pattern. Specifically, from 42 genes found to be more than twofold upregulated by CR treatment and present in the yeast cell wall chip, we observed about 72 % of them to be induced at least twofold in our system, plus an additional 14 % with inductions of between 1.7- and 2.0-fold. Moreover, when the comparisons were made in the inverse sense, we found that 30 out of 33 genes (91 %) upregulated (at least twofold) in the yeast cell wall chip data showed similar behaviour to that observed in the previous study. As expected, the consistency of the data between the two platforms was higher for genes with the highest transcriptional variations. Thus, upon scrutinizing those genes induced more than fourfold in at least one of the platforms, in general we observed a good correlation between both sets of data (Table 1), also in agreement with the previously published Q-RT-PCR expression values for some of these genes (García *et al.*, 2004) and those described in this work (Table 2). The most divergent data between the two platforms shown in Table 1 corresponded to *KTR2*.

**Table 1.** Comparison of genome-wide and yeast cell wall chip microarrays

Values show the ratios of genes upregulated more than fourfold in the presence of Congo red (CR) in at least one microarray platform.

ORF	Gene name	CR+/CR- (fold)	
		Genome-wide	Yeast cell wall chip
<i>YDR055W</i>	<i>PST1</i>	3.0	5.4
<i>YFL014W</i>	<i>HSP12</i>	4.2	2.9
<i>YGR032W</i>	<i>FKS2</i>	3.2	5.5
<i>YHR209W</i>		7.5	8.0
<i>YIL117C</i>	<i>PRM5</i>	3.8	5.0
<i>YKL096W</i>	<i>CWP1</i>	4.6	4.3
<i>YKL161C</i>	<i>MLP1</i>	9.4	15.9
<i>YKL163W</i>	<i>PIR3</i>	5.0	3.6
<i>YKR061W</i>	<i>KTR2</i>	1.8	5.2
<i>YKR091W</i>	<i>SRL3</i>	2.6	6.1
<i>YLR042C</i>		2.6	5.7
<i>YLR121C</i>	<i>YPS3</i>	5.5	5.4
<i>YLR194C</i>		7.9	3.5
<i>YPL088W</i>		7.7	8.9

**Table 2.** Confirmation by real-time quantitative RT-PCR (Q-RT-PCR) of some selected genes

Data correspond to the levels of induction of selected genes after 4 h of CR treatment. WT, wild-type.

ORF	Gene name	CR +/CR- (fold)			
		Arrays		Q-RT-PCR	
		WT	<i>hog1Δ</i>	WT	<i>hog1Δ</i>
<i>YFL014W</i>	<i>HSP12</i>	2.9	1.3	2.6	1.1
<i>YLR414C</i>		2.3	1.6	2.4	1.7

Q-RT-PCR experiments with this gene confirmed its upregulation to be about threefold (data not shown).

Bearing in mind the differences in the microarray platforms under evaluation and the use of different samples and operators, it may be concluded that the custom microarray described here is able to monitor transcriptional cell wall-related variations in an efficient, reproducible and accurate way.

### Characterization of the transcriptional response to cell wall damage in a *hog1* mutant strain

The cell integrity pathway, regulated by the MAPK Slt2p, has been shown to be the main pathway involved in the regulation of yeast cell wall construction (Firon *et al.*, 2004; García *et al.*, 2004; Jung & Levin, 1999; Smits *et al.*, 2001). In agreement, we have previously found that most of the transcriptional response to CR is dependent upon the MAPK Slt2p and the Rlm1p transcription factor activated by this MAPK (García *et al.*, 2004). However, *in silico* analysis of the regulatory sequences of the genes involved in the response to the two main groups of cell-wall-stress conditions, transient (García *et al.*, 2004) or constitutive (Lagorce *et al.*, 2003), highlighted the possible involvement in this regulation of several other signalling pathways, such as the calcineurin/Crz1p signalling pathway and the regulatory machinery from the general cellular stress response. The involvement of the Hog1p MAPK in the modulation of gene expression in the presence of osmotic stress has largely been characterized (O'Rourke & Herskowitz, 2004). However, neither the role in gene expression of this MAPK in response to cell wall damage nor the genes that can potentially be influenced by this pathway has been reported. Bearing this in mind, together with the fact that several other reports had also suggested the relationship between *HOG1* (the MAP kinase of the high-osmolarity glycerol pathway) and cell integrity (García-Rodríguez *et al.*, 2000; Kapteyn *et al.*, 2001; Klis *et al.*, 2002), we were prompted to examine the role of the MAPK Hog1p in the regulation of the transcriptional response to CR by using our yeast cell wall chip. The transcriptional pattern of a yeast strain lacking Hog1p

grown for 4 h in the presence and absence of CR was analysed. We observed that although the absence of *HOG1* does not affect the transcriptional activation of most of the genes, this MAPK is essential for the transcriptional activation of *HSP12* (*YFL014W*) and *YLR414C* (see Table 2) in response to CR. These data suggest a new role for the Hog1p MAPK pathway in the control of the response to cell wall damage, at least for the genes mentioned above. In order to validate the expression data obtained by microarray analysis, real-time Q-RT-PCR was performed. For this purpose, both Hog1p-dependent genes were analysed by this alternative method. As shown in Table 2, we found a very good correlation between both datasets, supporting the validity of the yeast cell wall chip experiments. The lack of Hog1p elicited a complete block in the upregulation of *HSP12*, while this effect was less dramatic in the case of *YLR414C* (Table 2). The evidence that the increased expression of these two genes is also completely dependent upon *SLT2* and *RLM1* (the cell-wall-integrity pathway transcription factor) (García *et al.*, 2004) supports the existence of genes controlled by different signal transduction pathways, at least dually, as a consequence of cell wall stress. The 800 bp non-coding upstream sequences of both genes were examined using the RSAT program (van Helden, 2003) searching for motifs of transcription factors related to different signal transduction pathways, including Crz1p, involved in Ca<sup>2+</sup>/calcineurin-regulated gene expression (Yoshimoto *et al.*, 2002), the general stress-related factor Msn2p/Msn4p (Martinez-Pastor *et al.*, 1996), the transcription factor for the pheromone response pathway Ste12p (also required for filamentous growth) and its inhibitor Dig1p (Schwartz & Madhani, 2004), the activator of pseudohyphal formation Tec1p (Schwartz & Madhani, 2004), Rlm1p (Dodou & Treisman, 1997), Hsf1p, which is related to heat stress (Sorger, 1991), and the HOG pathway transcription factor Sko1p (Proft & Struhl, 2002) (Table 3). The presence of this large number of putative regulatory sequences supports the hypothesis of a complex and coordinated regulation of these genes under different situations. Moreover, the dual dependence of *YLR414C* and *YFL014W* expression in response to CR on Slt2p and Hog1p could be explained by the presence within the regulatory region of these genes of Rlm1p and Sko1p or STRE binding sites, respectively, and therefore suggests the simultaneous involvement of different transduction pathways (the cell integrity and high osmolarity pathways in this case) in the regulation of the transcriptional response to this specific stress condition. How this control is governed and organized in relation to cell wall homeostasis remains unknown, but a similar situation of dual control has recently been described for the *HXT1* gene, a low-affinity glucose transporter, whose expression requires both the glucose-signalling and HOG pathways (Tomas-Cobos *et al.*, 2004). On the other hand, the transcriptional regulation of *FKS2* (encoding the alternative 1,3-β-glucan synthase subunit) is dependent upon both Rlm1p and Crz1p (Jung & Levin, 1999; Lagorce *et al.*, 2003). Additional work will be necessary to decipher

**Table 3.** *In silico* promoter analysis of Hog1p-dependent genes

Y=C/T; W=A/T; S=C/G; N=A, T, G or C. –, absence of sites of particular sequence motifs described in the other column.

Consensus	Sequence motif	Function	Number of sites	
			YFL014W	YLR414C
STRE (Msn2/Msn4)	AGGGG	General stress	7	1
RLM1	STAWWWWTAG	Cell wall integrity	2	1
STE12	TGAAACA	Mating/filamentous growth	–	2
TEC1	CATTCYY	Filamentous growth	1	3
DIG1	TGAAACA	Mating/filamentous growth	–	2
HSF1	TTCYN <sub>6</sub> TTC	Heat stress	2	1
CDRE (Crz1)	AGCCTC	Calcium metabolism	–	1
SKO1	ACGTCA	Osmotic stress	1	2

the putative participation of other signalling pathways on the expression pattern elicited by cell wall damage. In this context, the yeast cell wall chip could be a very useful tool to achieve this goal.

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