

Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants

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Abstract The cDNA from activated mutants of the homologous transcription factors Pdr1p and Pdr3p was used to screen DNA microarrays of the *Saccharomyces cerevisiae* complete genome. Twenty-six overexpressed targets of the *PDR1-3* and/or *PDR3-7* mutants were identified. Twenty-one are new targets, the majority of which are of unknown function. In addition to well known ABC transporters, these targets appear to be involved in transport or in membrane lipids and cell wall biosyntheses. Several of the targets seem to contribute to the cell defence against a variety of stresses. Pdr1p and Pdr3p do not act similarly on all targets. Unexpectedly, the expression of 23 other genes appeared to be repressed in the *PDR1-3* and/or *PDR3-7* mutants. In contrast to the majority of the activated genes, none of the repressed genes contains pleiotropic drug resistance binding sites in their promoter.

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1. Introduction

In 1973, Rank et al. [1] reported the existence of a single nuclear gene controlling the resistance of *Saccharomyces cerevisiae* to 18 mitochondrial and non-mitochondrial inhibitors. They proposed that this phenotype, called 'pleiotropic drug resistance' (PDR), results from modification of plasma membrane permeability [2]. In 1987, this gene was identified by Balzi et al. [3] as *PDR1*, a new member of the Zn2Cys6 family of transcription factors.

Another determinant of pleiotropic resistance was genetically identified in 1990 and called *PDR5* [4]. Sequencing of *PDR5* in 1994 [5–7] showed that the encoded protein belongs to the ABC transporter superfamily. It then became progressively clear that Pdr5p is a plasma membrane efflux pump of very broad specificity for amphiphilic drugs and that its expression is controlled by Pdr1p [5,8].

An outstanding feature of *PDR1* is that its efficiency as a pleiotropic drug resistance effector is considerably increased by a series of dominant point mutations [5,9]. One of the most powerful pleiotropic drug resistance mutations is *PDR1-3*, derived from a strain originally isolated by Guérineau et al.

[10]. This mutation dramatically increases the expression of *PDR5* mRNA [5,9,11,12], as well as that of other genes, such as those encoding the ABC transporters Snq2p [11–13], Yor1p [12,14], Pdr10p [12,15] and Pdr15p [12,15]. More importantly, in the *PDR1-3* mutant, the corresponding proteins are highly overexpressed and apparently correctly trafficked to the plasma membrane in an active form, able to confer drug resistance and to hydrolyze nucleoside triphosphates [11,12,16].

In addition to Pdr1p, yeast contains a homologue called Pdr3p [17], encoded by a gene originally identified and mapped by Subik et al. [18,19]. Pdr3p and Pdr1p share identical binding sites on the promoters of *PDR5*, *SNQ2* and *YOR1* [13,14,16,20–22]. Among other mutations, *PDR3-7* markedly enhances pleiotropic drug resistance [23].

Genome analysis by DNA microarray hybridization analysis is the tool of choice for identifying new targets of transcription factors in yeast. This approach has been recently validated by the study of the genes repressed in deletants of the *YAP1* gene [24]. We have used a similar technique to identify targets of the PDR transcription factors using the 'up'-mutations, *PDR1-3* and *PDR3-7*, which considerably amplify the sensitivity of the screen.

The identification of the overexpressed genes in the *PDR1-3* and *PDR3-7* mutants greatly contributes to the delineation of the complex network of genes involved in yeast pleiotropic drug resistance. The understanding of the regulation of this trait in *S. cerevisiae* should pave the way for the elucidation of putative similar networks operating in pathogenic yeasts and be relevant to the development of new fungicides.

2. Materials and methods

2.1. Strains and growth media

S. cerevisiae FY1679-28C/TDEC (*MATa ura3-52 leu2Δ1 his3Δ200 trpΔ63 GAL2+ pdr1Δ::TRP13 pdr3Δ::HIS3*) was the *pdr1*, *pdr3* double knock-out mutant used in this study. The strain was transformed either with the PRS315::pdr1-3 [9], or with the pFL36::pdr3-7 [23]. Plasmid transformation with PRS315 or pFL387 empty vectors was performed as a control. Yeast was grown on standard rich glucose (YPD) medium or on synthetic complete (SC) media lacking appropriate amino acids for plasmid maintenance.

2.2. Hybridizations

Total RNA was extracted from cells grown in synthetic medium containing 2% glucose and lacking appropriate amino acids for plasmid maintenance, harvested in the early exponential phase (10^7 cells/ml) followed by mRNA purification using the Promega PolyA-Tract mRNA Isolation System. Fabrication of DNA microarrays, synthesis of fluorescent labeled cDNA, hybridization to the microarrays and

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subsequent scanning were performed as previously described [24]. The current protocols for all DNA microarray-related procedures are available at <http://cmgm.stanford.edu/pbrown/protocols>.

2.3. Data analyses

Fluorescent images were analyzed using ScanAlyze software, available at <http://rana.stanford.edu/software>. Using the software of Eisen et al. [25], genes were clustered hierarchically into groups on the basis of the similarity of their expression profiles. The gene *HXT2* and *YGR212w* which yielded positive red signals were not considered significant because of a high similarity of *HXT2* with numerous hexose transporter gene sequences or because of putative interference of *YGR12w* with a vicinal gene.

3. Results

3.1. The up-regulated genes

Total mRNA was isolated from exponentially growing yeast cells and labeled with either the red fluorescent dye, Cy5, in the case of the *PDR1-3* or *PDR3-7* mutant strains or the green fluorescent dye, Cy3, in the case of the control strain. In the latter, both wild type genes *PDR3* or *PDR1* were deleted. The labeled cDNAs from each set were mixed and hybridized to the gene microarrays of all available *S. cerevisiae* genes [24]. For each gene represented in the array, the Cy5 fluorescence/Cy3 fluorescence ratio measured at the corresponding array element is a quantitative measurement of the relative abundance of the transcript of that gene in the two strains.

The mRNA levels for 26 genes were at least two-fold higher in the *PDR1-3* and/or *PDR3-7* mutants (Fig. 1, red squares). These genes could be grouped into three classes. The transcription of 19 genes was activated by both mutations, but to a greater extent by *PDR1-3* than by *PDR3-7*. Another group of four genes (*PDR15*, *COS10*, *REV1* and *FRE4*) showed greater activation by *PDR3-7* than by *PDR1-3*. A third group of three genes (*YPL088w*, *YLL056w* and *MET17*) seems to be exclusively activated by the *PDR1-3* mutants.

In vitro DNA binding studies and mutational analyses of the promoters of known targets, such as *PDR3*, *PDR5*, *SNQ2*, *YOR1*, *PDR10*, *PDR15* and *HXT11*, have previously identified several pleiotropic drug response elements called PDREs [13,17,20–22,26,27] (Table 1). Fifteen of the 26 over-expressed genes identified by the microarray hybridization experiment as being activated by the *PDR1-3* or *PDR3-7* mutations contain PDREs in their promoter (marked by the blue balls in Fig. 1). When the sequences upstream of the 26 *Pdr1p*- or *Pdr3p*-activated genes were subjected to an unbiased search for shared signature elements, a consensus se-

Table 1

The pleiotropic drug response elements (PDRE) in promoters controlled by *Pdr1p* and/or *Pdr3p*

PDRE-A	T	C	C*	G*	C*	G	G*	A*
PDRE-B	T	C	C	<u>A</u>	<u>T</u>	G*	G	A
PDRE-B'	T	C	C	<u>A</u>	C	G	G	A
PDRE-C	T	C	C	<u>G</u>	C	G	<u>C</u>	A
PDRE-D	T	C	C	G	C	G	<u>G</u>	<u>G</u>
Consensus	T	C	C	G/a	C/t	G	G/c	A/g

Asterisks denote residues which have been mutagenized and shown to be necessary for PDRE function. Residues shown to be specific for each variant are underlined. This table is a compilation from data reported in [20–22,26,27].

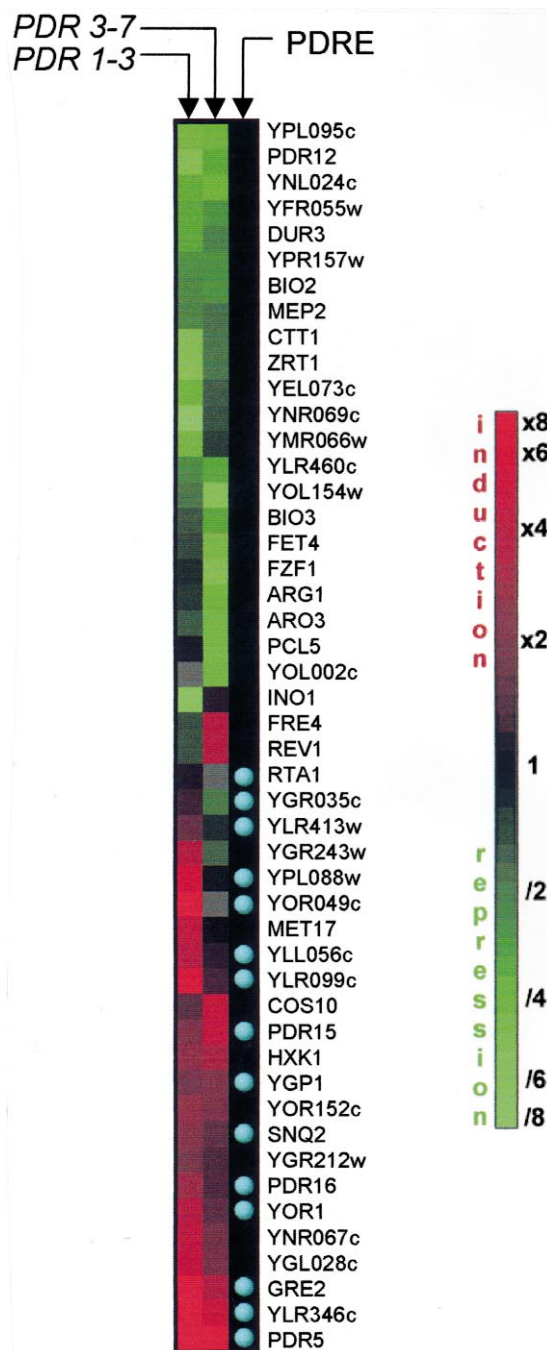


Fig. 1. Cluster image showing the different classes of gene expression profiles modified by PDR mutants. The *PDR1-3* or *PDR3-7* mutant alleles were carried on the centromeric plasmids pRS315 and pFL36, respectively, in the $\Delta PDR1\Delta PDR3$ double knock-out strain FY1679-28c/TDEC. Genes whose transcript levels were increased (red) or decreased (green) two-fold by the activating mutations *PDR1-3* (column 1) or *PDR3-7* (column 2) were selected. This subset of genes was clustered hierarchically on the basis of the similarity of their expression profiles [25]. The blue circles in column 3 identify genes whose promoters (within 800 bp upstream of the coding sequence) contain the consensus PDRE shown in Table 1.

quence (C/TCCGC/TGGA/G), which perfectly matched the known PDREs, was identified (Table 1).

However, several genes that were overexpressed in the *PDR1-3* or *PDR3-7* mutants had no recognizable PDRE in their promoters. It remains to be determined whether these

Table 2

Functional classification of gene products activated by the *PDR1*–3 and/or *PDR3*–7 mutations

Gene	Function	Phenotype
<i>ABC transporters</i>		
YOR153w/ <i>PDR5</i>	multidrug ATPase	multidrug resistance
YGR281w/ <i>YOR1</i>	multidrug ATPase	multidrug resistance
YDR011w/ <i>SNQ2</i>	multidrug ATPase	multidrug resistance
YDR406w/ <i>PDR15</i>	multidrug ATPase?	?
<i>MFS and other permeases</i>		
YLL028w/ <i>TP01</i>	MFS vacuolar polyamine transporter	multidrug resistance
YOR049c	transporter of unknown substrate?	homology to molasses resistance
YGR213c/ <i>RTA1</i>	transporter of unknown substrate?	7-amincholesterol resistance
<i>Lipid metabolism</i>		
YNL231c/ <i>PDR16</i>	lipids synthesis	multidrug resistance
YDR072c/ <i>IPT1</i>	mannosyl diphosphorylinositol ceramide synthase	multidrug resistance
<i>Cell wall metabolism</i>		
YLR099c/ <i>ICT1</i>	aromatic esterase	azoles, benomyl resistance
YPL088w	aryl-alcohol dehydrogenase	calcofluor resistance
YGL028c/ <i>SCW11</i>	soluble cell wall protein	cell wall maintenance
YNR067c	endo β -glucanase	cell wall maintenance
<i>Stress response</i>		
YNL160w/ <i>YGP1</i>	secreted glycoprotein	nutrient starvation-induced
YFR053c/ <i>HXX1</i>	hexokinase 1	glucose starvation-induced
YOL151w/ <i>GRE2</i>	dihydroflavonol-reductase?	osmotic stress-induced
YLR303w/ <i>MET17</i>	<i>O</i> -acetyl homoserine sulphydrylase	osmotic stress-induced
YNR060w/ <i>FRE4</i>	ferric reduction and transport	oxidized iron-induced
YOR346w/ <i>REV1</i>	DNA repair	DNA damage-induced
YLL056c	sugar epimerase?	mutagen-induced
<i>Unknown</i>		
YLR346c	?	?
YGR035c	?	?
YOR152c	?	?
YNR075w/ <i>COS10</i>	?	?
YLR413w	?	?
YGR243w	?	?

The functions were compiled from the SGD, YPD and MIPS protein databases. The question marks (?) indicate unknown functions or functions inferred only by sequence similarity.

genes contain Pdr1p or Pdr3p binding sites that differ from the known PDREs or whether they are activated by an alternative factor subject to regulation by Pdr1p or Pdr3p.

The 26 genes that showed significant increased expression in the *PDR1*–3 and *PDR3*–7 mutants can be divided into six groups according to the putative or recognized functions of their product (Table 2).

The first functional group is already well recognized. The ABC transporter genes, *PDR5* [5–7,16], *YOR1* [12,14] and *SNQ2* [11,13,28], are known to be major determinants of yeast multidrug resistance. The products of these three genes mediate the efflux of structurally diverse hydrophobic compounds [29]. The sequence of the *PDR15* protein is very similar to that of *PDR5*; its control by Pdr1p has previously been reported [12,15], but its function as multidrug pump has not yet been demonstrated.

The second group consists of three permeases. On the basis of phylogenetic arguments, plasma membrane permeases have been proposed to be involved in the cellular efflux of hydrophobic substrates [30]. However, none of the permeases listed in Table 2 have been previously reported to be controlled by *PDR1* or *PDR3*. This is the case for the polyamine transporter, *TP01* [31]. Tpo1p belongs to the DHA12 family (drug proton antiport with 12 predicted transmembrane spans) of the major facilitator superfamily and is involved in multidrug resistance (do Valle Matta, Université de Louvain-la-Neuve, Belgium, personal communication). Similarly, the gene *RTA1*, which is involved in resistance to toxic sterol derivative [32], is also a target of *PDR1*–3/*PDR3*–7. Very interestingly, another

new target, YORO49c, is a putative transporter homologous to a gene involved in resistance to unknown toxic constituents of sugar beet molasses [33]. It is thus tempting to suggest that all the new permease-type targets of *PDR1*–3/*PDR3*–7 listed in Table 2 are involved in the efflux of toxic, possibly hydrophobic, compounds.

Surprisingly some well characterized permease genes, known to be regulated by *PDR1* and *PDR3*, such as the glucose transporter genes *HXT9* and *HXT11* [27] and the multidrug transporter gene, *FLR1* [34], were not found in these analyses. The failure to detect by microarray analysis these *PDR1*/*PDR3* targets might be due to the limited sensitivity of the DNA microarray.

The third group comprises two genes with known or predicted roles in lipid metabolism. We have recently shown that *PDR16* controls the phospholipid composition of the plasma membrane which modifies the passive diffusion of hydrophobic drugs across the plasma membrane, thereby modulating multidrug resistance [35]. The *IPT1* gene product, involved in sphingosine synthesis [36], also modulates multidrug resistance (Lambert, Université de Louvain-la-Neuve, Belgium, personal communication). Thus, the lipid composition of the plasma membrane is a determinant of multidrug resistance.

The fourth group consists of genes that encode proteins with a weak sequence similarity to proteins involved in cell wall metabolism. This suggests that the cell wall is not a neutral barrier and that its composition, and possibly its permeability to hydrophobic compounds, might be modulated by *PDR1* or *PDR3*.

A coherent biochemical analysis of the genes classified in the fifth group (called 'stress') is more difficult. 'Guilt by association' suggests that an investigation of the possible role of these genes in defense against nutritional or other stresses is warranted.

The last group, named 'unknown', contains six genes without any known function. Of them, one of the strongest hyper-activated new Pdr1/3 targets is *YLR346c*; this gene, although of unknown function, has a homologue, *YGR035c*, which is also hyperinduced by *PDR1* and *PDR3* during late exponential growth (data not shown).

3.2. The down-regulated genes

The 23 genes for which transcript levels were at least two-fold lower in either the *PDR1-3* or the *PDR3-7* mutant than in their corresponding deletion mutants (green genes in Fig. 1) can also be divided into three classes. The transcript levels for 10 genes were reduced in both the *PDR1-3* and *PDR3-7* mutant strains, six genes showed reduced expression in the *PDR1-3* mutant, but were basically unaffected by the *PDR3-7* mutation, while seven genes showed significantly reduced expression in the *PDR3-7* mutant, but were essentially unaffected by the *PDR1-3* mutation. Here again, Pdr1p and Pdr3p act somewhat differently.

None of the genes for which transcript levels were reduced by the *PDR1-3* or *PDR3-7* mutations contained a recognizable PDRE. The observed Pdr1p/Pdr3p-dependent repression is therefore indirect and might be mediated by some as yet unidentified repressors activated by Pdr1p and Pdr3p or by another mechanism.

Since most of the genes that show *PDR1/PDR3*-dependent repression of expression do not contain well-identified PDRE elements in their promoters, their repression might not be directly relevant to the PDR phenotype. However, it is interesting to note that five of these genes (*DUR3*, *MEP2*, *PDR12*, *ZRT1* and *FET4*) code for membrane proteins involved in diverse transport processes. Of these, *PDR12* deserves a special mention, since the expression of this ABC transporter, which has been shown to confer resistance to permeable weak acids [37] was clearly enhanced when *PDR1/PDR3* are absent. Two other repressed genes, *FZF1* and *PCL5* code for regulatory elements involved in the developmental response to phosphorus-limited growth. Thus, several aspects of this Pdr1p/Pdr3p negative regulation seem to be related to transport functions in response to the cell environment.

4. Discussion

In addition to the 49 genes for which the transcript level changed, positively or negatively, by more than two-fold in response to the *PDR1-3* and *PDR3-7* regulatory mutations, several other genes showed smaller effects on transcript levels; a list of these genes is available on the web site <http://www.biologie.ens.fr/yeast-publi.html>. Some of these might turn out to be significant targets when tested under different physiological or genetic conditions. Indeed, the pattern of PDR-regulated genes here reported is only valid for the reported strains under the specific growth conditions used (synthetic glucose media and early exponential growth phase).

DNA microarray hybridization data must be considered only as an initial global screening investigation. The expression levels of each newly detected target must be confirmed by

other methods. Such confirmation measurements are under way (van den Hazel, Université de Louvain-la-Neuve, Belgium, personal communication). For each target, it also remains to be shown whether a single genomic copy of the wild type *PDR1* or *PDR3* genes suffices to significantly modulate its expression under physiological conditions. Nevertheless, almost all previously demonstrated positive targets of the wild type *PDR1* single copy gene have been confirmed by the present work which markedly increase the sensitivity of DNA microarray analysis by using up-regulating mutant.

Another transcription factor, the 'leucine zipper' Yap1p, controls certain aspects of yeast multidrug resistance [38]. The major function of Yap1p seems to be oxidative stress defense. At least three targets of *YAP1* are also controlled directly or indirectly by the PDR network [38]. The complex interactions between Pdr1p, Pdr3p, Yap1 and other transcription factors such as Yrr1p [39] are still to be experimentally dissected for a total understanding of the regulation of pleiotropic drug resistance in yeast. For that purpose, the DNA microarray approach used as presented here will probably be helpful.

In conclusion, our data show that, under the tested conditions, the Pdr1p/Pdr3p transcription factors renders the cell resistant to chemical and nutritional stress in several ways other than the well-known regulation of ABC efflux transporters. We show that many of the genes overexpressed by the *PDR1-3* and *PDR3-7* mutations encode proteins that reduce intracellular accumulation of hydrophobic compounds. At least four ABC transporters and three permeases seem to be involved in the cellular efflux of these compounds. Modulation of several enzymes involved in lipid synthesis and of others possibly involved in cell wall metabolism, may modify access of drugs to the plasma membrane. The coherence of this complex strategy for conferring drug resistance, the apparent functional redundancy of many genes involved and the complexity of the various transport pathways is remarkable. It will be interesting to investigate whether similar strategies for defense against noxious chemical agents are employed by other microorganisms, such as pathogenic yeasts and higher eukaryotes.

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