

Short Communication

Interplay among regulators of multidrug resistance in *Kluyveromyces lactis*

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Abstract. The *KIYAP1* and *KIPDR1* genes encode two main transcriptional regulators involved in the control of multidrug resistance in *Kluyveromyces lactis*. Deletion of *KIPDR1* or *KIYAP1* genes in *K. lactis* generated strain hypersusceptible to diamide, benomyl, fluconazole and oligomycin. Overexpression of genes *KIPDR1* or *KIYAP1* from a multicopy plasmid in the *Klprdr1Δ* mutant strain increased the tolerance of transformants to all the drugs tested. YRE response elements were found in the promoter of the *KIPDR1* gene. Gel retardation assays confirmed the binding of *KIYap1p* to the YREs in the *KIPDR1* gene promoter indicating that *KIYap1p* can control the *KIPDR1* gene expression.

Key words: Multidrug resistance — Oxidative stress — Transcriptional regulators — *Kluyveromyces lactis*

Abbreviations: MDR, multiple-drug resistance; MFS, major facilitator superfamily; PDR, pleiotropic-drug resistance; YAP, yeast AP-1-like; YNB medium, yeast nitrogen base medium; YPD medium, yeast extract/peptone/dextrose medium; YRE, yeast AP-1-like recognition element.

The cellular stress response is evolutionary conserved in all living organisms, a major role being attributed to the molecular mechanisms that confer stress protection. One of such mechanisms elicited by the cells in the presence of unrelated chemicals is referred to as multidrug resistance (MDR). The ability of cells to tolerate multiple-different drugs is a major concern for human health, as it leads to antibiotic resistance in pathogens and enables cancer cells to survive chemotherapy (Kane et al. 1990). MDR in eukaryotes often result from changes in expression of membrane transport systems (Ling 1997). The MDR regulatory pathway has been extensively studied in the model yeast *Saccharomyces cerevisiae*. The expression of the transporter encoding genes involved in MDR is under the control of two regulatory networks: pleiotropic drug resistance (PDR) and yeast AP-1-like (YAP). They include specific transcriptional regulatory proteins containing zinc cluster (typically exhibiting

a Zn(II)₂Cys₆ type of zinc finger) and basic region-leucine zipper (bZip)-sequence.

The PDR network in *S. cerevisiae* comprises 10 transcription factors regulating about 70 different target genes (Moye-Rowley 2003a; Fardeau et al. 2007). In this network, the Pdr1p transcription factor has the largest set of potential targets (about 50). Pdr1p and its functional homologue, Pdr3p, were identified as regulators of the basal level of drug resistance in yeast cells (Balzi et al. 1987; Delaveau et al. 1994). Gain- or loss-of-function alleles of *PDR1* and *PDR3* confer resistance or sensitivity to a large spectrum of unrelated drugs, through constitutive modifications to the expression of ATP binding cassette transporters, major facilitator superfamily (MSF) members or enzymes modifying the lipid composition of the plasma membrane (Kolaczowska and Goffeau 1999). Although *PDR1* and *PDR3* display some functional redundancy they seemed to have quantitatively different contributions to drug resistance (Delaveau et al. 1994; Katzmann et al. 1994). Pdr1p and Pdr3p recognize the same DNA consensus motif (PDRE – pleiotropic drug response element) found upstream of most genes regulated by these transcription factors (Katzmann et al. 1994; Dela-

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hodde et al. 1995). Putative orthologs of *PDR1* and *PDR3* genes were identified also in genomes of other sequenced hemiascomyces yeast species (Dujon et al. 2004). In species that diverged before the occurrence of the ancestral whole genome duplication, such as *Kluyveromyces lactis*, only one ortholog has been identified (Bussereau et al. 2006; Balkova et al. 2009).

The YAP network contains 8 members involved in the oxidative stress response and drug resistance regulation in *S. cerevisiae* (Rodrigues-Pousada et al. 2004). The *YAP1* gene encodes a 650 amino acid transcription factor that was initially isolated as a biochemical homologue of mammalian AP-1 (Harshman et al. 1988). Yap1p contains an amino-terminally located basic region-leucine zipper type of DNA-binding domain and has been extensively studied in regards to its role in oxidative stress tolerance. Yap1p binds to Yap1 recognition elements (YRE) TTA(C/G)T(A/C)A, located in the promoters of its target genes (Nguyen et al. 2001) and controls the expression of genes encoding the majority of antioxidants and thiol-oxidoreductases, such as the glutathione reductase *GLR1* and the thioredoxin reductase *TRR1* (Moye-Rowley 2003b; Herrero et al. 2008). Yap1p is also essential for the cell's response to cadmium or drug exposures and can be activated by chemicals (e.g. diamide) or antifungal agents (e.g. benomyl) (Lucau-Danila et al. 2005). Upon changes in cellular redox status, Yap1p is rapidly recruited to the nucleus, where it activates the genes involved in cellular antioxidant defences and thiol redox control (Delaunay et al. 2002; Azevedo et al. 2003; Rodrigues-Poussada et al. 2004). Recently, the Yap1p ortholog in *K. lactis* has been characterized and shown to be

involved in oxidative stress response (Billard et al. 1997). In the present work we studied the role of *KLYap1p* in the control of *K. lactis* MDR.

Experiments were performed using the following strains: *K. lactis* PM6-7A (*MATa uraA1 ade2 Rag⁺ pKDI*) and MW 179-1D (*MAT α lac4-8 uraA1-1 leu2 metA1-1 Ade-trp1 Rag⁺ pKDI*) (Chen et al. 1992) as well as PM6-7A/*pdr1* Δ (Balkova et al. 2009) and MW 179-1D/*yap1* Δ (Billard et al. 1997). Yeast strains were grown in YPD medium (in %: glucose 2, bactopectone 1, yeast extract 1) or in YNB minimal medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose and appropriate nutritional requirements. The media were solidified with 2% bactoagar. The *KIPDR1* and *KLYAP1* genes (Imrichova et al. 2005; Balkova et al. 2009) were cloned in multicopy plasmid pRS306K (2 μ m *URA3 ARS1 KARS2 ori Amp^r*) (Heus et al. 1994) under the control of their own promoter. Yeast cells were transformed by electroporation (Thompson et al. 1998).

The *Escherichia coli XL1-Blue* strain was used as a host for plasmid constructions and propagation. The bacterial cells were grown at 37°C in Luria-Bertani medium (in %: tryptone 1, NaCl 1, yeast extract 0.5; pH 7.0) supplemented with 100 μ g/ml ampicillin for selection of transformants.

Sensitivities of yeast cells to drugs were determined by spot assay. Yeast strains were grown in liquid medium to the early stationary phase (5×10^7 cells per ml). Serial dilutions (10-fold) of each strain were spotted onto solid minimal medium supplemented with various concentrations of the specific drug being tested. Qualitative growth differences among transformants were recorded

Table 1. *Kluyveromyces lactis* orthologues containing predicted YRE

ORF	Location relative to ATG	Matching sequence	Gene product role
KLLA0A09119g	-392 to -386	gttTTTGTAaAat	<i>KIPDR1</i> (transcription factor)
	-289 to -283	aaaTTACAAAgcc	
KLLA0C18931g	-454 to -448	acaTTAGTAAtga	<i>KNQ1</i> (MFS transporter)
	-721 to -715	gcgTTACTCActc	
KLLA0B09702g	-890 to -884	accTTACTCAatc	Similar to <i>S. cerevisiae PDR12</i> (ABC MDR transporter)
	-648 to -642	cggTTAGTCaggt	
KLLA0F18106g	-681 to -675	aaaTTAGTAAat	Similar to <i>S. cerevisiae TPO1</i> (polyamine transporter)
KLLA0E00462g	-791 to -785	tgaTTAGTAAaAat	Similar to <i>S. cerevisiae YBT1</i> (yeast bile transporter)
	-600 to -594	tagTTTGTAacgc	
KLLA0D19668g	-341 to -335	gaaTTTGTAagag	Similar to <i>S. cerevisiae PDR16</i> (protein involved in lipid biosynthesis and MDR)
KLLA0C04103g	-52 to -46	aaaTTAGTAAatg	Similar to <i>S. cerevisiae RPN4</i> (transcription factor, stimulates expression of proteasome genes)

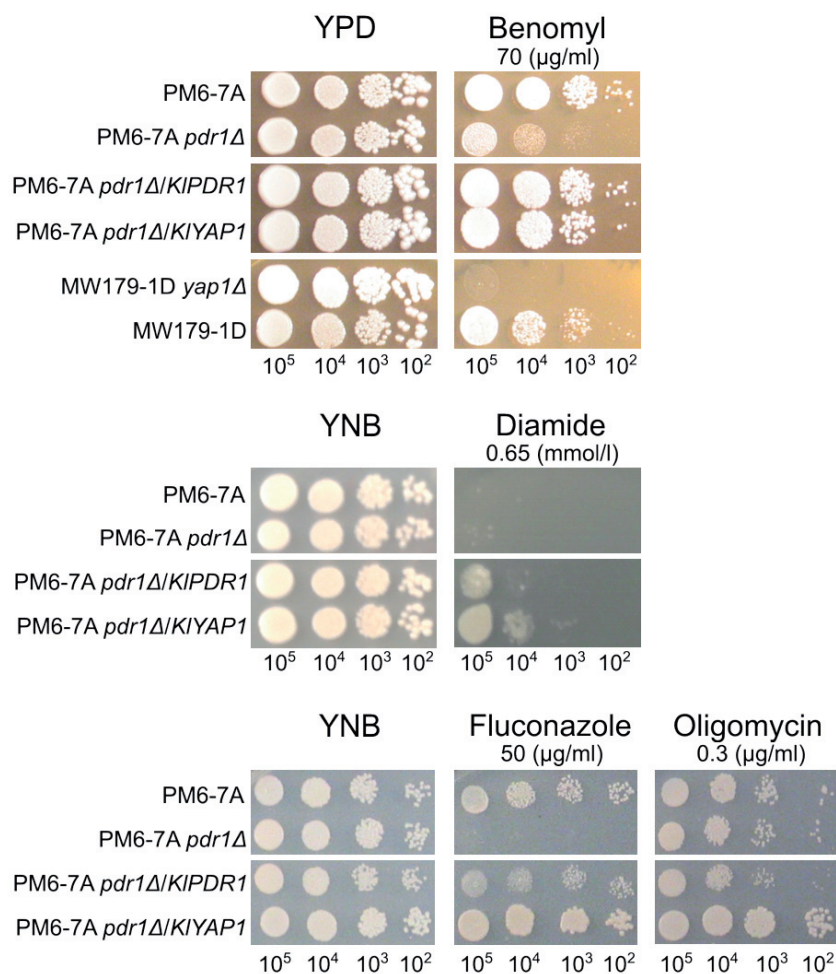


Figure 1. Drug susceptibility testing of *K. lactis* strains. Spotting assays were performed with serial dilutions of overnight cultures onto YPD (yeast-extract peptone dextrose) or YNB (yeast nitrogen base) minimal medium containing different drugs as indicated. Plates were incubated for 3 (YPD) or 5 (YNB) days at 28°C.

following the incubation of the plates at 28°C from three up to five days.

Formation of the *KIYap1p*-DNA complex in the protein-DNA binding assay was assayed by a gel retardation procedure according to Imrichova et al. (2005). The double stranded *HindII-HindIII* DNA fragment of 511 bp from *KIPDR1* promoter was labelled with ^{32}P and incubated with cell extracts (see below) in 20 μl of incubation buffer (in mmol/l: 20 Tris-Cl (pH 8.0), 5 MgCl_2 , 5 CaCl_2 , 90 KCl, 0.5 dithiothreitol, 1 Na_2EDTA , 7.5% glycerol and 2 μg of denaturated salmon sperm DNA as a nonspecific competitor). Binding reactions were carried out at 4°C for 15 min and the reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.5 \times TBE buffer, pH 8.3 (in mmol/l: 45 Tris base, 45 H_3BO_3 , 1.2 EDTA, 2% glycerol). After electrophoresis at 4°C, the gel was transferred to the surface of Whatman

3MM paper, dried and visualized by autoradiography. The cell extracts were prepared as follows: The yeast cells were grown in liquid minimal medium containing 2% glucose as carbon source. After the culture had reached the density of 2×10^7 cells per ml, cells were harvested by centrifugation and washed with extraction buffer (in mmol/l: 0.2 Tris-Cl (pH 8.0), 0.4 $(\text{NH}_4)_2\text{SO}_4$, 10 MgCl_2 , 1 Na_2EDTA , 7 β -mercaptoethanol, 1 phenylmethylsulfonyl fluoride (PMFS), 1 $\mu\text{g/ml}$ pepstatin A, 10% glycerol). The cells suspended in the extraction buffer (1 ml for 1 g cells) were disrupted using ice-chilled, acid washed glass beads (0.45 mm in diameter). The broken cells were centrifuged for 30 min at $27\,000 \times g$, and the supernatant fraction was collected and stored at -80°C until use.

The present work is focused on the role of the transcription regulators *KIPdr1p* and *KIYap1p* in *K. lactis* MDR.

Computer assisted analysis of the *KIPDR1* gene promoter region identified two potential YREs at positions -283 and -386 , respectively (Tab. 1). The presence of Yap1p recognition elements in the promoter of *KIPDR1* suggests a direct interaction of *KIYap1p* with this promoter. Yap1p is already known as an essential factor in the response to oxidative stress (Moye-Rowley 2003b; Rodrigues-Pousada et al. 2004). Yap1p confers azole resistance in *S. cerevisiae* by activating expression of MFS proteins encoded by the *ScFLR1* and *ScATR1* genes (Kanazawa et al. 1988; Coleman et al. 1997). We have previously demonstrated that the *KNQ1* gene, the structural and functional ortholog of the *ScATR1* gene encoding a multidrug transporter of the MFS in *K. lactis*, is a transcription target of *KIYap1p* (Takacova et al. 2004). The H_2O_2 and diamide-induced transcriptional activation of *KNQ1* is fully dependent on *KIYap1p* (Imrichova et al. 2005). As Fig. 1 shows, not only *Klyap1Δ* but also *Klpdr1Δ* mutant cells were sensitive to diamide and benomyl. On the other hand transformation of a drug sensitive *Klpdr1Δ* mutant strain which a plasmid-borne *KIPDR1* or *KIYAP1* genes, orthologs of which overexpress MDR transport genes in *S. cerevisiae* (deRisi et al. 2000), resulted in the tolerance of transformants to both drugs (Fig. 1). Although structurally unrelated,

both diamide and benomyl have thiol reactive properties (Azevedo et al. 2003) and so they might similarly activate Yap1p through the chemical modification of its C-terminal cysteines (Azevedo et al. 2003). Deletion of *KIPDR1* encoding the key transcription factor involved in *K. lactis* MDR resulted in an increased sensitivity not only to diamide and benomyl, but also to fluconazole and oligomycin. Transformants of the *Klpdr1Δ* mutant cells carrying the *KIPDR1* or *KIYAP1* genes on multicopy plasmids exhibited increased ability to tolerate the drugs tested. These results indicate that some of the target genes activated by *KIPdr1p* and/or *KIYap1p* transcription factors can participate in a process leading to a decreased intracellular level of the drugs in the *K. lactis* transformants. *KIYap1p* can alleviate the *KIPdr1p* deficiency and restore the normal level of *K. lactis* cell's tolerance to the tested compounds. Based on the presence of YREs in the promoter region of *KIPDR1* gene we tried to demonstrate the direct binding of *KIYap1p* to the *KIPDR1* promoter.

We performed an electrophoretic mobility shift assay with a ^{32}P labelled probe (511 bp) derived from the *KIPDR1* promoter overlapping the two putative YREs and protein extracts prepared from strains expressing the wild-type level of *KIYap1p* or containing deletion in the *KIYAP1* gene (*Klyap1Δ*). The presence of *KIYap1p* in the protein-DNA complex was demonstrated as follows: First, the complex was absent when protein extracts prepared from the *Klyap1Δ* strain were used (Fig. 2, lane C). Second, *KIYap1p* overexpression from a multicopy plasmid in the *Klyap1Δ* strain led to the appearance of a slowly-migrating complex (Fig. 2, lane D) that also appeared in the presence of protein extracts prepared from the wild-type strain (Fig. 2, lane B). The results suggest that the motifs identified by promoter analysis of the *KIPDR1* gene are important for *KIYap1p* binding *in vitro* and could indicate a close cooperation of *KIPdr1p* and *KIYap1p* in *K. lactis*.

Finally, we looked for the presence of YRE elements in the promoters of the all *K. lactis* genes. We found at least 150 different *KIYap1p* target genes. Along with the genes encoding the antioxidants and thiol-oxidoreductases, such as thioredoxin reductase *TRR1* ortholog (KLLAF15037g), we found the genes potentially involved in *K. lactis* MDR (Tab. 1) and more than 100 genes of unknown function.

In summary, this work describes novel regulatory association between transcriptional regulators in *K. lactis*. The association is presumably direct as suggested by the occurrence of putative *KIYap1p* transcription factor binding sites in the promoter region of *KIPDR1* gene. *KIYap1p* has been shown to drive elevated levels of transcription of *KIPDR1* as measured by use of a *KIPDR1-lacZ* reporter gene in *S. cerevisiae* (data not shown). We propose that *KIYap1p*, as a global regulator involves recruiting other zinc-cluster protein to fine-tune the regulation of MDR

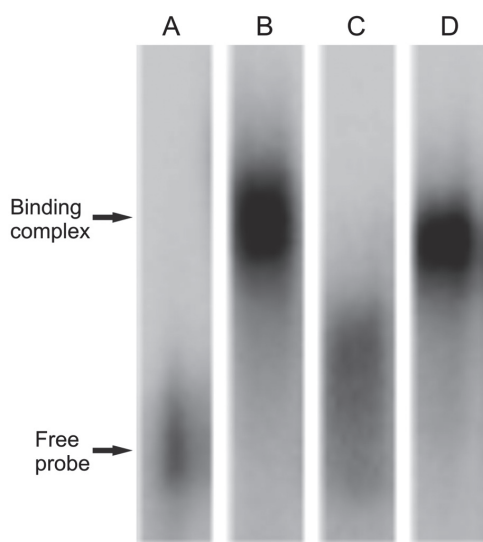


Figure 2. Binding of *KIYap1p* to the *KIPDR1* gene promoter. ^{32}P -labelled DNA probe overlapping both YREs was incubated with 30 μ g of protein extracts prepared from strains MW197-1D(*YAP1*) and MW197-1D/*yap1Δ* mutant or the MW197-1D/*yap1Δ* mutant transformed with multicopy plasmid containing *KIYAP1*. The resulting complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gels. A. Free DNA (without protein extract). B. MW197-1D(*YAP1*)-wild type. C. MW197-1D/*yap1Δ*. D. MW197-1D/*yap1Δ* + *KIYAP1*.

genes in this aerobic, biotechnologically important yeast species.

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