The yeast ABC transporter Pdr18 (ORF YNR070w) controls plasma membrane sterol composition, playing a role in multidrug resistance

Tânia Rodrigues Cabrito, Miguel Cacho Teixeira, Ashutosh Singh, Rajendra Prasad, Isabel Sá-Correia

The yeast ABC transporter Pdr18 (ORF *YNR070w*) controls plasma membrane sterol composition, playing a role in multidrug resistance

Tânia R Cabrito[1,2], Miguel C Teixeira[1,2], Ashutosh Singh[3], Rajendra Prasad[3] and Isabel Sá-Correia[1,2*]

[1]IBB - Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, 1049-001 Lisboa, Portugal.

[2]Department of Bioengineering, Instituto Superior Técnico, Technical University of Lisbon, 1049-001 Lisboa, Portugal.

[3] Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India.

Corresponding author: Prof. Isabel Sá-Correia, IBB - Institute for Biotechnology and Bioengineering, Biological Sciences Research Group, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; e-mail isacorreia@ist.utl.pt; Tel. +351-218417682; Fax +351-218419199.

Short title: The MDR transporter Pdr18 controls plasma membrane sterol composition

Keywords: multidrug resistance, ergosterol homeostasis, *Saccharomyces cerevisiae*, pleiotropic drug resistance transporters, herbicide resistance, PDR network

ABSTRACT

The action of multidrug efflux pumps in multidrug resistance (MDR) acquisition has been proposed to partially depend on the transport of physiological substrates which may indirectly affect drug partition and transport across cell membranes.

In this work, *PDR18* gene (ORF *YNR070w*), encoding a putative pleiotropic drug resistance (PDR) transporter of the ATP-binding cassette superfamily, was found to mediate plasma membrane sterol incorporation in yeast. Pdr18 physiological role is demonstrated to affect plasma membrane potential and is proposed to underlie its action as a MDR determinant, conferring resistance to the herbicide 2,4-D. The action of Pdr18 in yeast tolerance to 2,4-D, which was found to contribute to reduce [^{14}C]-2,4-D intracellular accumulation, may be indirect, given the observation that 2,4-D exposure deeply affects the sterol plasma membrane composition, this effect being much stronger in *Δpdr18* background. *PDR18* activation under 2,4-D stress is regulated by the transcription factors Nrg1, controlling carbon source availability and stress response, and, less significantly, Yap1, involved in oxidative stress and MDR, and Pdr3, a key regulator of the yeast PDR network, consistent with a broad role in stress defence. Altogether, our results suggest that Pdr18 plays a role in plasma membrane sterol incorporation, this physiological trait contributing to a MDR phenotype.

INTRODUCTION

Multidrug resistance (MDR), the ability to acquire simultaneous resistance to unrelated chemical compounds, is a widespread phenomenon that often results from the activity of multidrug efflux pumps of the ATP-Binding Cassette (ABC) Superfamily and of the Major Facilitator Superfamily (MFS). These transporters are proposed to actively extrude or compartmentalize a wide range of chemically and structurally disparate drugs and other xenobiotics, thus providing protection from these compounds. However, the prevalence and apparent redundancy of so many MDR transporters, which protect the cell against toxic compounds that are not present in its natural environment, has led to speculation concerning a natural physiological function [1-3].

In recent years, a physiological role for many of these transporters in the model eukaryote *Saccharomyces cerevisiae* has been proposed. Among MFS-MDR transporters, for example, Tpo1-4 and Qdr3 were suggested to mediate the export of polyamines [4, 5], while Qdr2 was proposed to catalyse K^+ influx [6]. These results suggest that the chemoprotection role of these MDR transporters may come, at least in some cases, as a result of their influence on plasma membrane potential and/or ΔpH control, which in turn can alter the partitioning and accumulation of drugs [2]. On the other hand, most of the yeast pleiotropic drug resistance (PDR) transporters, of the ABC superfamily, characterized as multidrug resistance determinants, have been associated to the control of lipid incorporation into cell membranes [3]. Interestingly, Pdr5 and its homologs from *Candida albicans*, Cdr1 and Cdr2, were shown to function as phospholipid translocators [7]. Pdr10 and Pdr15 were also implicated in membrane lipid organization [8, 9] and Aus1 and Pdr11 were found to mediate nonvesicular movement of plasma membrane sterol to the endoplasmic reticulum in *S. cerevisiae*, facilitating exogenous sterol uptake [10]. The deletion of yet another *S. cerevisiae* transporter encoding gene, *PDR16*, which is regulated by the PDR network transcription factor Pdr1, also leads to changes in yeast plasma membrane sterol composition [11]. Altogether, these results have been suggested to imply that the multidrug resistance phenotype conferred by PDR transporters may occur, to some extent, due to the changes they impose in the composition of all membranes, which in turn may affect drug partition or transport. This hypothesis is consistent with the fact that *ERG2, ERG4* and *ERG5* genes, encoding the final enzymes of the ergosterol biosynthetic pathway, were found to be determinants of multidrug resistance [12].

In this study, the functional analysis of *PDR18* (ORF *YNR070w*), encoding a putative yeast PDR transporter of the plasma membrane, was undertaken. Based on the fact that *PDR18* is up-regulated (1.6 fold) in yeast cells challenged by the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), as previously revealed by microarray analysis [13], *PDR18* gene was found to confer yeast resistance to the auxin-like herbicides 2,4-D and MCPA, among other xenobiotic compounds. Our previous studies on the yeast adaptive response mechanisms to the herbicide 2,4-D (reviewed in [14]) indicate that the expression of the drug:H^+ antiporter Tpo1, of the ABC transporter Pdr5, and of the *Arabidopsis thaliana* Tpo1 homolog At5g13750, confers 2,4-D resistance in *S. cerevisiae* and leads to a lower intracellular accumulation of the herbicide [15, 16]. In this work, the role and regulation of *PDR18* expression in yeast resistance to 2,4-D is

scrutinized. Given the previous implication of PDR transporters in phospholipid and ergosterol, the role of Pdr18 in yeast plasma membrane lipid composition was studied. *PDR18* expression was found to affect plasma membrane potential and sterol composition, allowing us to propose Pdr18 as mediator of non-vesicular ergosterol transport into the plasma membrane, this physiological role being, at least partially, responsible for the observed multidrug resistance phenotype.

Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

EXPERIMENTAL

Strains, plasmids and growth conditions. The parental *Saccharomyces cerevisiae* strain BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and the derived single deletion mutants *Δpdr18*, *Δnrg1*, *Δyap1* and *Δpdr3* used in this study, were obtained from the EUROSCARF collection. Plasmids pRS416_*PDR18* (*EUROSCARF*), expressing the *PDR18* gene from its natural promoter, pRS416_*PDR18Δp* and the cloning vector pRS416 (*EUROSCARF*) were also used. Plasmid pRS416_*PDR18Δp* was obtained through site-directed mutagenesis of pRS416_PDR18 using the QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene), and exhibit a substitution of 2 nucleotides in the putative Nrg1 binding site in the *PDR18* promoter region. The pair of mutagenic oligonucleotides used for this procedure were 5' GTTTCGTTTCATATGATTGCTTAGGTACCATTC 3' and its complementary sequence 5' GAATGGTACCTAAGCAATCATATGAAACGAAAC 3', in which the replacement nucleotides are underlined.

Yeast cells were cultivated at 30 ºC with 250 rpm agitation in minimal growth medium MM4, pH 3.5 (adjusted with HCl), containing (per litre): 1.7 g yeast nitrogen base without amino acids or (NH4)2SO4 (Difco), 20 g glucose (Merck), 2.65 g $(NH4)_2SO_4$ (Merck), 20 mg L-histidine (Merck), 20 mg L-methionine (Merck), 60 mg L-leucine (Sigma) and 20 mg L-uracil (Sigma). Solid medium, pH 4.0 (adjusted with HCl), was prepared by adding 20 gL^{-1} agar (Iberagar). Cells harbouring pRS416 or derived plasmids were grown in the same media conditions and without uracil (MM4-U) to assure plasmid segregational stability.

***Susceptibility assays*.** The susceptibility of the parental strain BY4741 and *Δpdr18* deletion mutant to toxic concentrations of 2,4D was assessed by comparing their growth curves or growth in spot assays in MM4 medium supplemented or not with inhibitory concentrations of 2,4-D (0.45mM in liquid medium and 1 - 2.5mM in solid medium). Cell suspensions used for the spot assays were prepared as described previously [5]. Besides 2,4-D, other chemical stress inducers (obtained from Sigma) were tested in the specified concentration ranges: the herbicides MCPA (2-methyl-4-chlorophenoxyacetic acid) (1 – 1.5 mM) and barban (0.08 – 0.1 mM); 2,4-dichlorophenol,the 2,4-D degradation intermediate 2,4-DCP (0.5 – 1 mM); the fungicides benomyl (17 – 35 mgL-1) and mancozeb (1 – 1.2 mgL-1); and salts of several toxic metal cations, namely, $CdSO_4$ (0.025 – 0.04 mM), $CuSO_4$ (0.5 – 1 mM), $MnSO_4$ (5 – 10 mM), $ZnSO_4$ (5 – 10 mM), $Al_2(SO_4)_3$ (0.5 – 1 mM), $TlCl_3$ (0.1 – 0.5 mM), $CoSO_4$ (1.5 – 2 mM) and $PbSO_4$ (1.5 – 2 mM).

PDR18 gene expression assays. The changes registered in the transcript levels from the *PDR18* gene in BY4741 and in derived mutants *Δpdr18*, *Δnrg1*, *Δyap1* and *Δpdr3*, upon yeast exposure to 0mM, 0.3mM or 0.45mM of 2,4-D were assessed by real time RT-PCR. RNA extraction from yeast cells were carried out as described previously [15]. The used RT-PCR protocol followed the manufacturer's instructions and was described elsewhere [5]. Primers for the amplification of the *PDR18* and *ACT1* cDNA were designed using Primer Express Software (Applied Biosystems) and are 3'-TTGGCAAGCCGGATCTGT-5', 3'- CCACGCGGATTGGGAAT -5' and 3'-CTCCACCACTGCTGAAAGAGAA -5', 3'- CCAAGGCGACGTAACATAGTTTT -5', respectively. The RT-PCR reaction was carried out using a thermal cycler block (7500 Real-Time PCR System - Applied Biosystems). The *ACT1* mRNA level was used as the internal control.

The relative values obtained for unstressed conditions were set as 1 and the remaining values are relative to that value.

[^{14}C]-2,4-D accumulation assays. The intracellular accumulation ratio of [^{14}C]-labelled 2,4-D in the parental strain BY4741 and the derived deletion mutant *Δpdr18* was assessed as described previously [16].

Plasma membrane potential (ΔΨ) estimation. To estimate the differences in BY4741 and derived mutant *Δpdr18* plasma membrane potential two methods were used: the [^{14}C]-Methylamine uptake assay [17] and the 3-3'-dihexyloxacarbocianine iodide ($DiOC_6(3)$) accumulation assay [18].

The uptake of [^{14}C]-methylamine in the parental strain BY4741 and the mutant strain *Δpdr18* was monitored as described previously [5, 17].

To estimate $DiOC_6(3)$ fluorescence, cells were harvested as described above, resuspended in MES-glucose buffer (10 mM MES, 0.1 mM $MgCl_2$ and 20 gL^{-1} glucose, pH 6), supplemented with $DiOC_6(3)$ (Molecular probes) at a final concentration of 0.25nM and incubated in the dark for 30 min at 30ºC with orbital agitation. After centrifugation, cells were immediately observed with a Zeiss Axioplan microscope equipped with adequate epifluorescence filters (BP450-490 and LP520). And fluorescence emission was collected with a CCD camera (Cool SNAPFX, Roper Scientific Photometrics). Bright-field images for determination of ΔΨ were obtained concurrently and recorded at 1 min intervals, each experiment being finished within 15 min. The images were analysed using MetaMorph 3.5. The fluorescence images were background corrected using dark-current images. The intensity values were calculated for a minimum of 80 cells per experiment. Individual cells were selected using regions of interest obtained from bright-field images recorded before or after the experiment. The value of fluorescence intensity emitted by each cell was obtained pixel-by-pixel in the region of interest. Fluorescence levels given by the software were expressed as a percentage.

Plasma membrane sterol composition assessment Total cell membranes (CMs) were extracted and prepared from yeast cells grown in MM4 medium (pH 3.5) and harvested in the exponential growth phase. For studying the effect of 2,4-D, exponential cells grown in MM4 medium were transferred to fresh medium supplemented with 0.45mM of 2,4-D, and grown for 1h at 30 ^{o}C. Cells were harvested by centrifugation,resuspended in homogenization buffer containing 50 mM Tris pH 7.5,2.5 mM EDTA and a protease inhibitor cocktail (1 mM PMSF, 1 µg/ml leupeptin, pepstatin A and aprotinin) andbroken by vortexing with glass beads (Glaperlon 0.40-0.60 mm).The CM were recovered by centrifugation at 1000 g to remove unbroken cells and finally the CM were pelleted by ultracentifugation at 25000 rpm for 1 hour. The CM were resuspended in a buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 20% glycerol and protease inhibitors at the concentrations mentioned above. Plasma membrane (PM) fractions were obtained from CM fractions by sucrose gradient centrifugation as described by Monk *et al.* (1991) [19]. The obtained CM and PM protein concentrations, measured by the BCA test, ranged between 8 – 19 µg/µl and 3 – 9 µg/µl respectively. Equal amounts of PMs were used for lipid extraction from each of the yeast strains using the method described by Bligh and Dyer (1959) with slight modifications [20]. Sterols were extracted and analyzed using the method described previously with slight modifications [21]. The saponified lipids were re-extracted using 5 ml hexane and vortexed several times. 1 ml of water was added to separate the

organic phase, which was then dried in N_2 at 60^{o}C. The extracted sterols were derivatized using 100 µl N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethyl-chlorosilane BSTFA/TMCS (Sigma) at 80ºC for 1 hr in N_2. The derivatized sterols were then analyzed using GCMS (Schimadzu QP2010 Plus, Japan) and aDB5-MS column 60 m X 0.2 mm, film thickness 0.20 µm. The carrier gas was helium with a flow rate of 1ml/min and a pressure of 80.8 kPa. Initial column temperature of 120^{o}C was held for 1 min. then programmed at 120^{o}C to 250^{o}C at 5^{o}C/min where it was held for 30 min. 1 µl injection was made using a split ratio of 10. Injection temperature was 300^{o}C. The total ion mass spectra were recorded in the mass range m/z 40-650 at the scan rate of 1 s/scan. The interface and detector temperature was 300^{o}C. Peak identification was based on relative retention time and total ion mass spectral comparison with an external standard. The sterol standards were obtained from Sigma-Aldrich.

RESULTS

The ABC transporter Pdr18, encoded by ORF YNR070w, confers yeast resistance to 2,4-D and other chemical stresses. The susceptibility towards 2,4-D imposed stress of the single deletion mutant *Δpdr18* was found to be higher compared to the parental strain, based on spot assays and liquid growth (Fig. 1). In the absence of Pdr18, yeast cells become susceptible to 2,4-D, even when supplemented in concentrations that hardly affect wild-type viability (Fig. 1A). For higher concentrations of the herbicide, the expression of this transporter becomes essential for survival under stress (Fig. 1A). *PDR18* deletion was also found to lead to a longer 2,4-D-induced lag-phase, and also a reduced value of final biomass concentration (Fig. 1B). The expression of *PDR18* from a centromeric plasmid was found to rescue the 2,4-D susceptibility phenotype of *Δpdr18* cells, to levels comparable to the parental strain, while no effect was detected in the control cells harbouring the corresponding cloning vector (Fig. 1C). The ability of this gene expression to confer resistance to other pesticides and chemical compounds of agroeconomical importance was further analyzed and *PDR18* was also found to be a determinant of yeast resistance to the herbicides MCPA and barban, to the 2,4-D degradation intermediate 2,4-dichlorophenol (2,4-DCP), to the agricultural fungicide mancozeb, and to the metal cations Zn^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} (Fig. 2). No protection was conferred by *PDR18* expression towards benomyl, Co^{2+}, Pb^{2+}, Al^{3+} and Tl^{3+} (data not shown).

PDR18 transcription is activated in response to 2,4-D imposed stress in a Nrg1-, Yap1- and Pdr3-dependent manner. A strong increase in the transcript levels of *Pdr18* (~8 fold) was registered after one hour of exposure of an un-adapted *S. cerevisiae* cell population to 0.3 mM of 2,4-D. This strong but transient increase was followed by a rapid decrease of transcript levels to basal values as cells adapt to growth in the presence of the herbicide. When exposed to a higher concentration of 2,4-D, 0.45 mM, *PDR18* transcriptional activation reached a maximum of up to 5-fold after 4h of stress exposure, correlating with the longer duration of the lag-phase imposed by this higher 2,4-D concentration tested (Fig 3). The fact that a higher herbicide concentration apparently leads to a lower activation of *PDR18* may be due to the fact that 0.45mM of 2,4-D induces viability loss, thus reducing the number of cells in the studied population with the ability to generate a stress response [22].

The YEASTRACT database (www.yeastract.com) [23, 24], was used to guide the analysis of the transcriptional control underlying *PDR18* activation. Five transcription factors were identified as documented *PDR18* regulators previously proven to bind to its promoter region, and, thus, were selected as candidates for, directly or indirectly, controlling 2,4-D-induced *PDR18* up-regulation (Fig. 4A). Of these, only Nrg1, Pdr3 and Yap1 were examined in this study, because mutants deleted for the other two genes are either unviable (*Δrap1*) or exhibits marked growth defects (*Δswi4*) (SGD, *Saccharomyces* Genome Database – www.yeastgenome.com). The three tested mutant strains, devoid of *PDR3*, *YAP1* or *NRG1*, exhibit nearly identical *PDR18* mRNA basal levels, as registered in unstressed parental strain cells. However, the *PDR18* up-regulation detected in the wild type cells after 4h of incubation with 2,4-D is abrogated in *Δnrg1* mutant or reduced in *Δyap1* and *Δpdr3* mutants (Fig. 4B), suggesting that both Pdr3 and Yap1 transcription factors are necessary to assure full *PDR18* activation.

Since Nrg1 has been described as a transcriptional repressor, its action as an activator of *PDR18* was hypothesized to be indirect. Nonetheless, this transcription factor was previously demonstrated, through genome-wide screenings [25, 26], to bind to the *PDR18* promoter region. Furthermore, according to the YEASTRACT database, a potential Nrg1 binding site can be found in the *PDR18* promoter at position -567. To evaluate whether or not the action of Nrg1 on *PDR18* transcriptional up-regulation might be direct, site-directed mutagenesis was used to abrogate the putative Nrg1 binding site found in the pRS416_*PDR18* expression plasmid. Both pRS416_*PDR18* and pRS416_*PDR18Δp* plasmids were transformed into *Δpdr18* cells, so that the genomic expression of *PDR18* from its natural promoter would not be accounted for. RT-PCR was used to measure the *PDR18* transcript levels in these cells, upon exposure to 0.45mM of 2,4-D. The abrogation of the Nrg1 binding site was seen to have only a moderate effect on herbicide-induced *PDR18* up-regulation (Fig. 4C) when compared to the full effect observed upon Nrg1 deletion. Altogether, these results suggest that the action of Nrg1 on *PDR18* expression appears to be indirect in this case.

Role of *PDR18* expression in 2,4-D intracellular accumulation. Given the presumed role of Pdr18 as a plasma membrane multidrug resistance transporter that confers resistance to yeast cells against 2,4-D imposed stress, the effect of *PDR18* expression in the intracellular accumulation of [^{14}C]-2,4-D was assessed. The accumulation of [^{14}C]-2,4-D in non-adapted yeast cells suddenly exposed to the presence of 0.3 mM 2,4-D (at pH 3.5), which induces a mild growth inhibition in both the parental strain and *Δpdr18* cells (data not shown), is 2.5 fold higher in cells devoid of *PDR18* than in parental cells (Fig. 5). This result strongly suggests that Pdr18 activity increases yeast resistance towards 2,4-D by reducing the accumulation of the 2,4-D anion within yeast cells, presumably by catalyzing the direct extrusion of the herbicide.

***PDR18* deletion causes changes in yeast plasma membrane sterol composition.** In the absence of 2,4-D supplementation, upon disruption of *PDR18*, a nearly 2-fold accumulation of squalene and lanosterol, the precursors of ergosterol biosynthetic pathway, and a 1,5 fold reduction of ergostatetraenol and ergosterol content, the end products of the ergosterol biosynthetic pathways, in the plasma membrane were detected (Fig. 6). Upon episomal complementation of *PDR18*, there was a partial complementation of the Δ*pdr18* plasma membrane sterol composition phenotype (Fig. 6).

The levels of ergosterol in the plasma membrane of *S. cerevisiae* BY4741 exposed to 2,4-D were found to decrease 1.5 fold compared with unstressed conditions, while the levels of squalene increased 5.5 fold. This effect is exacerbated in the absence of *PDR18* gene. Indeed, in *Δpdr18* cells 2,4-D exposure leads to a 4.3 fold increase in squalene and a 5.2 fold decrease in ergosterol plasma membrane concentrations (Fig. 6). Remarkably, under 2,4-D challenge the relative abundance of squalene becomes higher than the relative abundance of ergosterol in both wild type and *Δpdr18* backgrounds and the concentration of the other sterols detected in the plasma membrane of unstressed yeast cells become undetectable (Fig. 6).

Lower plasma membrane potential is observed in Δpdr18 cells. The role of *PDR18* expression in the maintenance of yeast membrane potential was also analyzed. Yeast plasma membrane potential was first estimated based on the uptake of methylammonium, a non-metabolizable ammonium analogue, whose influx is strongly

dependent on the maintenance of the transmembrane potencial [27]. The deletion of *PDR18* was found to decrease in around 60% the level of methylammonium uptake in yeast cells (Fig. 7A). Consistent with these results, the fluorescence intensity levels of cells loaded with the $DiOC_6(3)$ probe, whose accumulation inside yeast cells is dependent on the plasma membrane potential [18], is three fold higher in wild-type cells than in *Δpdr18* cells (Fig. 7B). Both methods indicate a strong depletion of the plasma membrane potential in the absence of *PDR18*.

DISCUSSION

This study provides the first functional report on the uncharacterized yeast PDR transporter, Pdr18, encoded by ORF *YNR070w*. Although this gene was not previously characterized, a microarray analysis from our group showed that *PDR18* is up-regulated in yeast cells exposed to inhibitory concentrations of the herbicide 2,4-D [13]. Guided by this preliminary result, the current study provides evidence showing that *PDR18* is a determinant of yeast resistance to 2,4-D, to MCPA, another auxin like herbicide, and to several other unrelated chemical stresses, including, barban, an herbicide of the carbanilate family, mancozeb, an agricultural fungicide and the soil contaminant metals cadmium, copper, manganese and zinc. This study provides evidence showing that *PDR18* gene expression decreases the intracellular accumulation of radiolabelled 2,4-D. The intracellular accumulation pattern observed for *Δpdr18* deletion mutant, compared with the wild-type strain, is similar to the one observed previously for *Δtpo1* deletion mutant [16]. Interestingly, the *PDR18* homolog in the plant model *Arabidopsis thaliana*, *AtPDR9*, was seen to confer 2,4-D resistance in plant, also contributing to decreased 2,4-D accumulation in plant roots [28].

During 2,4-D induced lag-phase period, preceding exponential growth resumption under herbicide stress, *PDR18* transcript levels were shown to increase transiently. This fact, together with the reduction of the duration of the lag phase induced by 2,4-D due to *PDR18* expression, indicates that the role of Pdr18 is preponderant during the period of adaptation to the herbicide, while the effect over the inhibition of specific growth exert by the herbicide is not significant. Consistent with a broad role in stress defence, the transcriptional up-regulation of *PDR18* was found to be partially reduced in mutants with either *PDR3* or *YAP1* genes deleted and completely abolished in a mutant devoid of *NRG1*. The partial effect of Pdr3 in *PDR18* activation resembles the effect described before exerted by Pdr3 over the transcriptional up-regulation of *TPO1* under 2,4-D stress [15] and places *PDR18* within the yeast PDR network. At the same time, the role of Yap1, the major regulator of *S. cerevisiae* oxidative stress response, in 2,4-D induced *PDR18* up-regulation may correlate with the observation that this herbicide exerts a pro-oxidant action in yeast [22]. Moreover, Yap1 also plays a role in the control of multidrug resistance, regulating the expression of at least ten other MDR proteins: the ABC drug efflux pumps Snq2, Ycf1 and Pdr5 and the drug-H^+ antiporters Flr1, Tpo1, Tpo2, Tpo4, Azr1, Yhk8 and Qdr3 (www.yeastract.com) [5, 23, 24]. On the other hand, in the absence of *NRG1* the 2,4-D-induced transcriptional up-regulation is completely abrogated. Consistent with the notion that Nrg1 acts as a transcriptional repressor, the action of Nrg1 in this case is proposed to be indirect, based on the fact that the abrogation of the Nrg1 binding site in the *PDR18* promoter led only to a slight change in its herbicide-dependent transcriptional up-regulation. The action of Nrg1 is likely to occur through the regulation of other genes, possibly other transcription factors. Interestingly, the transcript levels of the *NRG1* gene suffer a 5-fold increase in yeast cells exposed for 15 min to 0.3mM of 2,4-D, as described in a previous microarray analysis [13]. The same global analysis suggests that yeast cells challenged with toxic concentrations of 2,4-D experience a state of glucose and energy limitation, despite the saturating concentrations of this preferential carbon source in the surrounding medium, which could account for an Ngr1-mediated response [13].

Most significantly, in this work the deletion of *PDR18* in *S. cerevisiae* cells was found to lead to an accumulation of the precursors of ergosterol biosynthetic pathway, squalene and lanosterol, and to a decrease in the content of ergostatetraenol and ergosterol, the end products of the ergosterol biosynthetic pathway, in yeast plasma membrane. In the same conditions, no changes in the phospholipid composition of the yeast plasma membrane were registered upon *PDR18* deletion (results not shown). Although the exact role of Pdr18 in sterol homeostasis requires clarification, Pdr18 is proposed to play a direct role in the incorporation of ergosterol in the plasma membrane as part of the non-vesicular ER to plasma membrane ergosterol transport mechanism [10]. Both in mammalian and yeast cells, newly synthesized cholesterol/ergosterol has been shown to be transported from the endoplasmic reticulum to the plasma membrane via two mechanisms: one dependent on vesicular transport and the other dependent on ATP, but independent of vesicular transport [29]. However, no specific transporter has been implicated so far in the mediation of this non-vesicular ergosterol movement. Our results suggest that Pdr18 may contribute to this important physiological function. Given this proposed physiological role, the observed apparent inhibitory effect of *PDR18* deletion on sterol biosynthesis could result from probing local sterol concentrations, thus influencing the activity of ergosterol synthesizing enzymes, as suggested for Pdr16 [11].

The lipid composition of a cellular membrane has profound effects on its biophysical properties that may affect a membrane's fusibility, including intrinsic curvature, thickness, stiffness, and permeability [30-32]. Unlike intracellular membranes, the yeast plasma membrane is highly enriched in ergosterol. In various plant models, ergosterol induces changes in membrane potential [33, 34] and modifications of H^+ fluxes across the membranes [33, 35, 36], among other effects. A low level of ergosterol leads to disruption of the membrane lateral order [37], which results in membrane fluidization, compromising the physiological membrane potential. Consistent with the depletion of ergosterol in the plasma membrane of *Δpdr18* cells, *PDR18* expression was also found to be essential in the maintenance of yeast plasma membrane potential. Two probes were use to assess the differences between wild-type and *Δpdr18* plasma membrane potential to rule out the hypothesis that the observed variation might result from the direct action of Pdr18 in the excretion of one of the selected probes.

The action of Pdr18 in 2,4-D resistance can be explained in light of its contribution to sterol homeostasis. It is interesting to see that exposure to the herbicide 2,4-D leads to several changes in membrane sterol composition similar to those caused by *PDR18* deletion, including a decrease in ergosterol and an increase in squalene relative concentrations. These changes occurring under 2,4-D stress indicate a possible action of the herbicide as an inhibitor of ergosterol biosynthesis or transport into the plasma membrane and are consistent with the requirement for *PDR18* expression and the observed *PDR18* up-regulation registered in this study. Furthermore, in the absence of *PDR18* the effect of 2,4-D in the plasma membrane sterol content is even more pronounced than in wild-type cells. Such a reduced ergosterol content in *Δpdr18* cells is likely to increase the permeability of the plasma membrane towards 2,4-D and to affect the active export of 2,4-D to the outer medium, through dedicated transporters, eventually including Tpo1, Pdr5 [15] and Pdr18 itself,

consistent with the observed increase accumulation of 2,4-D in yeast cells devoid of *PDR18*.

Altogether, based on the results presented in this paper a physiological role for Pdr18 in the control of sterol homeostasis, specifically, in maintaining ergosterol physiological levels in the plasma membrane is proposed. Pdr18 role as a multidrug resistance determinant is suggested to derive, at least partially, from its physiological role, which is expected to affect drug partition and transport across cell membranes. These results are expected to increase current knowledge on the action of this family of transporters with impact in the design of strategies to deal with MDR. Given the particular role of Pdr18 in pesticide resistance, these results may also guide the design of new pesticide resistant crops of agroeconomic interest.

FUNDING

This work was supported by FEDER and "Fundação para a Ciência e a Tecnologia" (FCT) (Contracts PTDC/AGR-AAM/102967/2008 and PTDC/BIA-MIC/72577/2006 and PhD grant SFRH/BD/40885/2007 to TRC). This work is also supported in part by grants to R.P. from Department of Biotechnology (BT/PR9100/Med/29/03/2007, BT/PR9563/BRB/10/567/2007, BT/PR11158/BRB/10/640/2008, BT/PR13641/MED/29/175/2010). We acknowledge Advanced Instrumentation Research Facility (AIRF), JNU for providing GC-MS.

REFERENCES

1 Roepe, P. D., Wei, L. Y., Hoffman, M. M. and Fritz, F. (1996) Altered drug translocation mediated by the MDR protein: direct, indirect, or both? J. Bioenerg. Biomembr. **28**, 541-555

2 Sá-Correia, I., Santos, S., Teixeira, M., Cabrito, T. and Mira, N. (2009) Drug:H^+ antiporters in chemical stress response in yeast. Trends Microbiol. **17**, 22-31

3 Jungwirth, H. and Kuchler, K. (2006) Yeast ABC transporters-- a tale of sex, stress, drugs and aging. FEBS Lett. **580**, 1131-1138

4 Tomitori, H., Kashiwagi, K., Asakawa, T., Kakinuma, Y., Michael, A. J. and Igarashi, K. (2001) Multiple polyamine transport systems on the vacuolar membrane in yeast. Biochem. J. **353**, 681-688

5 Teixeira, M. C., Cabrito, T. R., Hanif, Z. M., Vargas, R. C., Tenreiro, S. and Sá-Correia, I. (2011) Yeast response and tolerance to polyamine toxicity involving the drug:H+ antiporter Qdr3 and the transcription factors Yap1 and Gcn4. Microbiology. **157**, 945-956

6 Vargas, R. C., Garcia-Salcedo, R., Tenreiro, S., Teixeira, M. C., Fernandes, A. R., Ramos, J. and Sá-Correia, I. (2007) *Saccharomyces cerevisiae* Multidrug Resistance Transporter Qdr2 Is Implicated in Potassium Uptake, Providing a Physiological Advantage to Quinidine-Stressed Cells. Eukaryot. Cell. **6**, 134-142

7 Smriti, Krishnamurthy, S., Dixit, B. L., Gupta, C. M., Milewski, S. and Prasad, R. (2002) ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. Yeast. **19**, 303-318

8 Schuller, C., Mamnun, Y. M., Wolfger, H., Rockwell, N., Thorner, J. and Kuchler, K. (2007) Membrane-active compounds activate the transcription factors Pdr1 and Pdr3 connecting pleiotropic drug resistance and membrane lipid homeostasis in saccharomyces cerevisiae. Mol. Biol. Cell. **18**, 4932-4944

9 Rockwell, N. C., Wolfger, H., Kuchler, K. and Thorner, J. (2009) ABC transporter Pdr10 regulates the membrane microenvironment of Pdr12 in *Saccharomyces cerevisiae*. J. Membr. Biol. **229**, 27-52

10 Li, Y. and Prinz, W. A. (2004) ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum. J. Biol. Chem. **279**, 45226-45234

11 van den Hazel, H. B., Pichler, H., do Valle Matta, M. A., Leitner, E., Goffeau, A. and Daum, G. (1999) *PDR16* and *PDR17*, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J. Biol. Chem. **274**, 1934-1941

12 Hillenmeyer, M. E., Fung, E., Wildenhain, J., Pierce, S. E., Hoon, S., Lee, W., Proctor, M., St Onge, R. P., Tyers, M., Koller, D., Altman, R. B., Davis, R. W., Nislow, C. and Giaever, G. (2008) The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science. **320**, 362-365

13 Teixeira, M. C., Fernandes, A. R., Mira, N. P., Becker, J. D. and Sá-Correia, I. (2006) Early transcriptional response of *Saccharomyces cerevisiae* to stress imposed by the herbicide 2,4-dichlorophenoxyacetic acid. FEMS Yeast Res. **6**, 230-248

14 Teixeira, M. C., Duque, P. and Sá-Correia, I. (2007) Environmental genomics: mechanistic insights into toxicity of and resistance to the herbicide 2,4-D. Trends Biotechnol. **25**, 363-370

15 Teixeira, M. C. and Sá-Correia, I. (2002) *Saccharomyces cerevisiae* resistance to chlorinated phenoxyacetic acid herbicides involves Pdr1p-mediated transcriptional activation of *TPO1* and *PDR5* genes. Biochem. Biophys. Res. Commun. **292**, 530-537

16 Cabrito, T. R., Teixeira, M. C., Duarte, A. A., Duque, P. and Sá-Correia, I. (2009) Heterologous expression of a Tpo1 homolog from *Arabidopsis thaliana* confers resistance to the herbicide 2,4-D and other chemical stresses in yeast. Appl. Microbiol. Biotechnol. **84**, 927-936

17 Hoeberichts, F. A., Perez-Valle, J., Montesinos, C., Mulet, J. M., Planes, M. D., Hueso, G., Yenush, L., Sharma, S. C. and Serrano, R. (2010) The role of K(+) and H(+) transport systems during glucose- and H_2O_2-induced cell death in *Saccharomyces cerevisiae*. Yeast. **27**, 713-725

18 Madrid, R., Gomez, M. J., Ramos, J. and Rodriguez-Navarro, A. (1998) Ectopic potassium uptake in trk1 trk2 mutants of Saccharomyces cerevisiae correlates with a highly hyperpolarized membrane potential. J. Biol. Chem. **273**, 14838-14844

19 Monk, B. C., Kurtz, M. B., Marrinan, J. A. and Perlin, D. S. (1991) Cloning and characterization of the plasma membrane H(+)-ATPase from Candida albicans. J. Bacteriol. **173**, 6826-6836

20 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911-917

21 Sharma, M., Dhamgaye, S., Singh, A. and Prasad, R. (in press) Lipidome analysis reveals antifungal polyphenol curcumin affects membrane lipid homeostasis: targets Δ5,6 desaturase *ERG3* of *Candida albicans*. Frontiers in Bioscience

22 Teixeira, M. C., Telo, J. P., Duarte, N. F. and Sá-Correia, I. (2004) The herbicide 2,4-dichlorophenoxyacetic acid induces the generation of free-radicals and associated oxidative stress responses in yeast. Biochem. Biophys. Res. Commun. **324**, 1101-1107

23 Abdulrehman, D., Monteiro, P. T., Teixeira, M. C., Mira, N. P., Lourenco, A. B., dos Santos, S. C., Cabrito, T. R., Francisco, A. P., Madeira, S. C., Aires, R. S., Oliveira, A. L., Sá-Correia, I. and Freitas, A. T. (2011) YEASTRACT: providing a programmatic access to curated transcriptional regulatory associations in *Saccharomyces cerevisiae* through a web services interface. Nucleic. Acids Res. **39**, D136-140

24 Teixeira, M. C., Monteiro, P., Jain, P., Tenreiro, S., Fernandes, A. R., Mira, N. P., Alenquer, M., Freitas, A. T., Oliveira, A. L. and Sá-Correia, I. (2006) The YEASTRACT database: a tool for the analysis of transcription regulatory associations in *Saccharomyces cerevisiae*. Nucleic. Acids Res. **34**, D446-451

25 Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., Jennings, E. G., Zeitlinger, J., Pokholok, D. K., Kellis, M., Rolfe, P. A., Takusagawa, K. T., Lander, E. S., Gifford, D. K., Fraenkel, E. and Young, R. A. (2004) Transcriptional regulatory code of a eukaryotic genome. Nature. **431**, 99-104

26 Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T., Thompson, C. M., Simon, I., Zeitlinger, J., Jennings, E. G., Murray, H. L., Gordon, D. B., Ren, B., Wyrick, J. J., Tagne, J. B., Volkert, T. L., Fraenkel, E., Gifford, D. K. and Young, R. A. (2002) Transcriptional regulatory networks in *Saccharomyces cerevisiae*. Science. **298**, 799-804

27 van de Mortel, J. B., Mulders, D., Korthout, H., Theuvenet, A. P. and Borst-Pauwels, G. W. (1988) Transient hyperpolarization of yeast by glucose and ethanol. Biochim. Biophys. Acta. **936**, 421-428

28 Ito, H. and Gray, W. M. (2006) A gain-of-function mutation in the *Arabidopsis* pleiotropic drug resistance transporter *PDR9* confers resistance to auxinic herbicides. Plant Physiol. **142**, 63-74

29 Sullivan, D. P., Ohvo-Rekila, H., Baumann, N. A., Beh, C. T. and Menon, A. K. (2006) Sterol trafficking between the endoplasmic reticulum and plasma membrane in yeast. Biochem. Soc. Trans. **34**, 356-358

30 Hancock, J. F. (2006) Lipid rafts: contentious only from simplistic standpoints. Nat. Rev. Mol. Cell Biol. **7**, 456-462

31 Mukherjee, S. and Maxfield, F. R. (2004) Membrane domains. Annu. Rev. Cell Dev. Biol. **20**, 839-866

32 Dupont, S., Beney, L., Ferreira, T. and Gervais, P. (2011) Nature of sterols affects plasma membrane behavior and yeast survival during dehydration. Biochim. Biophys. Acta. **1808**, 1520-1528

33 Amborabe, B. E., Rossard, S., Perault, J. M. and Roblin, G. (2003) Specific perception of ergosterol by plant cells. C. R. Biol. **326**, 363-370

34 Rossard, S., Luini, E., Perault, J. M., Bonmort, J. and Roblin, G. (2006) Early changes in membrane permeability, production of oxidative burst and modification of PAL activity induced by ergosterol in cotyledons of *Mimosa pudica*. J. Exp. Bot. **57**, 1245-1252

35 Granado, J., Felix, G. and Boller, T. (1995) Perception of Fungal Sterols in Plants (Subnanomolar Concentrations of Ergosterol Elicit Extracellular Alkalinization in Tomato Cells). Plant Physiol. **107**, 485-490

36 Kasparovsky, T., Blein, J. P. and Mikes, V. (2004) Ergosterol elicits oxidative burst in tobacco cells via phospholipase A2 and protein kinase C signal pathway. Plant Physiol. Biochem. **42**, 429-435

37 Tierney, K. J., Block, D. E. and Longo, M. L. (2005) Elasticity and phase behavior of DPPC membrane modulated by cholesterol, ergosterol, and ethanol. Biophys. J. **89**, 2481-2493

FIGURE LEGENDS

Figure 1 – Comparison of the susceptibility to 2,4-D of *S. cerevisiae* BY4741 and the derived deletion mutant *Δpdr18*, through cultivation in liquid medium (B) or spot assays (A and C). A: Spot assays were carried as described in the M&M section. The cell suspensions used to prepare the spots in lanes b) and c) were 1:5 and 1:25 serial dilutions, respectively, of the suspensions with an OD_{600nm}=0.05±0.005 spotted in lane a). Pictures were taken after 2 days of incubation. B: Growth curves of wild-type (wt) (■,□) and *Δpdr18* (▲,△) cells in MM4 liquid medium, pH 3.5, (■,▲) or in this medium supplemented with 0.45mM of 2,4-D (□,△). Cells of the inocula were grown in the absence of the herbicide and the growth curves are representative of at least three independent experiments. C: Spot assays were carried as described in the M&M section. The cell suspensions used to prepare the spots in lanes b) and c) were 1:5 and 1:25 serial dilutions, respectively, of the suspensions with an OD_{600nm}=0.05±0.005 spotted in lane a). Pictures were taken after 4 days of incubation.

Figure 2 - Susceptibility to the herbicides 2,4-D, MCPA and barban, to the 2,4-DCP, to the agricultural fungicide mancozeb, and to the metal ions Zn^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} induced stress of deletion mutant *Δpdr18* compared to the wt, through spot assays. Cells used for the spot assays were prepared as described in the M&M section. The cell suspensions used to prepare the spots in lanes b) and c) were 1:5 and 1:25 serial dilutions, respectively, of the suspensions with an OD_{600nm}=0.05±0.005 spotted in lane a). Pictures were taken after 2 or 3 days of incubation.

Figure 3 – *PDR18* transcript levels in yeast cells exposed to 2,4-D imposed stress. A: Comparison of the susceptibility to 2,4-D induced stress of *S. cerevisiae* parental strain BY4741 exposed to 0 (◆), 0.3 (■) or 0.45mM (▲) of 2,4-D through cultivation in MM4 liquid medium, pH 3.5. Cells used to prepare the inocula were previously grown in the absence of 2,4-D untill mid-exponential phase. Growth curves are representative of at least three independent growth experiments. B: Comparison of the relative transcript values of *PDR18* mRNA/*ACT1* mRNA, in cells of parental BY4741 during the period of adaptation to 2,4-D by RT-PCR. The *PDR18* mRNA value for the control conditions (0h, unsuplemented medium) was set as 1 and the remaining values were relative values. Values are the mean of at least three independent experiments and error bars indicate the standard deviation.

Figure 4 – A: Representation of the putative regulatory network controlling *PDR18* transcription, according to the information in the YEASTRACT database (www.yeastract.com). B: Relative values of *PDR18* mRNA in wild type strain (wt) and *Δnrg1*, *Δpdr3* and *Δyap1* mutant cells before and four hours following an yeast cell population exposure to 0.45 mM of 2,4-D. The mRNA relative value for the wild type strain immediately before exposure to the herbicide (control) was set as 1. Values are means of at least three independent experiments and error bars indicate the standard deviation. C: Relative values of *PDR18* mRNA in *Δpdr18* cells transformed with pRS416_*PDR18*, pRS416_*PDR18Δp* or the corresponding empty vector before and four hours following an yeast cell population exposure to 0.45 mM of 2,4-D. The mRNA relative value for the *Δpdr18* strain, harboring the pRS416_*PDR18* plasmid, immediately before exposure to the herbicide was set as 1. Values are means of at least three independent experiments and error bars indicate the standard deviation.

Figure 5 - Comparison of [^{14}C]-2,4-D accumulation in non-adapted cells of *S. cerevisiae* BY4741 (■) and the derived deletion mutant *Δpdr18* (▲), during cultivation for 30min in MM4 liquid medium (pH 3.5) supplemented with 0.3 mM of cold 2,4-D (Sigma) and 0.5 μM of [^{14}C]-2,4-D.

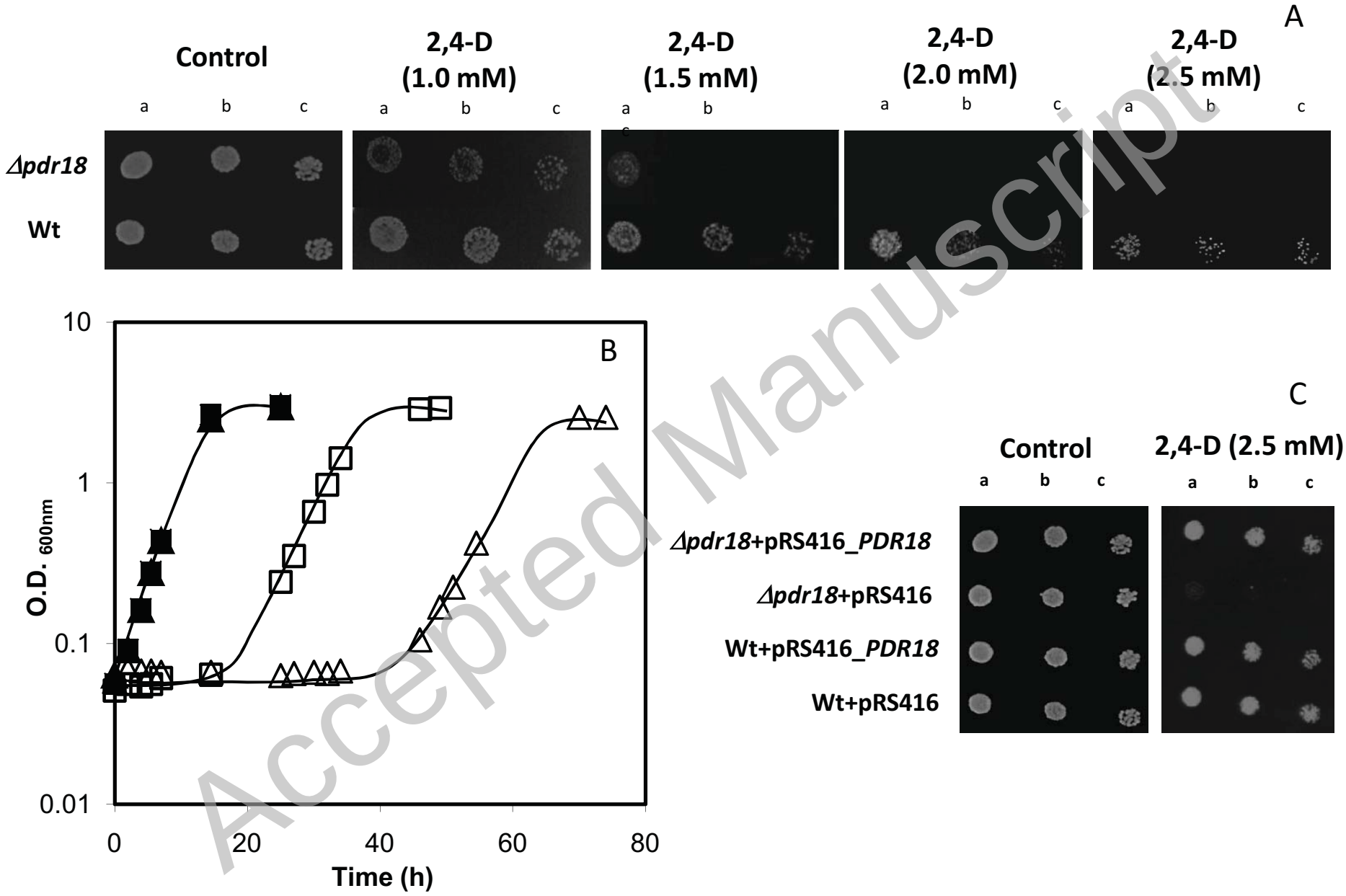
The accumulation (2,4-D* intra/2,4-D* extra) values are the means of at least three independent experiments, error bars indicating standard deviation.

Figure 6 – Comparison of the relative abundance of sterol content in yeast plasma membrane of the *S. cerevisiae* BY4741 and the derived deletion mutant *Δpdr18* harboring the *PDR18* expression plasmid or the corresponding empty vector, both grown under control conditions or after 1h of exposure to 0.45 mM of 2,4-D, measured by GCMS. Values are means of at least three independent experiments and error bars indicate the standard deviation.

Figure 7 – *PDR18* gene expression is important to maintain plasma membrane potential in yeast cells. A: Time course accumulation of [^{14}C]-Methylammonium was followed during incubation of *S. cerevisiae* BY4741 (■) and the derived deletion mutant *Δpdr18* (▲), at 30ºC, in growth MM4 liquid medium, pH 3.5, supplemented with the radiolabelled ammonium analogue. Relative levels of [^{14}C]-Methylammonium, assessed as described in M&M, are average ± standard deviation of at least three independent experiments. B: Comparison of the average membrane potential using the fluorescent probe $DiCO_6(3)$. Values of membrane potential are set as the percentage of the value obtained for wild-type cells and result of at least three independent experiments.

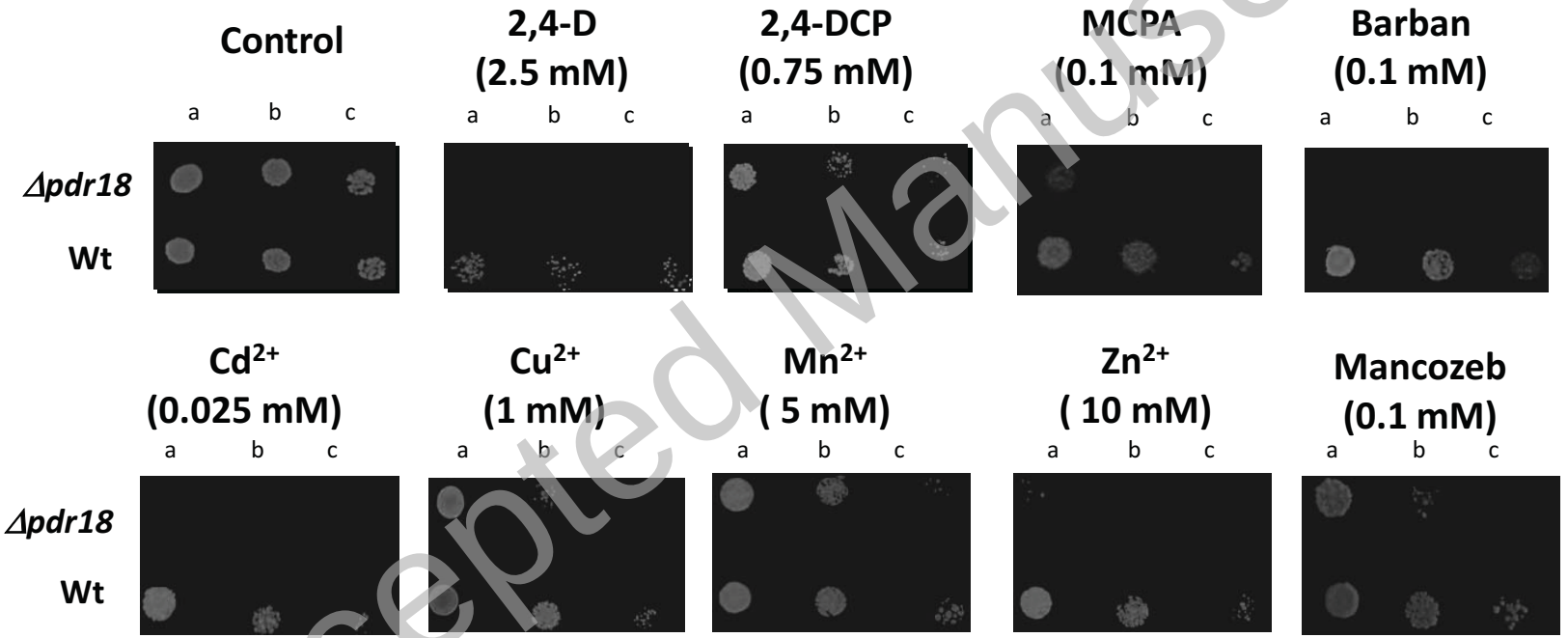
Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 1



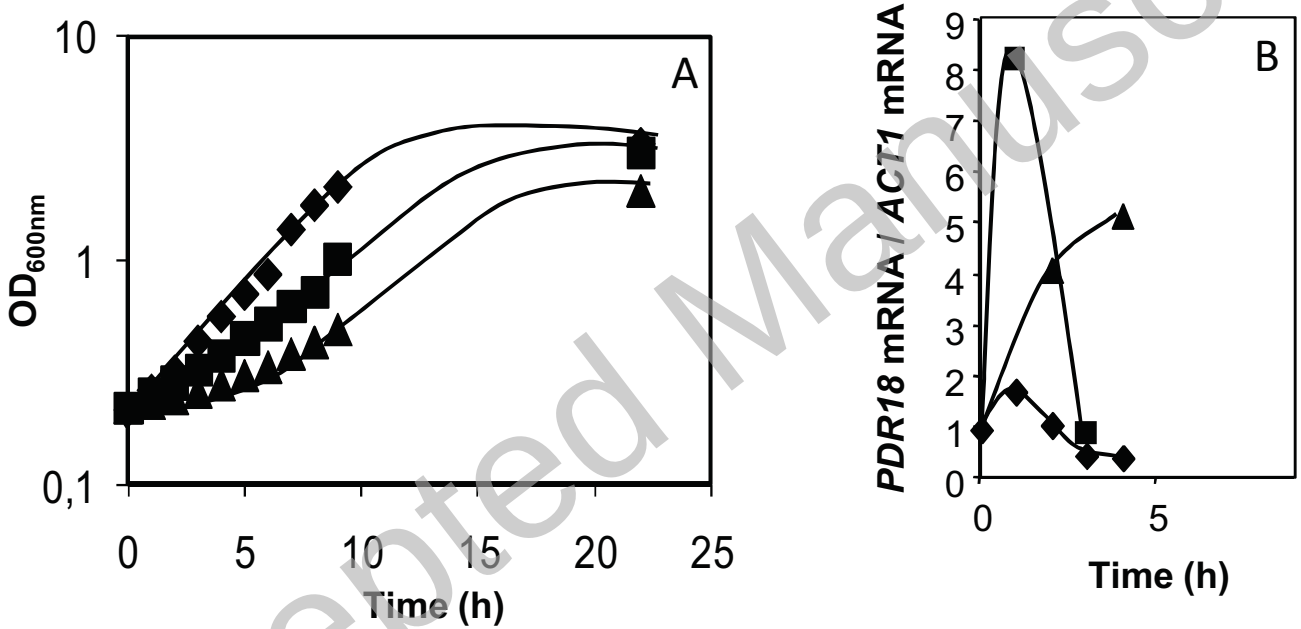
Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 2



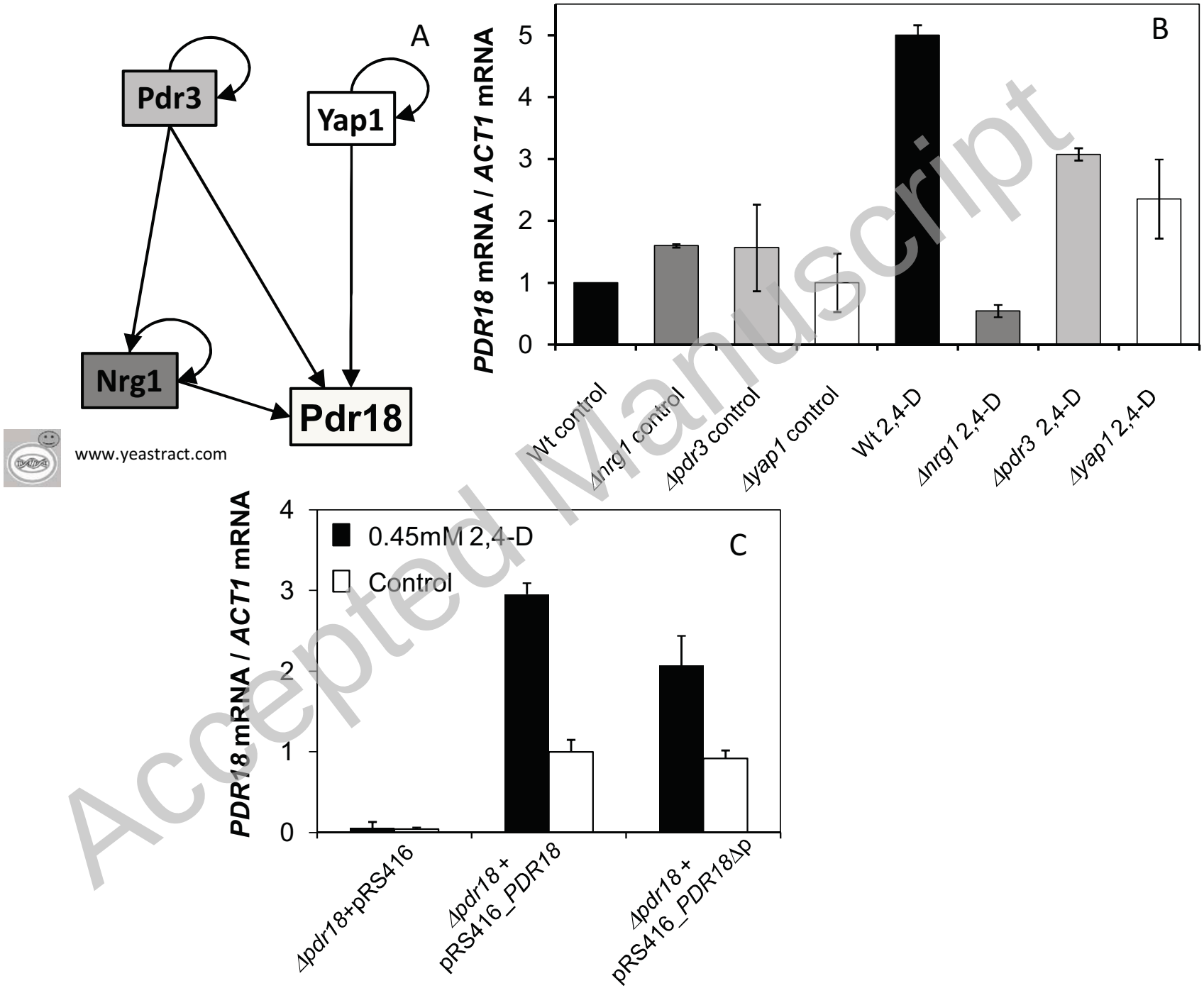
Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 3



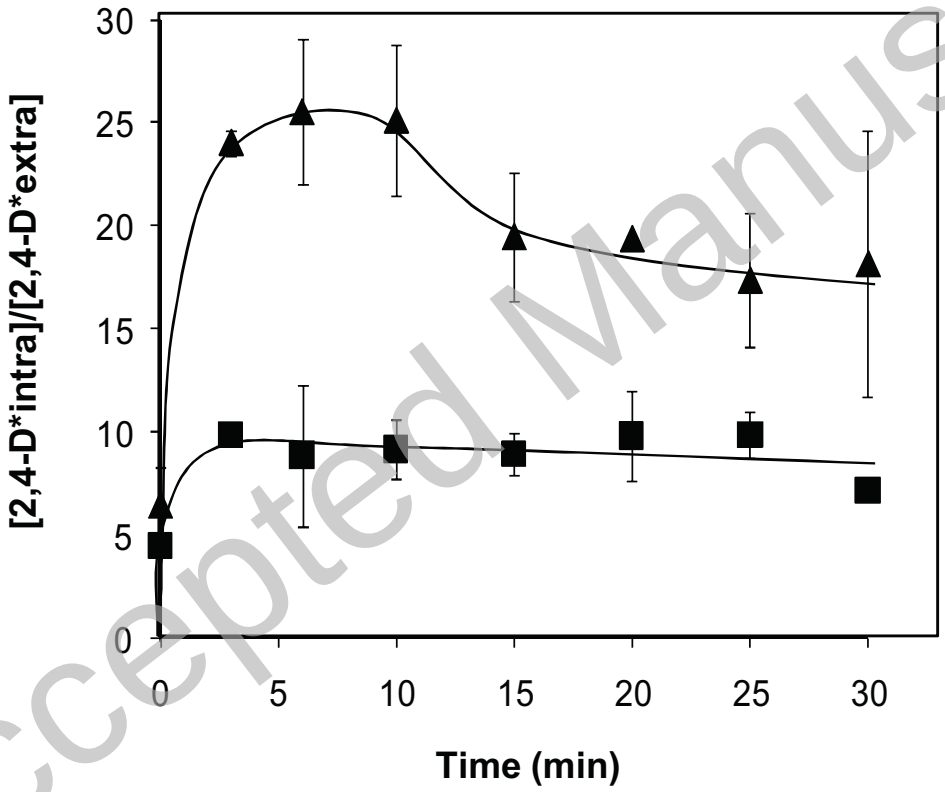
Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 4



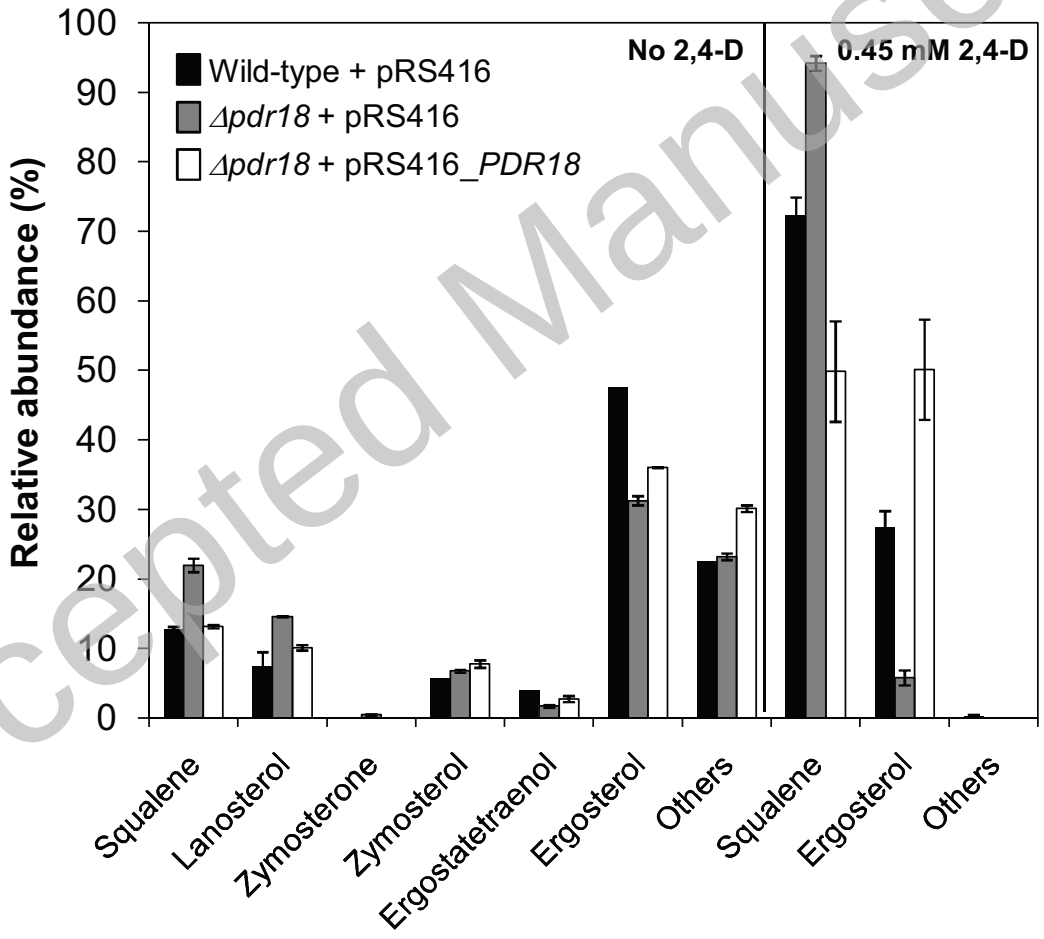
Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 5



Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 6



Biochemical Journal Immediate Publication. Published on 10 Aug 2011 as manuscript BJ20110876

Figure 7