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

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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 [14C]-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 Aup1 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.
EXPERIMENTAL

Strains, plasmids and growth conditions. The parental *Saccharomyces cerevisiae* strain BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and the derived single deletion mutants Δpdr18, Δnrp1, 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' GTTTCGTTCTATGATTTGCTTAGGTACCTTC 3' and its complementary sequence 5' GAATGTCATCTCAGCATCATGAAACGAAAAC 3', in which the replacement nucleotides are underlined.

Yeast cells were cultivated at 30 °C 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)2SO4 (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 g L-1 agar (Ibferagar). 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 2,4-D 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 fenomyl (17 - 35 mg/L) and mancozeb (1 - 1.2 mg/L); and salts of several toxic metal cations, namely, CdSO4 (0.025 - 0.04 mM), CuSO4 (0.5 - 1 mM), MnSO4 (5 - 10 mM), ZnSO4 (5 - 10 mM), Al2(SO4)3 (0.5 - 1 mM), TiCl3 (0.1 - 0.5 mM), CoSO4 (1.5 - 2 mM) and PbSO4 (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, Δnrp1, Δyap1 and Δpdr3, upon yeast exposure to 0mM, 0.3mM or 0.45mM of 2,4-D were assayed 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' - TGGGCAACGGGATGTCGTG-5', 3' - CCAACCGGATGGGAAT -5' and 3' - CTCACCACCTGTGAAGAGGA -5', 3' - CCAAGGCGAGTTACATAGTTTT -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.

**[14C]-2,4-D accumulation assays**. The intracellular accumulation ratio of [14C]-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 [14C]-Methylamine uptake assay [17] and the 3-3'-dihexyloxacarbocanine iodide (DiOC₆(3)) accumulation assay [18].

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

To estimate DiOC₆(3) fluorescence, cells were harvested as described above, resuspended in MES-glucose buffer (10 mM MES, 0.1 mM MgCl₂ and 20 μL⁻¹ glucose, pH 6), supplemented with DiOC₆(3) (Molecular Probes) at a final concentration of 0.25nM and incubated in the dark for 30 min at 30°C 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 0.45μM of 2,4-D, and grown for 1h at 30°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) and broken 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 ultracentrifugation 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 N₂ at 60°C. The extracted sterols were
derivatized using 100 μl N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethyl-
chlorosilane BSTFA/TMCS (Sigma) at 80°C for 1 hr in N₂. The derivatized sterols were
then analyzed using GCMS (Shimadzu 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°C was held for
1 min. then programmed at 120°C to 250°C at 5°C/min where it was held for 30 min. 1
μl injection was made using a split ratio of 10. Injection temperature was 300°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°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 agro economical 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 Ti\(^{3+}\) (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.yeasttract.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 inviable (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 Δnrp1 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 [14C]-2,4-D was assessed. The accumulation of [14C]-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 ergosteryl 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 Δpdr18 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 steroids 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 potential [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 DiOC₆(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 Δtop1 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 TOP1 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.yeastact.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 used 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.
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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 OD₆₀₀nm=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 OD₆₀₀nm=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 Zn²⁺, Mn²⁺, Cu²⁺ and Cd²⁺ 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 OD₆₀₀nm=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 until 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, unsupplemented 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 Δmrg1, Δpdr3 and Δypa1 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 [¹⁴C]-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 [¹⁴C]-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 [14C]-Methylammonium was followed during incubation of S. cerevisiae BY4741 (■) and the derived deletion mutant Δpdr18 (▲), at 30°C, in growth MM4 liquid medium, pH 3.5, supplemented with the radiolabelled ammonium analogue. Relative levels of [14C]-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 DiCO4(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.
Figure 1

A

Δpdr18

Control

2,4-D (1.0 mM)

2,4-D (1.5 mM)

2,4-D (2.0 mM)

2,4-D (2.5 mM)

Wt

B

Δpdr18+pRS416_PDR18

Δpdr18+pRS416

Wt+pRS416_PDR18

Wt+pRS416

C

Control

2,4-D (2.5 mM)
Figure 2

| Treatment         | Δpdr18 Control | Δpdr18 2,4-D (2.5 mM) | Δpdr18 2,4-DCP (0.75 mM) | Δpdr18 MCPA (0.1 mM) | Δpdr18 Barban (0.1 mM) |
|-------------------|---------------|-----------------------|--------------------------|----------------------|------------------------|
|                   | a             | b                     | c                        | a                    | b                      |
| Δpdr18            |               |                       |                          |                      |                        |
| Wt                |               |                       |                          |                      |                        |
| Cd$^{2+}$ (0.025 mM) |               |                       |                          |                      |                        |
| Cu$^{2+}$ (1 mM)  |               |                       |                          |                      |                        |
| Mn$^{2+}$ (5 mM)  |               |                       |                          |                      |                        |
| Zn$^{2+}$ (10 mM) |               |                       |                          |                      |                        |
| Mancozeb (0.1 mM) |               |                       |                          |                      |                        |

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Figure 3

(A) Growth kinetics of yeast strain S288C (solid line) and its topoisomerase mutants (open symbols) as measured by OD600. 

(B) Expression of PDR18 mRNA/ACT1 mRNA ratio as a function of time.
Figure 4

A

B

C

www.yeastact.com
Figure 5

![Graph showing the change in [2,4-D^*intra]/[2,4-D^*extra] over time. The graph displays two lines: one that increases and then decreases with time, and another that remains relatively flat. Error bars are shown for each data point. The x-axis represents time in minutes (0 to 30), and the y-axis represents the ratio of [2,4-D^*intra]/[2,4-D^*extra].]
Figure 6

![Bar chart showing the relative abundance of different compounds under different conditions.](chart)

- **Wild-type + pRS416**
- **Δpdr18 + pRS416**
- **Δpdr18 + pRS416_PDR18**

**Conditions:**
- No 2,4-D
- 0.45 mM 2,4-D

**Compounds:**
- Squalene
- Lanosterol
- Zymosterol
- Ergosta-4,7,14-trien-3-ol
- Ergosterol
- Others

The chart illustrates the relative abundance of these compounds under different conditions, showing a clear distinction in the abundance profile.
Figure 7

A

Methylammonium uptake (mmol/μL) \times 10^{-5}

| Time (min) | 0 | 20 | 40 | 60 | 80 |
|------------|---|----|----|----|----|
|            | 1 | 2  | 3  | 4  |    |

B

% Intensity

|          | Wild-type | Δpdh18 |
|----------|-----------|--------|
|          | 100       | 20     |