

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

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The action of multidrug efflux pumps in MDR (multidrug resistance) 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 the present study, the *PDR18* gene [ORF (open reading frame) *YNR070w*], encoding a putative PDR (pleiotropic drug resistance) transporter of the ATP-binding cassette superfamily, was found to mediate plasma membrane sterol incorporation in yeast. The physiological role of Pdr18 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 (2,4-dichlorophenoxyacetic acid). 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 a $\Delta pdr18$ background. *PDR18* activation under 2,4-D stress is regulated by the transcription factors Nrg1, controlling carbon source availability and the 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. Taken together, the results of the present study suggest that Pdr18 plays a role in plasma membrane sterol incorporation, this physiological trait contributing to an MDR phenotype.

Key words: ergosterol homeostasis, herbicide resistance, multidrug resistance, pleiotropic drug resistance, *Saccharomyces cerevisiae*.

INTRODUCTION

MDR (multidrug resistance), 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 ABC (ATP-binding cassette) superfamily and of the MFS (major facilitator superfamily). 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], whereas 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 Δ pH control, which in turn can alter the partitioning and accumulation of drugs [2]. On the other hand, most of the yeast PDR (pleiotropic drug resistance) transporters of the ABC superfamily, characterized as MDR determinants, have been associated with the control of lipid incorporation into cell membranes [3]. Interestingly, Pdr5 and its homologues 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 non-vesicular 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]. Taken together, these results have been suggested to imply that the MDR phenotype conferred by PDR transporters may occur, to some extent, due to the changes they impose on the composition of cell 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 MDR [12].

In the present study, the functional analysis of *PDR18* [ORF (open reading frame) *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 2,4-D (herbicide 2,4-dichlorophenoxyacetic acid), as previously revealed by microarray analysis [13], the *PDR18* gene was found to confer yeast resistance to the auxin-like herbicides 2,4-D and MCPA (2-methyl-4-chlorophenoxyacetic acid), 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 homologue At5g13750, confers

Abbreviations used: ABC, ATP-binding cassette; CM, cell membrane; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; ER, endoplasmic reticulum; MCPA, 2-methyl-4-chlorophenoxyacetic acid; MDR, multidrug resistance; MFS, major facilitator superfamily; ORF, open reading frame; PDR, pleiotropic drug resistance; RT, reverse transcription.

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2,4-D resistance in *S. cerevisiae* and leads to a lower intracellular accumulation of the herbicide [15,16]. In the present study, 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 metabolism, 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 MDR phenotype.

EXPERIMENTAL

Strains, plasmids and growth conditions

The parental *S. cerevisiae* strain BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and the derived single deletion mutants *Δpdr18*, *Δnrg1*, *Δyap1* and *Δpdr3* used in the present 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 two nucleotides in the putative Nrg1-binding site in the *PDR18* promoter region. The pair of mutagenic oligonucleotides used for this procedure were 5'-GTTTCGTTTCATATGATTGC-TTAGGTACCATTC-3' and its complementary sequence 5'-GATGGTACCTAAGCAATCATATGAAACGAAAC-3', in which the replacement nucleotides are underlined.

Yeast cells were cultivated at 30°C with agitation at 250 rev./min in minimal growth medium MM4 [pH 3.5 (adjusted with HCl)], containing 1.7 g/l yeast nitrogen base without amino acids or (NH₄)₂SO₄ (Difco), 20 g/l glucose (Merck), 2.65 g/l (NH₄)₂SO₄ (Merck), 20 mg/l L-histidine (Merck), 20 mg/l L-methionine (Merck), 60 mg/l L-leucine (Sigma) and 20 mg/l L-uracil (Sigma). Solid medium [pH 4.0 (adjusted with HCl)] was prepared by adding 20 g/l agar (Iberagar). Cells harbouring pRS416 or derived plasmids were grown in the same medium conditions and without uracil (MM4-U) to ensure 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 (0.45 mM in liquid medium and 1–2.5 mM 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 (1–1.5 mM) and barban (0.08–0.1 mM); 2,4-DCP (2,4-dichlorophenol; a 2,4-D degradation intermediate) (0.5–1 mM); the fungicides benomyl (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*, *Δnrg1*, *Δyap1* and *Δpdr3*, upon yeast exposure to 0 mM, 0.3 mM or 0.45 mM 2,4-D, were assessed by real-time RT (reverse transcription)–PCR. RNA extraction from yeast cells was carried out as described previously [15]. The RT–PCR protocol used followed the manufacturer's instructions and has been described previously [5]. Primers for amplification of the *PDR18* and *ACT1* cDNA were designed using Primer Express Software (Applied Biosystems) and were 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.

[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 ($\Delta\Psi$) 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 DiOC₆(3) (3-3'-dihexyloxacarbocyanine iodide) 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 g/l glucose (pH 6)], supplemented with DiOC₆(3) (Molecular Probes) at a final concentration of 0.25 nM and incubated in the dark for 30 min at 30°C with orbital agitation. After centrifugation (8600 g for 5 min at 4°C), cells were immediately observed with a Zeiss Axioplan microscope equipped with adequate epifluorescence filters (BP450-490 and LP520). Fluorescence emission was collected with a CCD (charge-coupled device) camera (Cool SNAPFX, Roper Scientific Photometrics). Bright-field images for determination of $\Delta\Psi$ 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 CMs (cell membranes) 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 0.45 mM 2,4-D, and grown for 1 h at 30°C. Cells were harvested by centrifugation, resuspended in homogenization buffer [50 mM Tris/HCl (pH 7.5), 2.5 mM EDTA

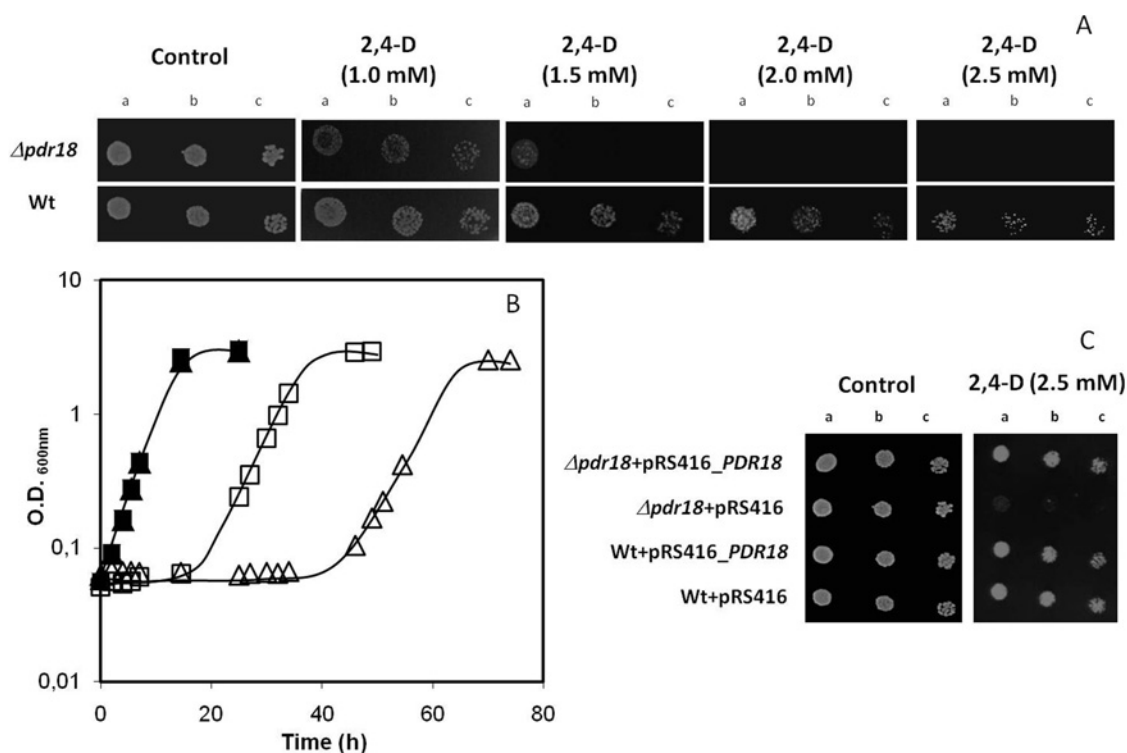


Figure 1 Comparison of the susceptibility to 2,4-D of *S. cerevisiae* BY4741 and the derived deletion mutant $\Delta pdr18$, through cultivation in liquid medium (B) or spot assays (A and C)

(A) Spot assays were carried out as described in the Experimental 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 a $D_{600} = 0.05 \pm 0.005$ spotted in lane a. Pictures were taken after 2 days of incubation. (B) Growth curves of wild-type (wt) (■, □), and $\Delta pdr18$ (▲, △) cells in MM4 liquid medium, pH 3.5 (■, ▲) or in this medium supplemented with 0.45 mM 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 Experimental 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 $D_{600} = 0.05 \pm 0.005$ spotted in lane a. Pictures were taken after 4 days of incubation.

and a protease inhibitor cocktail (1 mM PMSF and 1 $\mu\text{g}/\text{ml}$ each of leupeptin, pepstatin A and aprotinin) and broken by vortex mixing with glass beads (Glaperlon 0.40–0.60 mm). The CMs were recovered by centrifugation at 1000 g to remove unbroken cells and finally the CMs were pelleted by ultracentrifugation at 25000 rev./min (rotor type SW41Ti, Beckman Coulter) for 1 h. The CMs were resuspended in a buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 20% glycerol and protease inhibitors at the concentrations mentioned above. Plasma membrane fractions were obtained from CM fractions by sucrose gradient centrifugation as described by Monk et al. [19]. The CM and plasma membrane protein concentrations obtained, measured using the BCA (bicinchoninic acid) test, ranged from 8 to 19 $\mu\text{g}/\mu\text{l}$ and 3 to 9 $\mu\text{g}/\mu\text{l}$ respectively. Equal amounts of plasma membranes were used for lipid extraction from each of the yeast strains using the method described by Bligh and Dyer [20] with slight modifications. Sterols were extracted and analysed using a method described previously with slight modifications [21]. The saponified lipids were re-extracted using 5 ml of hexane and vortex mixed several times. Water (1 ml) was added to separate the organic phase, which was then dried in nitrogen at 60 °C. The extracted sterols were derivatized using 100 μl of BSTFA/TMCS [N,O-Bis(trimethylsilyl) trifluoroacetamide with trimethyl-chlorosilane; Sigma] at 80 °C for 1 h in nitrogen. The derivatized sterols were then analysed using GC-MS (Schimadzu QP2010 Plus, Japan) and a DB5-MS column 60 m \times 0.2 mm with a film thickness of 0.20 μm . The carrier gas was helium with a flow rate of 1 ml/min and a pressure of 80.8 kPa. The initial column temperature of 120 °C was held for 1 min and then

programmed at 120–250 °C at 5 °C/min where it was held for 30 min. A 1 μl injection was made using a split ratio of 10. The injection temperature was 300 °C. The total ion mass spectra were recorded in the mass range m/z 40–650 at a 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 $\Delta pdr18$ was found to be higher compared with the parental strain, based on spot assays and liquid growth (Figure 1). In the absence of Pdr18, yeast cells become susceptible to 2,4-D, even when supplemented at concentrations that hardly affected wild-type viability (Figure 1A). For higher concentrations of the herbicide, the expression of this transporter become essential for survival under stress (Figure 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 (Figure 1B). The expression of *PDR18* from a centromeric plasmid was found to rescue the 2,4-D-susceptibility phenotype of $\Delta pdr18$ cells, to levels comparable with the parental strain, whereas no effect was detected in the control cells harbouring the corresponding cloning vector (Figure 1C). The ability of this gene expression to

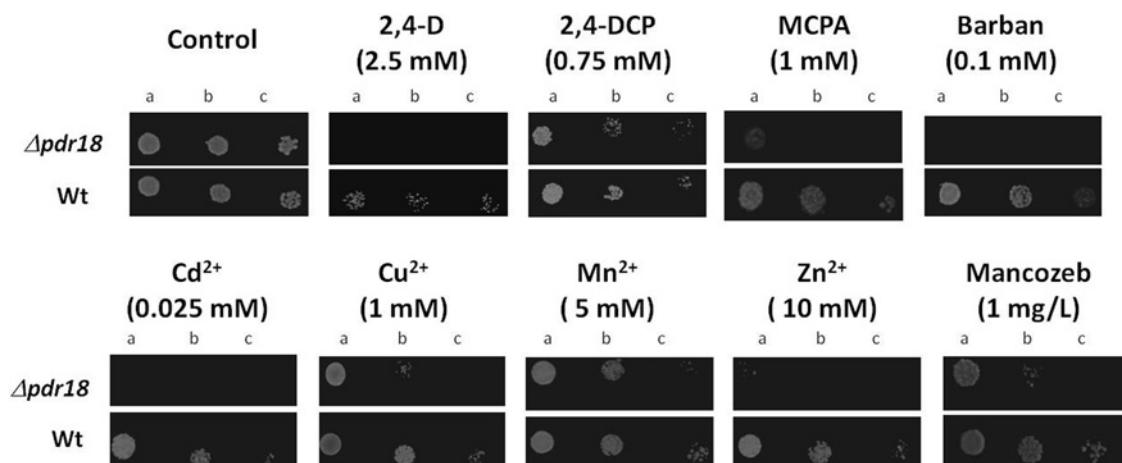


Figure 2 Susceptibility to the herbicides 2,4-D, MCPA and barban, to 2,4-DCP, to the agricultural fungicide mancozeb, and to the metal ions Zn^{2+} , Mn^{2+} , Cu^{2+} and Cd^{2+} induced stress of the deletion mutant $\Delta pdr18$ compared with the wild-type, assessed by spot assays

Cells used for the spot assays were prepared as described in the Experimental 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 $D_{600} = 0.05 \pm 0.005$ spotted in lane a. Pictures were taken after 2 or 3 days of incubation. wt, wild-type.

confer resistance to other pesticides and chemical compounds of agro-economic importance was further analysed, 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-DCP, to the agricultural fungicide mancozeb, and to the metal cations Zn^{2+} , Mn^{2+} , Cu^{2+} and Cd^{2+} (Figure 2). No protection was conferred by *PDR18* expression towards benomyl, Co^{2+} , Pb^{2+} , Al^{3+} or Tl^{3+} (results not shown).

PDR18 transcription is activated in response to 2,4-D-imposed stress in an *Nrg1*-, *Yap1*- and *Pdr3*-dependent manner

A strong increase in the transcript levels of *PDR18* (~8-fold) was registered after 1 h of exposure of an unadapted *S. cerevisiae* cell population to 0.3 mM 2,4-D. This strong, but transient, increase was followed by a rapid decrease of transcript levels to basal values as cells adapted 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 4 h of stress exposure, correlating with the longer duration of the lag-phase imposed by this higher 2,4-D concentration (Figure 3). The fact that a higher herbicide concentration apparently leads to a lower activation of *PDR18* may be due to the fact that 0.45 mM 2,4-D induces viability loss, thus reducing the number of cells in the population with the ability to generate a stress response [22].

The YEASTRACT database (<http://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 (Figure 4A). Of these, only *Nrg1*, *Pdr3* and *Yap1* were examined in the present study because mutants deleted for the other two genes were either unviable ($\Delta yap1$) or exhibited marked growth defects ($\Delta swi4$) [SGD (*Saccharomyces* Genome Database); <http://www.yeastgenome.com>]. The three mutant strains tested, 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 4 h of incubation

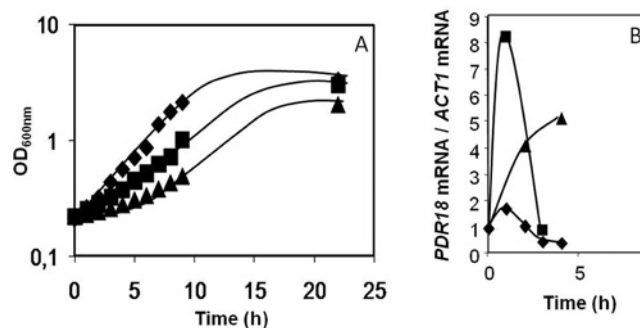


Figure 3 *PDR18* transcript levels in yeast cells exposed to 2,4-D-imposed stress

(A) Comparison of the susceptibility to 2,4-D-induced stress of *S. cerevisiae* parental strain BY4741 exposed to 0 (\blacklozenge), 0.3 (\blacksquare) or 0.45 (\blacktriangle) mM 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 strain BY4741 during the period of adaptation to 2,4-D by RT-PCR. The *PDR18* mRNA value for the control conditions (0 h, unsupplemented medium) was set as 1 and the remaining values were relative values. Values are the means \pm S.D. for at least three independent experiments.

with 2,4-D was abrogated in the $\Delta nrg1$ mutant or reduced in $\Delta yap1$ and $\Delta pdr3$ mutants (Figure 4B), suggesting that both *Pdr3* and *Yap1* transcription factors are necessary to ensure full activation of *PDR18*.

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_*PDR18* Δp plasmids were transformed into $\Delta pdr18$ cells, so that the genomic expression of *PDR18* from its natural

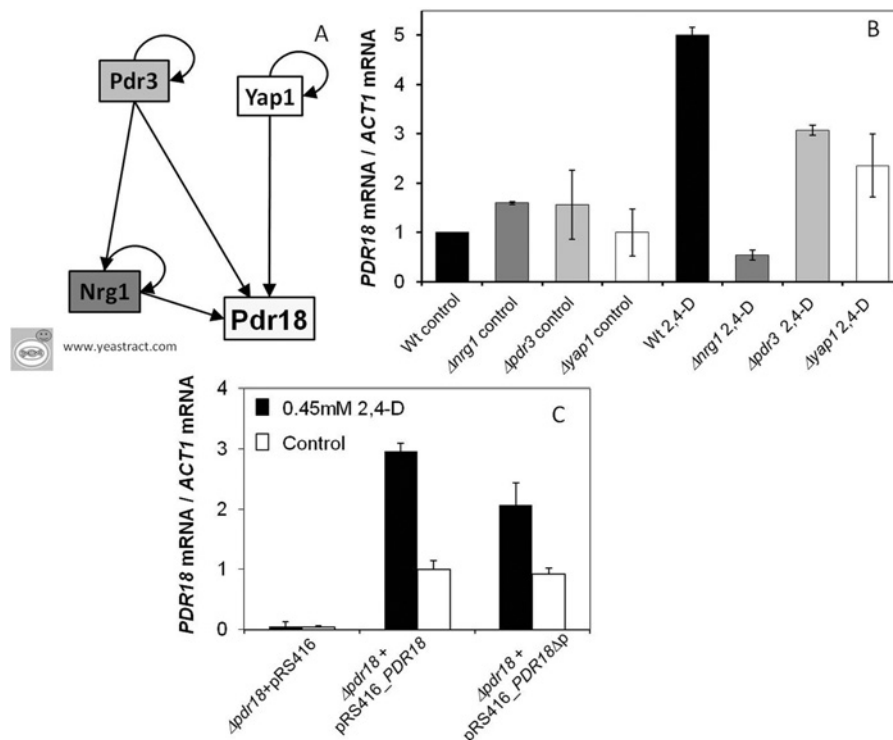


Figure 4 *PDR18* expression regulation under 2,4-D-imposed stress

(A) Representation of the putative regulatory network controlling *PDR18* transcription, according to the information in the YEASTRACT database (<http://www.yeasttract.com>). (B) Relative values of *PDR18* mRNA in wild-type strain (wt) and $\Delta nrg1$, $\Delta pdr3$ and $\Delta yap1$ mutant cells before and 4 h following a yeast cell population exposure to 0.45 mM 2,4-D. The relative value of mRNA for the wild-type strain immediately before exposure to the herbicide (control) was set as 1. Values are means \pm S.D. for at least three independent experiments. (C) Relative values of *PDR18* mRNA in $\Delta pdr18$ cells transformed with pRS416_ *PDR18*, pRS416_ *PDR18* Δp or the corresponding empty vector before and 4 h following a yeast cell population exposure to 0.45 mM 2,4-D. The relative value of mRNA for the $\Delta pdr18$ strain, harbouring the pRS416_ *PDR18* plasmid, immediately before exposure to the herbicide was set as 1. Values are means \pm S.D. for at least three independent experiments.

promoter would not be accounted for. RT-PCR was used to measure the *PDR18* transcript levels in these cells upon exposure to 0.45 mM 2,4-D. The abrogation of the Nrg1-binding site was seen to have only a moderate effect on herbicide-induced *PDR18* up-regulation (Figure 4C) when compared with the full effect observed upon Nrg1 deletion. Taken together, 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 MDR transporter that confers resistance on yeast cells against 2,4-D-imposed stress, the effect of *PDR18* expression on 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 $\Delta pdr18$ cells (results not shown), was 2.5-fold higher in cells devoid of *PDR18* than in parental cells (Figure 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 catalysing 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-

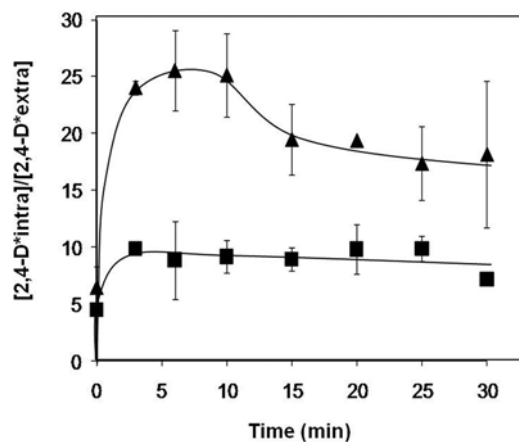


Figure 5 Accumulation of [14 C]2,4-D

Comparison of [14 C]2,4-D accumulation in non-adapted cells of *S. cerevisiae* BY4741 (■) and the derived deletion mutant $\Delta pdr18$ (▲), during cultivation for 30 min in MM4 liquid medium (pH 3.5) supplemented with 0.3 mM non-radioactive 2,4-D (Sigma) and 0.5 μ M [14 C]2,4-D. The accumulation [2,4-D* intra]/[2,4-D* extra] values are the means \pm S.D. for at least three independent experiments.

fold reduction of ergostatetraenol and ergosterol content, the end-products of the ergosterol biosynthetic pathways, were detected in the plasma membrane (Figure 6). Upon episomal complementation of *PDR18*, there was a partial complementation

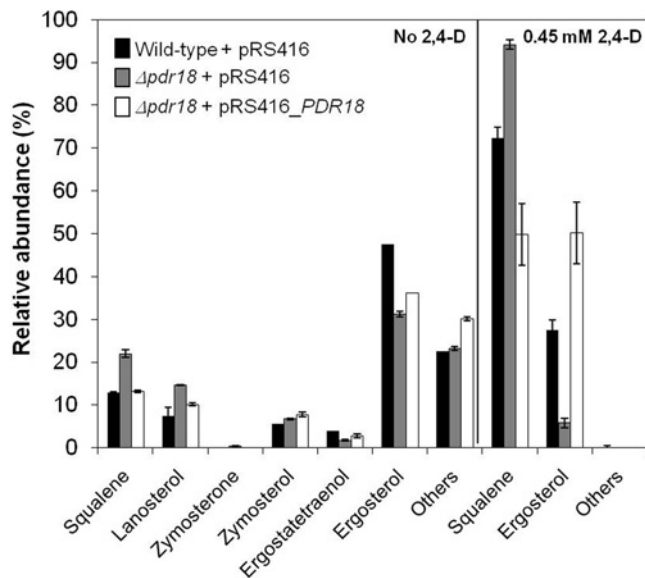


Figure 6 *PDR18* expression affects plasma membrane sterol composition

Comparison of the relative abundance of sterol content in yeast plasma membrane of the *S. cerevisiae* BY4741 strain and the derived deletion mutant $\Delta pdr18$ harbouring the *PDR18* expression plasmid or the corresponding empty vector, both grown under control conditions or after 1 h of exposure to 0.45 mM 2,4-D, measured by GC-MS. Values are means \pm S.D. for at least three independent experiments.

of the $\Delta pdr18$ plasma membrane sterol composition phenotype (Figure 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, whereas the levels of squalene increased 5.5-fold. This effect was exacerbated in the absence of the *PDR18* gene. Indeed, in $\Delta pdr18$ cells 2,4-D exposure led to a 4.3-fold increase in squalene and a 5.2-fold decrease in ergosterol plasma membrane concentrations (Figure 6). Remarkably, under 2,4-D challenge, the relative abundance of squalene became higher than the relative abundance of ergosterol in both wild-type and $\Delta pdr18$ backgrounds, and the concentration of the other sterols detected in the plasma membrane of unstressed yeast cells became undetectable (Figure 6).

A lower plasma membrane potential is observed in $\Delta pdr18$ cells

The role of *PDR18* expression in the maintenance of yeast membrane potential was also analysed. 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 to approximately 60% the level of methylammonium uptake in yeast cells (Figure 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], was 3-fold higher in wild-type cells than in $\Delta pdr18$ cells (Figure 7B). Both methods indicate a strong depletion of the plasma membrane potential in the absence of *PDR18*.

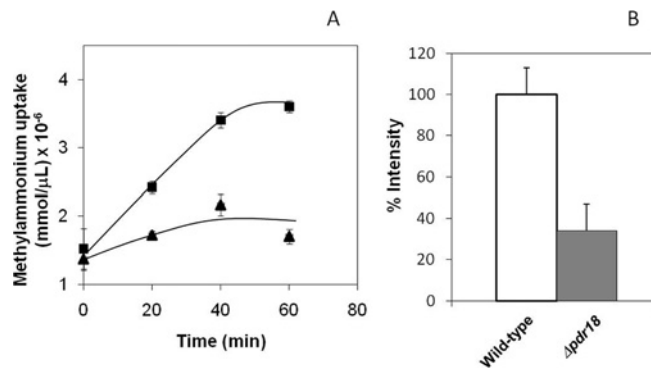


Figure 7 *PDR18* gene expression is important to maintain plasma membrane potential in yeast cells

(A) Time-course accumulation of [¹⁴C]methylammonium was followed during incubation of *S. cerevisiae* BY4741 (■) and the derived deletion mutant $\Delta pdr18$ (▲), 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 the Experimental section, are means \pm S.D. for at least three independent experiments. (B) Comparison of the average membrane potential using the fluorescent probe DiOC₆(3). Values of membrane potential are set as the percentage of the value obtained for wild-type cells and are means \pm S.D. for at least three independent experiments.

DISCUSSION

The present 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 present 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 the $\Delta pdr18$ deletion mutant, compared with the wild-type strain, is similar to the one observed previously for the $\Delta tpo1$ deletion mutant [16]. Interestingly, the *PDR18* homologue in the plant model *Arabidopsis thaliana*, *AtPDR9*, was seen to confer 2,4-D resistance in plants, also contributing to decreased 2,4-D accumulation in plant roots [28].

During the 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, whereas the effect over the inhibition of specific growth exerted 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 the *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 exerted by Pdr3 over the transcriptional up-regulation of *TPO1* under 2,4-D stress described previously [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 MDR, 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 (<http://www.yeasttract.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 encoding other transcription factors. Interestingly, the transcript levels of the *NRG1* gene suffer a 5-fold increase in yeast cells exposed for 15 min to 0.3 mM 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 Nrg1-mediated response [13].

Most significantly, in the present study the deletion of *PDR18* in *S. cerevisiae* cells was found to lead to an accumulation of the precursors of the ergosterol biosynthetic pathway, squalene and lanosterol, and to a decrease in the content of ergostetraenol and ergosterol, the end-products of the ergosterol biosynthetic pathway, in yeast plasma membrane. Under 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 (endoplasmic reticulum)-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 ER 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 so far been implicated in the mediation of this non-vesicular ergosterol movement. The results of the present study 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 which may affect the fusibility of a membrane, 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 $\Delta pdr18$ 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 $\Delta pdr18$ 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 the present 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 $\Delta pdr18$ 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*.

On the basis of 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. The role of Pdr18 as an MDR determinant is suggested to derive, at least partially, from its physiological role, which is expected to affect drug partition and transport across cell membranes. The results of the present study are expected to increase current knowledge on the action of this family of transporters with an effect on 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 agro-economic interest.

AUTHOR CONTRIBUTION

Tânia Cabrito participated in the design and optimization of the experiments, and carried out most of the experimental work. Miguel Teixeira carried out the first preliminary experiments, contributing to the conception and co-ordination of the study. Ashutosh Singh performed the plasma membrane sterol composition assessment assays and contributed to the writing of this part of the paper, under the supervision of Rajendra Prasad. Tânia Cabrito, Miguel Teixeira and Isabel Sá-Correia participated in the writing of the paper. Isabel Sá-Correia co-ordinated the study and conceived and supervised all of the work. All authors read and approved the final paper.

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