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 Δpdr18 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 homoeostasis, herbicide resistance, multidrug resistance, pleiotropic drug resistance, Saccharomyces cerevisiae.
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, Δ yap1 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 putative oligonucleotides used for this procedure were 5′-GGTGACTTTACATGATGGTT-3′ and its complementary sequence 5′-GAATGGTACCTAAGCAGAATCCGGAATAAAAAGG-3′, in which the replacement nucleotides are underlined.

Yeast cells were grown at 30°C with agitation at 250 rev./min in minimal growth medium MM4 [pH 5.5 (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 2,4-DCP (2,4-dichlorophenoxyacetic acid), containing 1.7 g/l yeast nitrogen base without amino acids or (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 and 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; 2,4-D degradation intermediate) (0.5–1 mM); the fungicides benomyl (0.08–0.1 mM); 2,4-DCP (2,4-dichlorophenoxyacetic acid); and the herbicides MCPA (1–1.5 mM) and 2,4-DCP (2,4-dichlorophenoxyacetic acid); and the herbicides MCPA (1–1.5 mM) and 2,4-DCP (2,4-dichlorophenoxyacetic acid). The susceptibility of 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 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), the cells were immediately observed with a Zeiss Axioskop microscope equipped with adequate epifluorescence filters (BP450-490 and LP520). Fluorescence emission was collected with a CCD (charge-coupled device) camera (Cool SNAP FX, 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 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/Cl (pH 7.5), 2.5 mM EDTA and 1 mM PMSF), and lysed as described above. CMs were then separated from non-soluble material by centrifugation, washed and treated with a solution containing 1.0 mM CaCl2 and 10 mg/ml proteinase K (Sigma) at 30°C overnight. After centrifugation, the CMs were resuspended in Mes/glucose buffer (10 mM Mes, 0.1 mM MgCl2, and 20 g/l glucose (pH 6)).

**PDR18 gene expression assays**

The changes registered in the transcript levels from the *PDR18* gene in BY4741 and in derived mutants Δpdr18, Δnrg1, Δ yap1 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 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′-TTGGCAAGCGCGATCTGT-5′, 3′-CCACGCCGATTGGGAAT-5′ and 3′-CTCCACACTGCTGAAAGAGAA-5′, 3′-CCAAAGCCGACATAGATGGTA-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.
and a protease inhibitor cocktail (1 mM PMSF and 1 μg/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 25 000 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 μg/μl and 3 to 9 μg/μl 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. Sterols were extracted and analysed using a 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 the hexane layer and the whole was mixed with glass beads (Glaperlon 0.40–0.60 mm) and broken by vortex mixing. The extracted sterols were derivatized using 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 (Shimadzu QP2010 Plus, Japan) and a DB5-MS column 60 m × 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 Δpdr18 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 2,4-D, the expression of this transporter becomes 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 Δpdr18 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...
confer resistance to other pesticides and chemical compounds of agroeconomic 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 Ti\(^{3+}\) (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 baseline values as cells adapted to growth in the presence of 2,4-D. 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.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 (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 (Δrap1) or exhibited marked growth defects (Δswi4) [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 with 2,4-D was abrogated in the Δnrg1 mutant or reduced in Δyap1 and Δpdr3 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_PDR18Δp plasmids were transformed into Δpdr18 cells, so that the genomic expression of PDR18 from its natural
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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.yeastract.com). (B) Relative values of PDR18 mRNA in wild-type strain (wt) and Δnrg1, Δpdr3 and Δyap1 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 ± S.D. for at least three independent experiments. (C) Relative values of PDR18 mRNA in Δpdr18 cells transformed with pRS416_PDR18, pRS416_PDR18Δp 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 Δpdr18 strain, harbouring the pRS416_PDR18 plasmid, immediately before exposure to the herbicide was set as 1. Values are means ± 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 [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 (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-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

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of the Δpdr18 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 Δpdr18 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 Δpdr18 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 Δpdr18 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 Δpdr18 cells (Figure 7B). Both methods indicate a strong depletion of the plasma membrane potential in the absence of PDR18.

**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 carbamate 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 Δpdr18 deletion mutant, compared with the wild-type strain, is similar to the one observed previously for the Δpdr1 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...
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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.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 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 Ngr1-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 ergostatetraenol 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 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 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 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.

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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