Membrane hyperpolarization and salt sensitivity induced by deletion of *PMP3*, a highly conserved small protein of yeast plasma membrane

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Yeast plasma membranes contain a small 55 amino acid hydrophobic polypeptide, Pmp3p, which has high sequence similarity to a novel family of plant polypeptides that are overexpressed under high salt concentration or low temperature treatment. The *PMP3* gene is not essential under normal growth conditions. However, its deletion increases the plasma membrane potential and confers sensitivity to cytotoxic cations, such as Na⁺ and hygromycin B. Interestingly, the disruption of *PMP3* exacerbates the NaCl sensitivity phenotype of a mutant strain lacking the Pmr2p/Enap Na⁺-ATPases and the Nha1p Na⁺/H⁺ antiporter, and suppresses the potassium dependency of a strain lacking the K⁺ transporters, Trk1p and Trk2p. All these phenotypes could be reversed by the addition of high Ca²⁺ concentration to the medium. These genetic interactions indicate that the major effect of the *PMP3* deletion is a hyperpolarization of the plasma membrane potential that probably promotes a non-specific influx of monovalent cations. Expression of plant RCI2A in yeast could substitute for the loss of Pmp3p, indicating a common role for Pmp3p and the plant homologue.

Keywords: membrane potential/Pmp3p/*Saccharomyces cerevisiae*/toxic cation tolerance

Introduction

Proteolipids are a class of extremely hydrophobic proteins distinguished by their solubility in mixtures of organic solvents such as chloroform–methanol (Folch and Lees, 1951). We have previously purified the proteolipidic fraction extracted from yeast plasma membranes and sequenced two small (38 residues) and highly hydrophobic isoproteins, named Pmp1p and Pmp2p (Navarre *et al.*, 1992, 1994). Deletion of both genes, *PMP1* and *PMP2*, significantly decreases the rate of ATP hydrolysis of Pma1p, the plasma membrane H⁺-ATPase (Navarre *et al.*, 1994). Recently, mutations in Pma1p were reported to be Na⁺ resistant partly by reducing Na⁺ uptake, but also by up-regulating the activity of an intracellular Na⁺/H⁺ exchanger (Nass *et al.*, 1997; Withee *et al.*, 1998).

Na⁺ homeostasis in yeast is achieved by the coordinate activity of plasma membrane efflux and influx systems, and by an efficient Na⁺ sequestration system (see reviews by Gaber, 1992; Rodriguez-Navarro *et al.*, 1994; Niu *et al.*,

1995; Serrano, 1996; Schachtman and Liu, 1999; Serrano *et al.*, 1999).

Na⁺ efflux is mediated mainly by several P-type ATPases encoded by the *PMR2/ENA* locus, containing up to five nearly identical genes (Garcia-deblas *et al.*, 1993; Wieland *et al.*, 1995). Disruption of the whole *PMR2/ENA* locus makes cells hypersensitive to NaCl (Wieland *et al.*, 1995), but does not abolish Na⁺ efflux completely (Haro *et al.*, 1991), indicating the existence of another plasma membrane Na⁺ efflux system. The plasma membrane Na⁺/H⁺ antiporter, Nha1p, which is highly homologous to the fission yeast Sod2p antiporter, mediates the exchange of cytoplasmic Na⁺ and K⁺ with external H⁺ (Prior *et al.*, 1996; Bañuelos *et al.*, 1998). In addition to the plasma membrane Na⁺ efflux systems, Nhx1p/Nha2p plays an important role in halotolerance by mediating Na⁺ sequestration in a pre-vacuolar compartment (Nass *et al.*, 1997; Nass and Rao, 1998; Numata *et al.*, 1998). Finally, *YJL094c* encodes a putative Na⁺/H⁺ permease similar to the *Enterococcus hirae* Na⁺/H⁺ permease, NapA (Miosga *et al.*, 1994), although it functions rather as a K⁺/H⁺ exchanger since its deletion results in an increased K⁺ content (Ramirez *et al.*, 1998).

Moreover, Na⁺ is believed to be taken up by the K⁺ transporters Trk1p and Trk2p (Gaber *et al.*, 1988; Ko and Gaber, 1991; Ramos *et al.*, 1994). At high Na⁺ concentrations, Trk1p increases its selectivity, favouring K⁺ over Na⁺ (Haro *et al.*, 1993; Mendoza *et al.*, 1994). This change depends upon the function of the phosphatase calcineurin (Mendoza *et al.*, 1994). A non-specific plasma membrane Na⁺ and K⁺ uptake channel, independent of Trk1p and Trk2p, has been shown in whole-cell patch-clamp experiments (Bihler *et al.*, 1998; Roberts *et al.*, 1999). The corresponding monovalent cation current NSC1 is highly sensitive to divalent cations (Roberts *et al.*, 1999).

In this report, a novel polypeptide, named Pmp3p, was purified from *Saccharomyces cerevisiae* plasma membranes after extraction with organic solvent. Deletion of *PMP3* confers NaCl hypersensitivity to a mutant strain lacking the major Na⁺ efflux systems, Pmr2p/Enap and Nha1p. It also restores growth of a strain deficient in K⁺ uptake, particularly at low pH. We show that these phenotypes may be due to an increased cation uptake resulting from a hyperpolarization of the plasma membrane. The loss of Pmp3p function can be replaced by the expression of a plant homologue. This result confirms the suitability of the yeast system to study the molecular mechanisms underlying halotolerance in higher plants, as previously described (see recent reviews by Schachtman and Liu, 1999; Serrano *et al.*, 1999).

Fig. 1. Pmp3p is a novel 55 amino acid proteolipid of the yeast plasma membrane. **(A)** Yields (in pmol) of phenylthiohydantoin derivatives recovered after each cycle of Edman degradation for the proteolipid fraction extract derived from *S.cerevisiae* W303-1B plasma membranes. **(B)** Alignment of Pmp3p with RCI2A (*A.thaliana*), RCI2B (*A.thaliana*), ESI3 (*L.elongatum*) and BLT101 (*Hordeum vulgare*): amino acids identical in Pmp3p and one of the plant 54 amino acid polypeptides are boxed. **(C)** Silver-stained Tricine-SDS-polyacrylamide gels of purified plasma membrane preparations (50 µg/gel lane). Lane 1, molecular weight markers; lane 2, *PMP3* wild-type strain W303-1B; lane 3, *pmp3Δ* mutant strain CN3.

Table I. Yeast strains used

Strain	Genotype	Reference
W303-1B	<i>MATα ade 2-1 can 1-100 his3-11,15 leu 2-3,112 trp1-1 ura3-1</i>	Wallis <i>et al.</i> (1989)
CN3	<i>W303-1B Δpmp3::TRP1</i>	this work
CN12	<i>W303-1B Δpmp1::URA3 Δpmp2::HIS3</i>	Navarre <i>et al.</i> (1994)
CN123	<i>W303-1B Δpmp1::URA3 Δpmp2::HIS3 Δpmp3::TRP1</i>	this work
YR93	<i>MATα ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 gal2 Δpmp2-2::HIS3</i>	Wieland <i>et al.</i> (1995)
YR93-3	<i>YR93 Δpmp3::URA3</i>	this work
YR93-1	<i>YR93 Δnha1::LEU2</i>	this work
YR93-31	<i>YR93 Δpmp3::URA3 Δnha1::LEU2</i>	this work
YR93-31-RCI2A	<i>YR93 Δpmp3::PMP3PROM-RCI2A-PMP3STOP Δnha1::LEU2</i>	this work
CY162	<i>MATα ura3-52 trk1Δ his 4-15 trk2Δ1::pCK64</i>	Ko and Gaber (1991)
CY162-3	<i>CY162 Δpmp3::URA3</i>	this work

Results

Isolation and sequence of the *PMP3* gene

Microsequencing of the chloroform-methanol extract of the yeast plasma membranes has led to the identification of two small (38 residues) and highly similar polypeptides, Pmp1p and Pmp2p (Navarre *et al.*, 1992, 1994). A close examination of these preparations revealed the presence of a third hydrophobic polypep-

tide beginning with the sequence Met-Asp-Ser-Ala-Lys. This protein, named Pmp3p, was unnoticed initially because it represented <10% of the total protein content of this fraction (Figure 1A). The first 25 residues of Pmp3p were then determined unambiguously, using the organic solvent extract prepared from the mutant strain CN12, containing a deletion of both *PMP1* and *PMP2* genes (see Table I). A search for identity between this amino acid sequence and yeast

expressed sequence tags (ESTs) revealed a match between EST 101712 (accession No. T36719) and the C-terminal end of the microsequenced Pmp3p.

A PCR product of *PMP3* was amplified from yeast DNA by using a degenerate primer encoding the N-terminal sequence of Pmp3p and a second primer found in the 3'-non-coding region of EST 101712. The PCR product was labelled and used as a probe to screen an *EcoRI* genomic DNA library by hybridization. Positive clones containing the *PMP3* gene in a 2 kb *EcoRI* fragment were subcloned and sequenced. The deduced open reading frame (ORF) was found to encode a highly hydrophobic 55 amino acid protein ($M_r = 6109$), which is predicted to contain two membrane-spanning segments. Unlike Pmp1p and Pmp2p (Navarre *et al.*, 1992, 1994), Pmp3p conserves its first N-terminal methionine residue, as indicated by microsequencing. Searching for homologues of Pmp3p in protein databases revealed 40% sequence identity with several 54 residue plant polypeptides whose expression is induced by high salinity or low temperature (Figure 1B). For instance, ESI3 is encoded by a gene that belongs to a group of 11 members referred to as ESI (for early salt stress-induced) because their expression is induced in the roots of the salt-tolerant wheatgrass *Lophopyrum elongatum* upon addition of 250 mM NaCl (Gulick and Dvorak, 1992; Galvez *et al.*, 1993). ESI3 (Gulick *et al.*, 1994) is 100% identical to the polypeptide encoded by the *blt101* gene of winter barley. Transcription of *blt101* is induced in all tissue types tested (shoot meristem, mature leaves and roots) by low temperature treatment (Goddard *et al.*, 1993). Pmp3p, moreover, is also highly similar to two related 54 amino acid *Arabidopsis thaliana* polypeptides, RCI2A and RCI2B, whose synthesis is induced transiently by low temperatures (Capel *et al.*, 1997).

Once the complete sequence of the *S.cerevisiae* genome was available, we were able to locate the *PMP3* gene on the right arm of chromosome IV (*YDR276c*), between genes *YDR275w* and *MTH1* (*YDR277c*), from position 1 013 467 to position 1 013 634. Northern blot analysis of total RNA showed the *PMP3* transcript to be ~0.5 kb long and fairly abundant (data not shown), in agreement with the presence of a perfect TATA box (TATAATA) located 54 bp upstream from the AUG codon, a high codon adaptation index value (0.37) and the analysis of the yeast transcriptome (Velculescu *et al.*, 1997) indicating the presence of 56 mRNA copies per cell.

NaCl sensitivity associated with deletion of *PMP3*

To determine the physiological function of Pmp3p in yeast, the chromosomal copy of *PMP3* was disrupted by homologous gene recombination. Haploid strains CN3 (*pmp3Δ*) and CN123 (*pmp1Δ pmp2Δ pmp3Δ*) were obtained, indicating that Pmp3p is not essential under normal growth conditions. Figure 1C (Tricine-SDS-PAGE) shows that plasma membranes isolated from the CN3 (*pmp3Δ*) mutant lack a silver-stainable band of 6.2 kDa, corresponding exactly to the predicted molecular mass of Pmp3p.

The CN3 strain was more sensitive to high concentrations of NaCl (>1 M) than the *PMP3* wild-type strain (W303-1B) (Figures 2A and 3). The NaCl sensitivity phenotype is not explained by an osmotic effect, since the

Fig. 2. Effect of *PMP3* deletion on NaCl tolerance. (A) Increased sensitivity to NaCl of a *pmp3*-deleted strain. Serial 10-fold dilutions of saturated cultures of wild-type W303-1B (*PMP3*) and CN3 (*pmp3Δ*) were spotted onto YD plates supplemented with the indicated concentrations of NaCl. The plates were photographed after a 2 day incubation at 30°C. (B) Effect of *PMP3* deletion on the time course of intracellular Na⁺ accumulation during salt stress. Exponentially growing CN3 (*pmp3Δ*; open squares) and W303-1B (*PMP3*; closed squares) cells in YD medium were stressed by adding NaCl at time zero to a final concentration of 0.9 M. Aliquots were taken at the times indicated for determination of the intracellular Na⁺ concentration as described in Materials and methods.

CN3 strain cells grew as well as the wild type on 2.0 and 2.5 M sorbitol (data not shown). The wild-type and mutant strains were incubated with 1 M NaCl and the intracellular Na⁺ concentrations were determined at various time intervals. At each sampling time, the intracellular Na⁺ concentration was nearly twice as high in CN3 (*pmp3Δ*) as in W303-1B (*PMP3*) (Figure 2B), indicating that the defect of *pmp3Δ* cells in NaCl tolerance results from a higher intracellular Na⁺ concentration.

To examine further the contribution of Pmp3p to Na⁺ tolerance, we analysed the effect of the *PMP3* deletion on the Na⁺ sensitivity of a mutant deficient in the Enap/Pmr2p plasma membrane Na⁺-ATPases. In comparative growth assays, YR93-3 (*pmr2Δ pmp3Δ*) and YR93 (*pmr2Δ PMP3*) cells showed half-maximal inhibition at 200 and 300 mM NaCl, respectively (Figure 3). Thus, deletion of *PMP3* in the *pmr2Δ* background markedly increased the sensitivity to Na⁺.

Fig. 3. Deletion of *PMP3* exacerbates the Na^+ sensitivity phenotype of strains lacking Pmr2p/Enap and Nha1p. Strains W303-1B (*PMR2 NHA1 PMP3*; closed squares), CN3 (*PMR2 NHA1 pmp3Δ*; open squares), YR93 (*pmr2Δ NHA1 PMP3*; closed triangles), YR93-3 (*pmr2Δ NHA1 pmp3Δ*; open triangles), YR93-1 (*pmr2Δ nha1Δ PMP3*; closed circles) and YR93-31 (*pmr2Δ nha1Δ pmp3Δ*; open circles) were grown in YD medium to saturation. Then, 10 μl aliquots were transferred to 3 ml of YD medium supplemented with various NaCl concentrations and grown for 15 h at 30°C. Relative growth is the ratio of the OD_{600} of a given culture to the value obtained with control cells grown in YD. The experiment was repeated three times with similar results.

It has been reported previously that, in a *pmr2Δ* background, the loss of the plasma membrane Na^+/H^+ antiporter, Nha1p, renders yeast more sensitive to Na^+ (Prior *et al.*, 1996; Bañuelos *et al.*, 1998). We confirmed this observation with strain YR93-1 (*pmr2Δ nha1Δ PMP3*), which showed half-maximal growth inhibition at ~200 mM NaCl (Figure 3). YR93-1 (*pmr2Δ nha1Δ PMP3*) and YR93-3 (*pmr2Δ NHA1 pmp3Δ*) thus behave similarly in this respect. To test whether the effects of deleting *NHA1* and *PMP3* are additive in this sensitivity test, we constructed strain YR93-31 (*pmr2Δ nha1Δ pmp3Δ*). This strain showed half-maximal inhibition at 125 mM NaCl (Figure 3). The additivity of the NaCl sensitivities conferred by *PMP3* deletion and *PMR2* and/or *NHA1* deletions suggests that Pmp3p does not interfere with either of the two plasma membrane Na^+ efflux transporters and that the NaCl sensitivity phenotype of *pmp3Δ* cells might be correlated with an increase in Na^+ uptake.

Potassium transport associated with *PMP3* deletion

To examine further the role of the *PMP3* deletion in cation uptake, we analysed the effect of deleting *PMP3* in a strain lacking the Trk1p and Trk2p K^+ transporters, and which grows only on media supplemented with high K^+ concentrations (Ko and Gaber, 1991). Growth curves of CY162 (*trk1Δ trk2Δ PMP3*) and CY162-3 (*trk1Δ trk2Δ pmp3Δ*) in YD medium without any added KCl showed that deletion of *PMP3* considerably enhanced the growth of the *trk1Δ trk2Δ* cells (Figure 4A). As a matter of fact, the CY162 (*trk1Δ trk2Δ PMP3*) cells started to grow after a lag phase of ~48 h, the culture eventually reaching a higher cell density than that of CY162-3 (*trk1Δ trk2Δ pmp3Δ*). It is likely that spontaneous suppressor mutations were selected that restored the ability to grow on low potassium medium, as observed previously with glucose and amino

acid transporter mutants (Ko *et al.*, 1993; Wright *et al.*, 1997; Liang *et al.*, 1998; Madrid *et al.*, 1998). Isolated colonies were indeed detected after 3 days when the CY162 strain was plated on YD medium (Figure 4B).

Deletion of *TRK1,2* confers hypersensitivity to low pH, even in the presence of a high concentration of K^+ (Ko and Gaber, 1991). In our assay, the growth defect of the *trk1Δ trk2Δ PMP3* (CY162) was observed at a pH <4.0. Interestingly, deletion of *PMP3* in the *trk1Δ trk2Δ* background (strain CY162-3) restored growth at pH 3.0–4.5 (Figure 4C).

The phenotypes obtained with the *trk1,2Δ* cells could be explained by an increase in K^+ uptake caused by *PMP3* deletion.

Sensitivity to toxic cations and membrane hyperpolarization associated with *PMP3* deletion

Deletion of *PMP3* confers sensitivity to toxic cations other than Na^+ , such as hygromycin B and tetramethylammonium (TMA). Indeed, *pmp3Δ* (CN3) cells failed to grow on media containing 100 $\mu\text{g}/\text{ml}$ hygromycin B or 700 mM TMA, whereas the growth of the *PMP3* (W303-1B) cells was not modified under the same conditions (Figure 5A). The plasma membrane electric potential has been reported to be a major determinant of toxic cation tolerance. It is therefore possible that the *PMP3* deletion causes a modification of the membrane potential, which might increase the rate of cation uptake into the cell and, consequently, the cell's sensitivity to toxic cations. To compare the membrane potential of the W303-1B (*PMP3*) and CN3 (*pmp3Δ*) strains, we measured [^{14}C]methylammonium uptake as an indicator of plasma membrane potential (Vallejo et Serrano, 1989; Mulet *et al.*, 1999). Uptake of methylammonium was increased in CN3 (*pmp3Δ*) when compared with W303-1B (*PMP3*) (Figure 5B), consistent with a membrane potential hyperpolarization in *pmp3Δ* cells.

The Pmp1p and Pmp2p isoproteolipids are required for maximal activity of Pma1p (Navarre *et al.*, 1994). One can therefore imagine that the hyperpolarization observed in the *pmp3Δ* strain might result from an activation of Pma1p. This appears unlikely as deletion of *PMP3* had no effect on Pma1p activity. Purified plasma membranes from CN3 (*pmp3Δ*) or W303-1B (*PMP3*) displayed similar Pma1p-mediated MgATP hydrolysis activity at each MgATP concentration tested (Figure 6A). As a control, plasma membranes were also purified from a *pmp1Δ pmp2Δ pmp3Δ* (CN123) mutant. As previously reported for CN12 (*pmp1Δ pmp2Δ PMP3*) (Navarre *et al.*, 1994), the ATPase activity was decreased 2-fold, in comparison with that of CN3 or W303-1B (Figure 6A). Asolectin vesicles were reconstituted from plasma membranes prepared from W303-1B (*PMP3*) and CN3 (*pmp3Δ*). No difference in MgATP-dependent H^+ pumping was observed between the two strains: proton pumping, expressed as a percentage of ACMA (9-amino-6-chloro-2-methoxy-acridine) quenching, showed comparable dependence on specific ATPase activity (Figure 6B). It seems likely therefore that, in the *pmp3Δ* mutants (CN3), the increasing Na^+ , K^+ and hygromycin B uptake is associated with membrane hyperpolarization through a mechanism that does not require the function of Pma1p. Note, however, the absence of any ACMA quenching by

Fig. 4. Growth assays of *trk1Δ trk2Δ* and *trk1Δ trk2Δ pmp3Δ*. (A) Growth of CY162 (*trk1Δ trk2Δ PMP3*) (closed symbols) and CY162-3 (*trk1Δ trk2Δ pmp3Δ*) (open symbols) in YD medium without any potassium added supplemented with 1 mM EGTA (squares) or with 10 mM CaCl₂ (triangles). (B) Serial 10-fold dilutions of overnight cultures of W303-1B (*TRK1 TRK2 PMP3*), CN3 (*TRK1 TRK2 pmp3Δ*), CY162 (*trk1Δ trk2Δ PMP3*) and CY162-3 (*trk1Δ trk2Δ pmp3Δ*) in YD medium containing 100 mM KCl were spotted onto YD plates without any KCl added. The plates were photographed after a 3 day incubation at 30°C. (C) Strains CY162 (*trk1Δ trk2Δ PMP3*; closed squares) and CY162-3 (*trk1Δ trk2Δ pmp3Δ*; open squares) were grown to saturation in YD medium containing 100 mM KCl. Then, 10 μ l aliquots were transferred to 3 ml of YD containing 100 mM KCl at the indicated pH, and the OD₆₀₀ was measured after 15 h at 30°C. Relative growth is expressed as the ratio of the OD₆₀₀ of a given culture at the indicated pH to the OD₆₀₀ of the control culture at pH 5.5. The experiment was repeated three times with similar results. (D) Serial 10-fold dilutions of overnight cultures of W303-1B (*TRK1 TRK2 PMP3*), CN3 (*TRK1 TRK2 pmp3Δ*), CY162 (*trk1Δ trk2Δ PMP3*) and CY162-3 (*trk1Δ trk2Δ pmp3Δ*) in YD medium containing 100 mM KCl were spotted onto YD plates containing 100 mM KCl at pH 3.0 and 10 mM CaCl₂ when indicated. The plates were photographed after a 2 day incubation at 30°C.

the membrane vesicles of the *pmp3Δ* strain at low MgATP concentrations (<0.1 mM), in comparison with the wild type (Figure 6B).

Calcium reverses the phenotypes of *pmp3*-deleted strains

We tested whether the increased uptake of Na⁺ and K⁺ in *pmp3Δ* mutants is inhibited by divalent cations. In YD medium without any added potassium, the growth of CY162-3 (*trk1Δ trk2Δ pmp3Δ*) was impaired in the presence of 10 mM CaCl₂, similarly to that of CY162 (*trk1Δ trk2Δ PMP3*) in the absence of CaCl₂ (Figure 4A). Similarly, the growth of CY162-3 (*trk1Δ trk2Δ pmp3Δ*) in a pH 3.0 medium containing 100 mM KCl was totally inhibited by 10 mM CaCl₂ (Figure 4D). To investigate how calcium affects the Na⁺ sensitivity caused by the *PMP3* deletion, the Na⁺ sensitivity of YR93 (*pmr2Δ PMP3*) and YR93-3 (*pmr2Δ pmp3Δ*) were compared in the presence of either 10 mM CaCl₂ or 1 mM EGTA. At 10 mM CaCl₂, YR93-3 (*pmr2Δ pmp3Δ*) and YR93

(*pmr2Δ PMP3*) displayed similar sensitivity to NaCl, which is comparable to that seen with YR93 in the absence of added CaCl₂ (Figure 7A). To examine further the effect of divalent cations on the Na⁺ sensitivity of YR93-3 (*pmr2Δ pmp3Δ*), the cells were grown in YD medium containing 0.2 M NaCl and increasing concentrations of CaCl₂ or MgCl₂. The NaCl sensitivity phenotype of YR93-3 (*pmr2Δ pmp3Δ*) decreased continuously with the CaCl₂ concentrations ranging from 0 to 7 mM (Figure 7B). Above this concentration, a plateau is reached (Figure 7B). Mg²⁺ had a lesser protective effect at concentrations up to 10 mM, although millimolar Mg²⁺ concentrations clearly did improve the strain's growth (Figure 7B).

Expression of *Arabidopsis thaliana RCI2A* in yeast

To determine whether the 54 residue plant polypeptides are functional homologues of the yeast Pmp3p, we placed the *A. thaliana RCI2A* cDNA clone under the control of the *PMP3* transcription promoter and introduced it by homologous recombination into the yeast chromosomal

Fig. 5. Effect of *PMP3* deletion on membrane potential indicators. (A) Increased sensitivity to toxic cations of *pmp3*-deleted strains. Serial 10-fold dilutions of saturated cultures of wild-type W303-1B (*PMP3*) and CN3 (*pmp3Δ*) were spotted onto YD plates supplemented with the indicated concentrations of hygromycin B, TMA or H^+ . The plates were photographed after a 2 day incubation at 30°C. (B) Increased methylammonium uptake caused by deletion of *PMP3*. W303-1B (*PMP3*; closed squares) and CN3 (*pmp3Δ*; open squares) cells were grown to exponential phase in MM and washed. The uptake of 2 mM [^{14}C]methylammonium was determined at the times indicated as described in Materials and methods. The experiment was repeated twice with similar results.

PMP3 locus. The resulting yeast strain, YR93-31-RCI2A, expresses the *A.thaliana* *RCI2A* gene instead of the *S.cerevisiae* *PMP3* gene. Na^+ sensitivity of YR93-1 (*pmr2Δ nha1Δ PMP3*), YR93-31 (*pmr2Δ nha1Δ pmp3Δ*) and YR93-31-RCI2A (*pmr2Δ nha1Δ pmp3Δ RCI2A*) cells was tested in liquid medium containing increasing concentrations of NaCl. Expression of the plant *RCI2A* gene in the *pmr2Δ nha1Δ* background conferred the same sodium sensitivity as expression of the yeast *PMP3* gene in the same background (Figure 8A), demonstrating that the yeast Pmp3p and the plant RCI2A are functionally interchangeable with regard to Na^+ tolerance. Moreover, expression of the plant *RCI2A* gene is capable of partially suppressing the hygromycin B sensitivity of the *pmp3* mutant (Figure 8B), suggesting that the plant RCI2A abolishes the hyperpolarization resulting from *PMP3* deletion.

Fig. 6. Deletion of *PMP3* has no effect on plasma membrane H^+ -ATPase activity. (A) ATP dependence of ATPase activity of plasma membranes from W303-1B (*PMP1 PMP2 PMP3*; closed squares), CN3 (*PMP1 PMP2 pmp3Δ*; open squares) and CN123 (*pmp1Δ pmp2Δ pmp3Δ*; open triangles). Each curve represents the average of three independent preparations. (B) ATP-dependent fluorescence quenching of ACMA versus ATP hydrolysis. The initial rate of fluorescence quenching in sealed plasma membranes from W303-1B (*PMP3*; closed squares) and CN3 (*pmp3Δ*; open squares) was determined in the presence of varying MgATP concentrations (from 0.05 to 4 mM) and plotted as a function of the ATP hydrolysis activity measured under the same conditions.

Discussion

Sequence comparison of yeast Pmp3p, a novel and small proteolipid of plasma membrane, shows high homology

Fig. 7. Effect of divalent cations on the NaCl sensitivity due to the *PMP3* deletion. (A) Strains YR93 (*pmr2Δ PMP3*; closed symbols) and YR93-3 (*pmr2Δ pmp3Δ*; open symbols) were grown in YD medium to saturation, then 10 μ l aliquots were transferred to 3 ml of YD medium containing either 1 mM EGTA (circles) or 10 mM CaCl₂ (triangles) supplemented with the indicated concentration of NaCl. After 15 h at 30°C, the OD₆₀₀ of all cultures was measured. Relative growth is the ratio between the OD₆₀₀ obtained for a given NaCl concentration and the OD₆₀₀ of a control culture in YD. The experiment was repeated twice with similar results. (B) Strains YR93 (*pmr2Δ PMP3*; closed symbols) and YR93-3 (*pmr2Δ pmp3Δ*; open symbols) were grown in YD medium to saturation, then 10 μ l aliquots of each culture were transferred to 3 ml of YD medium containing 0.2 M NaCl and either MgCl₂ (squares) or CaCl₂ (triangles) at the indicated concentration. Relative growth is the ratio between the OD₆₀₀ obtained for a given divalent ion concentration and the OD₆₀₀ of the corresponding culture containing 10 mM CaCl₂. The experiment was repeated twice with similar results.

with *Escherichia coli* P77240, with *Caenorhabditis elegans* P34655, Q20516, Q22702, Q22701 and Q22700 putative proteins and with several 54 residue plant polypeptides. All these proteins belong to the upf0057 family (Milton Saier, personal communication) and may share a common function, since at least one of them, the product of the *A.thaliana RC12A* gene, can functionally replace Pmp3p in yeast. In the plant *L.elongatum*, the expression level of *ESI3* mRNA, encoding a Pmp3p homologue, increases 3-fold after only 6 h of treatment with 250 mM NaCl or KCl. Then, it gradually declines to the basal level, even though the plant is kept on a high-salt medium (Galvez *et al.*, 1993). In *A.thaliana*, an increase in *RC12* transcripts is detectable after 12 h of exposure to low temperature (4°C). Maximal induction (up to 10 times the basal level) occurs after 24 h and then begins to decrease (Capel *et al.*, 1997). In barley, the *blt101* transcript level rises after 24 h of low-temperature treatment and peaks

Fig. 8. The *A.thaliana* cDNA T20621 complements the *PMP3* deletion with regard to NaCl and hygromycin B sensitivity. (A) Strains YR93-1 (*pmr2Δ nha1Δ PMP3*; closed circles), YR93-31 (*pmr2Δ nha1Δ pmp3Δ*; open circles) and YR93-31-RC12A (*pmr2Δ nha1Δ pmp3Δ RC12A*; closed triangles) were grown in YD medium to saturation, then 10 μ l aliquots were transferred to 3 ml of YD supplemented with NaCl at the indicated concentration. After 15 h at 30°C, the OD₆₀₀ of all cultures was measured. Relative growth is the ratio of the OD₆₀₀ of a given culture to the OD₆₀₀ of a control culture in YD. The experiment was repeated twice with similar results. (B) Serial 10-fold dilutions of saturated cultures of YR93-1 (*pmr2Δ nha1Δ PMP3*), YR93-31 (*pmr2Δ nha1Δ pmp3Δ*) and YR93-31-RC12A (*pmr2Δ nha1Δ pmp3Δ RC12A*) were spotted onto YD plates supplemented with 100 μ g/ml hygromycin B. The plate was photographed after a 2 day incubation at 30°C.

after 7 days. If the plant is transferred back to the control temperature (20/15°C), the transcript level decreases rapidly to the basal level within 24 h (Goddard *et al.*, 1993; Dunn *et al.*, 1994; Phillips *et al.*, 1997). The rapid but transient rise in the corresponding transcripts under conditions of high salinity or low temperature suggests that the plant homologues of Pmp3p mediate an early minimal protection against sudden stress, before an overall and more permanent response is initiated. Gene replacement of yeast *PMP3* by *A.thaliana RC12A* indicates that the two genes are functionally interchangeable, and therefore share a conserved function. However, we failed to detect an induction of *PMP3* transcription during a 150 min incubation of *pmr2Δ* (YR93) and *pmr2Δ nha1Δ* (YR93-1) cells in the presence of 0.15–0.3 M NaCl (data not shown), probably because the basal level is already very high in non-stressed cells.

In yeast, deletion of *PMP3* increases Na⁺ sensitivity in a strain deficient in the major plasma membrane Na⁺ efflux systems: the Pmr2p/Enap Na⁺-ATPases and Nha1p

Na⁺/H⁺ antiporter. Loss of *PMP3* also partially suppresses the K⁺ requirement of the *trk1Δ trk2Δ* CY162 mutant and reduces its hypersensitivity to low pH. We showed that *pmp3Δ* strains were sensitive to toxic cations such as hygromycin B or TMA. Methylammonium uptake is increased in the *pmp3Δ* cells, consistent with a hyperpolarization of the plasma membrane potential. Taken together, these results indicate that the absence of Pmp3p facilitates a non-specific monovalent cation uptake through an increase in the membrane potential, which is mediated by an uptake system different from Trk1,2p.

The plasma membrane potential is a major determinant of toxic cation tolerance. Mutations in the electrogenic H⁺-ATPase, Pma1p, lead to increased Na⁺ resistance, partly because of the reduced Na⁺ uptake linked to a reduction in the membrane potential (Nass *et al.*, 1997; Withee *et al.*, 1998). *pma1* mutants having reduced Pma1p activity exhibit increased tolerance to hygromycin B (McCusker *et al.*, 1987), as a consequence of their membrane potential defect (Perlin *et al.*, 1988). The membrane hyperpolarization seen in *pmp3* mutants could not be explained by an up-regulated plasma membrane H⁺-ATPase activity. Deletion of *PMP3* indeed has no effect on ATP hydrolysis, ATP-dependent H⁺ pumping in asolectin vesicles reconstituted from plasma membranes and medium acidification by intact cells (data not shown).

In addition to the plasma membrane H⁺-ATPase, inactivation of Trk1p and Trk2p has been reported to affect membrane hyperpolarization (Madrid *et al.*, 1998), resulting in increased sensitivity not only to Na⁺ and H⁺, but also to hygromycin B and TMA (Madrid *et al.*, 1998; Mulet *et al.*, 1999). Deletion of *TRK1,2* (Madrid *et al.*, 1998) or *HAL4,5*, genes for two isokinases regulating the K⁺ transporters (Mulet *et al.*, 1999), results in membrane hyperpolarization that would subsequently activate low-affinity cation uptake pathways (Madrid *et al.*, 1998; Mulet *et al.*, 1999), including several permeases involved in glucose, amino acid, inositol or choline uptake. This hypothesis is supported by the ability of mutated permeases to suppress the phenotype of *trk1Δ trk2Δ* mutants on K⁺-limiting medium (Ko *et al.*, 1993; Wright *et al.*, 1997; Liang *et al.*, 1998; Madrid *et al.*, 1998). However, our results indicate that the membrane potential defect in the *pmp3* mutants is independent of the Trk1,2p K⁺ transporter activity, since deletion of *PMP3* and *TRK1,2* had additive phenotypes.

Patch-clamp experiments on *trk1Δ trk2Δ* spheroplasts have confirmed the existence of a low-affinity inward K⁺ current that is sensitive to divalent cations (Bihler *et al.*, 1998; Roberts *et al.*, 1999). This current, named NSC1, is detected at extracellular Ca²⁺ concentrations <0.1 mM. Interestingly, extracellular K⁺ could be replaced by Na⁺ with only a slight modification of the magnitude of the Ca²⁺-sensitive low-affinity current (Roberts *et al.*, 1999), suggesting that the uptake systems apparently can also take up Na⁺. The presumptive monovalent cation transport system that is triggered through the membrane hyperpolarization caused by the loss of Pmp3p shares several properties with the NSC1 system, as millimolar concentrations of Ca²⁺ suppress the increased Na⁺ and K⁺ uptake observed in Pmp3p-deficient strains. Alternatively, it is possible that high Ca²⁺ concentrations act independently

of the NSC1 system by dissipating the membrane hyperpolarization mediated by *PMP3* deletion.

The *PMP3* deletion suppresses the growth defect of the *trk1Δ trk2Δ* mutant at low pH, although the *pmp3Δ* deletion alone has no effect on cell tolerance to acidic pH (Figure 5A), despite the hyperpolarized state of the *pmp3Δ* cells. This observation could be explained if *trk1Δ trk2Δ pmp3Δ* CY162-3 show decreased proton permeability in comparison with *trk1Δ trk2Δ PMP3* CY162 membranes. Preliminary measurements of intracellular pH after the cells were incubated for 3 h at pH 3.0 showed increased acidification of CY162 cells' interior when compared with CY162-3 cells (data not shown), as if the deletion of *PMP3* prevents acidification of the cell's interior under conditions of low external pH.

In conclusion, the loss of Pmp3p induces a hyperpolarization of the membrane potential. It is possible that Pmp3p activates a depolarizing cation leak (different from Trk1,2p) or mediates the cation leak itself. The growth of *trk1Δ trk2Δ pmp3Δ* cells on acidic medium is consistent with a decrease of plasma membrane proton permeability, suggesting that Pmp3p possibly could mediate a proton leak. If Pmp3p encodes a proton leak, the similarity between the growth of W303-1B and CN3 in acidic medium probably results from the non-hyperpolarized state of wild-type cells that correlates with a lesser sensitivity to low pH. Further analysis of the proton permeability of *pmp3* mutants will be necessary to understand the contribution of Pmp3p in the regulation of membrane potential, a conserved function that could be of crucial importance in halotolerance.

Materials and methods

Media and growth conditions

Yeast strains were grown at 30°C either in rich medium (YD) containing 2% glucose and 2% yeast extract (KAT) or in minimal medium (MM) containing 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco) and the required amino acids. Solid media additionally contained 2% agar (Sigma). NaCl, KCl, CaCl₂ and tetramethylammonium chloride were added as indicated. Hygromycin B was added to the medium after autoclaving. Solid YD medium at pH 3.0 or 2.2 was prepared by mixing a 2-fold concentrated YD medium adjusted to the desired pH with HCl to a concentrated agar solution after autoclaving them separately. The ability of yeast strains to grow in various liquid conditions was tested by adding equal amounts of cells from overnight cultures in YD to 3 ml of YD supplemented with 100 mM KCl adjusted to the desired pH with HCl or with various amounts of NaCl in the presence of EGTA, CaCl₂ or MgCl₂, as indicated. After 15 h at 30°C, the OD₆₀₀ was measured for each culture. The relative growth of a strain in a given medium was expressed as the ratio of the OD₆₀₀ obtained with this strain to the OD₆₀₀ of a control culture.

Isolation and sequencing of the *PMP3* gene

A 60 ng aliquot of genomic DNA was amplified using a degenerate 23mer oligonucleotide corresponding to the N-terminal amino acid sequence Met-Asp-Ser-Ala-Lys-Ile-Ile-Asn of Pmp3p and the oligonucleotide 5'-GAGGGTGACCCGAACATGTTGATACCG-3', found in the 3'-non-coding region of the EST 101712 clone. The amplification reaction was carried out in the presence of 100 pmol of each oligonucleotide primer, 100 μM dATP, 100 μM dGTP, 100 μM dTTP, 20 μM dCTP and 8 μl of [α-³²P]dCTP for 25 cycles (denaturation at 93°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 3 min). The radiolabelled PCR product was used as a probe to screen a library (2 × 10⁴ clones) by DNA hybridization. The library consists of *EcoRI* fragments of DNA from W303-1B that were ligated into pTZ19U. Positive clones containing *PMP3* in a 2 kb *EcoRI* fragment were subcloned and sequenced.

Yeast strains

The yeast strains used in this study are listed in Table I. They are derived from the haploid strains W303-1B (Wallis *et al.*, 1989), YR93 (Wieland *et al.*, 1995) and CY162 (Ko and Gaber, 1991).

The promoter and 3' end regions of *PMP3* were amplified by PCR and cloned into the pTZ vector as *HindIII*–*BamHI* and *BamHI*–*EcoRI* fragments, respectively. The resulting plasmid pTZ::PMP3PROM–PMP3STOP was then cut with *BamHI* and ligated to a *BglII* fragment containing either the *TRP1* or the *URA3* gene, respectively, from the pFL39 or pFL34 vector (François Lacroute, Gif-sur-Yvette, France). The promoter and 3' end regions of *NHA1* were amplified by PCR on genomic DNA and cloned, respectively, into the *EcoRI*–*BamHI* and *BamHI*–*XbaI* sites of the pSK vector. A *BamHI* fragment containing the *LEU2* gene was isolated from the YDpL vector (Berben *et al.*, 1991) and cloned into the *BamHI* site of the resulting plasmid. The plasmids containing the null allele of *PMP3* and *NHA1* were restricted with *EcoRI*–*KpnI* and *EcoRI*–*XbaI*, respectively, and the generated DNA was transformed into yeast cells. Gene deletion was checked by PCR screening as described by Decottignies *et al.* (1998).

RC12A DNA was prepared as follows. The 0.4 kb *A.thaliana* cDNA clone 88O1477 (a kind gift from the Arabidopsis Biological Resource Center) was cut with *HindIII* and *EcoRI*. The resulting 369 bp fragment starts 41 bp upstream from the initiation ATG and extends 183 bp beyond the stop codon of the *RC12A* ORF. This fragment was blunted with Klenow and cloned into the *BamHI* site (blunted with Klenow) of pTZ::PMP3PROM–PMP3STOP. Correct integration of *RC12A* was checked by *SspI*–*EcoRV* restriction mapping. The resulting pTZ::PMP3PROM–*RC12A*–PMP3STOP plasmid was cut with *EcoRI* to generate a 2.3 kb fragment. This fragment was used to transform YR93-31, where *PMP3* is disrupted by the *URA3* marker. Loss of the *URA3* marker was monitored by plating one-tenth of an overnight culture in YD on solid YD medium containing 1 g/l of 5-fluoroorotic acid. The resistant cells were screened by PCR to make sure that *RC12A* had replaced the *URA3* marker at the chromosomal *PMP3* locus.

Measurement of the intracellular sodium concentration

Yeast were grown in rich medium (2% yeast extract KAT, 2% glucose) and harvested in the exponential growth phase (final OD₆₀₀ = 0.8). Cells (0.5 mg/ml) were incubated for 1 h at 4°C in H₂O and transferred to a solution containing 10 mM MES buffer pH 6.0, 50 mM glucose and 1 M NaCl. After incubation at 28°C, 50 mg of cells were harvested by centrifugation for 3 min at 8000 g, resuspended twice in a cold solution containing 20 mM MgCl₂ and 1.5 M sorbitol, centrifuged as described above and resuspended in 2 mM CTAB (cetyltrimethylammonium bromide). After 10 min at room temperature, the supernatant was diluted 10 times in H₂O and analysed for Na with an atomic emission spectrometer (ICP model JY48, Jobin Yvon).

Measurement of methylammonium uptake

Cells were grown in MM at low cell density (final OD₆₀₀ = 0.8), washed twice, resuspended with water and stored on ice. For uptake measurements, the cells were diluted to a final OD₆₀₀ of 5 in a reaction medium containing 50 mM glucose and 10 mM MES adjusted to pH 6.0 with KOH. After a 5 min incubation, [¹⁴C]methylamine hydrochloride (2.0 mM and 2.5 µCi/ml final concentration) was added. At the indicated times, 100 µl samples were withdrawn and diluted into 10 ml of ice-cold 20 mM MgCl₂ to stop the transport reaction, filtrated onto a 0.45 µm nitrocellulose filter (Millipore HA) and washed three times with 10 ml of the same solution. Moist filters were transferred to a scintillation cocktail and the radioactivity was measured using a Beckman LS 1701 liquid scintillation counter.

Other methods

Plasma membranes were prepared as described by de Kerchove d'Exaerde *et al.* (1995) and the proteolipid fraction was extracted with chloroform–methanol as described by Navarre *et al.* (1992). The amino acid sequence of Pmp3p was determined with an Applied Biosystems sequenator (model 477A) equipped with an on-line phenylthiohydantoin derivative analyser (model 120A). The protein content was measured as described by Lowry *et al.* (1951) with bovine serum albumin as the standard. The protein samples were electrophoresed on Tricine gels (Schägger and von Jagow, 1987) and silver stained. ATPase assays were performed at pH 6.0, using a free Mg²⁺ concentration of 1 mM and an ATP-regenerating system (1 mM phosphoenolpyruvate and 200 µg/ml pyruvate kinase) (Supply *et al.*, 1993). The ATP concentration was varied from 0.15 to 5.2 mM. The preparation of sealed plasma membranes and the measurement of ATP-dependent transport were performed as

described by Morsomme *et al.* (1996), except that the pH was adjusted to 6.0 for vesicle reconstitution and ACMA quenching measurements.

Accession number

PMP3 sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession No. X91499.

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