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The *PMR2* gene cluster encodes functionally distinct isoforms of a putative Na^+ pump in the yeast plasma membrane

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We report a structural and functional analysis of the *PMR2* gene cluster in yeast. We found that several strains of *Saccharomyces cerevisiae* contain multiple *PMR2* genes repeated in tandem, whereas most phylogenetically related yeasts appear to possess only a single *PMR2* gene. This unusual tandem array of nearly identical genes encodes putative ion pumps involved in Na^+ tolerance. Pmr2a and Pmr2b, the proteins encoded by the first two repeats, differ by only 13 amino acid exchanges. Both proteins share localization to the plasma membrane, but represent distinct isoforms of a putative Na^+ pump. When expressed under identical conditions *in vivo*, Pmr2a and Pmr2b cause different tolerances to Na^+ and Li^+. Finally, we show that the Na^+ tolerance mediated through these pumps is regulated by calmodulin via a calcineurin-independent mechanism which activates the Pmr2 ion pumps post-transcriptionally.

Keywords: calcineurin/calmodulin/P-type ATPase/salt tolerance/sodium transport

Introduction

All forms of life have evolved mechanisms to achieve and maintain cellular ion homeostasis. Primary ion pumps transport ions across membranes and generate ion gradients from the energy of ATP, redox reactions or light (Skou, 1957; Jagendorf and Uribe, 1966; Mitchell, 1966). Antiports and symports utilize these ion gradients for uphill transport of a second ion or solute (Mitchell, 1973). Finally, ion channels mediate the passive flow of ions down the electrochemical gradient (Lazdunski *et al.*, 1979; Urry, 1979). The controlled flux of ions along these routes enables cells to adjust ion concentrations in any subcompartment and to regulate the membrane potential at each cellular membrane. A critical challenge for all cells is to maintain a low intracellular Na^+ concentration in the face of a large gradient across the plasma membrane. Eukaryotic cells make use of a wide range of strategies in order to couple cell metabolism to Na^+ extrusion.

In the plasma membrane of animal cells (Na^+, K^+)-ATPase (Skou, 1957) and cation exchangers are coordinately regulated to maintain pH and characteristic intracellular ion concentrations. In fungi and plants a H^+-ATPase generates the membrane potential and drives down extracellular pH in order to promote secondary transport coupled to H^+ (reviewed in Serrano, 1985). In *Schizosaccharomyces pombe* a Na^+/H^+ antiport encoded by *sod2* is responsible for Na^+ extrusion at the plasma membrane (Jia *et al.*, 1992) and mediates tolerance to Na^+ and Li^+, a toxic analog of Na^+ (Fleishman, 1991). Transport of Na^+ at the plasma membrane of *Saccharomyces cerevisiae* may occur through a similar antiport, but a specific protein or gene has not been identified.

In a search for new ion pumps in budding yeast, the *PMR2* locus (Rudolph *et al.*, 1989) was originally identified in a screen for genes related to *PMA1*, the gene for the plasma membrane H^+-ATPase (Serrano *et al.*, 1986). This H^+ pump contains several sequence motifs characteristic of a family of highly conserved ion pumps present in both prokaryotes and eukaryotes, the so-called P-type ion pumps (reviewed in Pedersen and Carafoli, 1987a,b). P-type ATPases couple hydrolysis of ATP to transport of cations against electrochemical gradients and form a characteristic phosphoenzyme intermediate. These pumps, closely regulated and specific for different cations, comprise the major determinants of ion gradients across the plasma membrane and across many intracellular membranes. All P-type ATPases share several highly conserved functional regions and possess similar predicted transmembrane topologies.

Post-translational regulation of P-type ATPases has been described in several systems. In some cases an auto-inhibitory domain at the C-terminus limits the activity of the pump. This auto-inhibitory domain may be an important target for cell signaling to modulate ATPase activity. Human plasma membrane Ca^{2+}-ATPase is activated by binding of calmodulin to sequester the C-terminal auto-inhibitory domain (James *et al.*, 1988). Exchange of the C-terminal domains confers this calmodulin-dependent regulation on the (Na^+,K^+)-ATPase (Ishii and Takeyasu, 1995). In yeast, activation of the H^+-ATPase in media with glucose occurs concomitantly with a rapid phosphorylation of the pump (Chang and Slayman, 1991). The target of this regulation may well be the extreme C-terminal residues of Pma1; truncations and point mutations at the C-terminus lead to a constitutive, fully active enzyme, independent of glucose activation (Portillo *et al.*, 1989, 1991).

The *PMR2* locus, independently isolated and called *ENA* by Haro *et al.* (1991), consists of an unusual tandem array of nearly identical genes (termed *PMR2A*, *PMR2B*, etc.) which encode large predicted polypeptides with the signature features of ion-transporting P-type ATPases. Remarkably, DNA sequencing revealed almost complete identity between the open reading frames (ORFs) of the first two repeat units: the deduced proteins Pmr2a and Pmr2b are of identical length and exhibit only 13

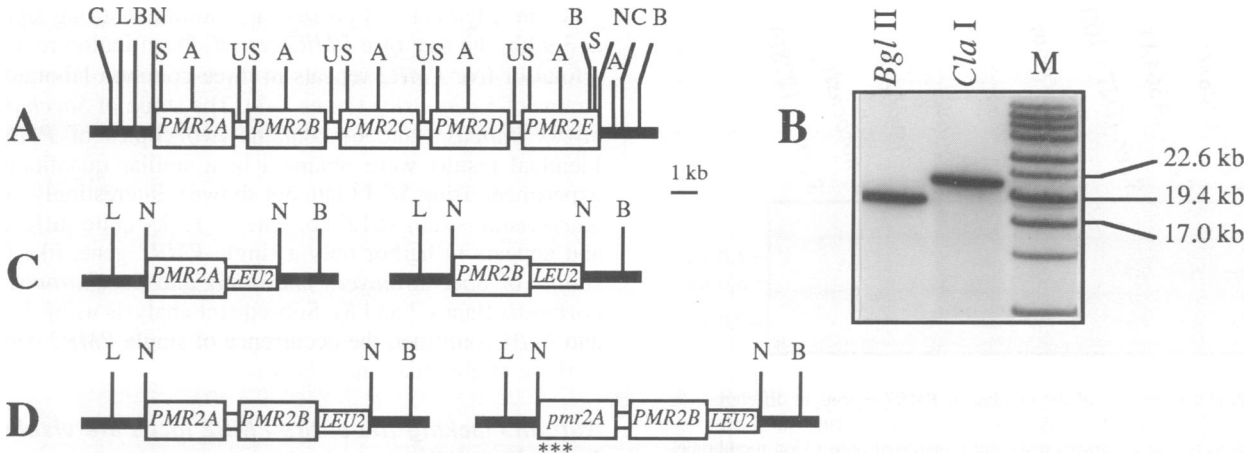

Fig. 1. The *PMR2* locus in strains used for this study. (**A**) Restriction map of wild-type strain S288C with five repeats, *PMR2A–PMR2E*. Large boxes mark ORFs, small boxes indicate internal transcriptional control regions. Restriction sites are: A, *Acc*I; B, *Bgl*II; C, *Cla*I; L, *Sal*I; N, *Sna*BI; S, *Sac*I; U, *Mlu*I. (**B**) Southern blot of genomic DNA from S288C cut with *Bgl*II or *Cla*I and probed with ^{32}P-labeled *PMR2* DNA (see Materials and methods). M, high molecular weight marker (Gibco BRL). (**C**) Strains YW13 (left) and YW15 (right) harbor the alleles *PMR2A* and P_{PMR2A}::*PMR2B* respectively and possess the same authentic *PMR2A* promoter region at the *PMR2* locus. (**D**) Strain YW17 (left) harbors an authentic *PMR2A*::*PMR2B* tandem. In strain YW19 (right) a 4 bp deletion was introduced into *PMR2A* to produce allele *pmr2A-1*::*PMR2B*. Due to the frameshift, three stop codons (marked *) block *PMR2A* translation 81 bp downstream of the ATG. From all strains shown in (C) and (D) epitope-tagged derivatives were constructed to monitor expression of specific gene products (see Table II, Materials and methods).

exchanges within 1091 amino acids (Rudolph and Fink, 1990; Garciadeblas *et al.*, 1993). Studies on whole cells loaded with Na^+ or Li^+ show that the putative pumps are involved in efflux of Na^+ and Li^+ and mediate tolerance to these ions (Garciadeblas *et al.*, 1993). Mutants lacking *PMR2A* are supersensitive to these ions. Using reporter gene fusions to the *PMR2A* promoter region, Garciadeblas and colleagues observed significant induction of *PMR2A* expression in cells adapting to high Na^+ or a high media pH. Differential gene expression seems to account for the large differences observed between *PMR2A* and *PMR2B* in these studies. *PMR2B* is poorly expressed, but functions well when fused to the *PMR2A* promoter. Full induction of *PMR2A* by Na^+ or high pH depends upon function of the phosphatase calcineurin (Mendoza *et al.*, 1994).

Calcineurin, a protein phosphatase which requires Ca^{2+} and calmodulin for full activity (Klee *et al.*, 1988; Stemmer and Klee, 1994), is a key component in the T cell activation pathway that couples plasma membrane-associated events with transcription in the nucleus (Liu *et al.*, 1991a; Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). Heterodimeric calcineurin consists of a catalytic subunit (CN-A) and a regulatory subunit (CN-B). Up to four Ca^{2+} ions bind to CN-B and stimulate phosphatase activity of the A–B complex, which is essentially inactive in the absence of Ca^{2+}. Additional binding of Ca^{2+}/calmodulin to CN-A results in a fully active trimeric enzyme. In yeast, though not essential for vegetative growth, calcineurin is important for recovery from pheromone arrest (Cyert *et al.*, 1991), adaptation to high salt stress conditions (Nakamura *et al.*, 1993; Mendoza *et al.*, 1994) and the regulation of Ca^{2+} homeostasis (Cunningham and Fink, 1994). Two genes (*CNA1*, *CNA2*) have been identified (Cyert *et al.*, 1991; Liu *et al.*, 1991b) for the catalytic subunit. A single gene (*CNB1*) encodes the regulatory subunit (Kuno *et al.*, 1991; Cyert and Thorner, 1992). Calmodulin is encoded by *CMD1* (Davis *et al.*, 1986). Yeast calmodulin possesses only three functional EF hands to bind Ca^{2+} with high affinity. Mutations in yeast calmodulin can eliminate all Ca^{2+} binding without compromising vegetative growth. However, the calmodulin protein is essential; it appears to have diverse essential functions for which calcium binding seems not to be required (Geiser *et al.*, 1991, 1993; Ohya and Botstein, 1994).

Here we have analyzed the *PMR2* locus in several strains of *S.cerevisiae* and phylogenetically related yeasts. Most *S.cerevisiae* strains harbor multiple *PMR2* genes repeated in tandem, while most other yeast genera tested seem to possess only a single copy of *PMR2*. We show that Pmr2a and Pmr2b, the proteins encoded by the first two repeats, are isoforms of a putative Na^+ pump. In addition to their near sequence identity, both proteins share localization to the plasma membrane. Nonetheless, the two pumps have distinct ion specificities and thereby mediate distinct degrees of tolerance to Na^+ and Li^+ challenge. Furthermore, we show that in addition to its role in activating calcineurin-dependent induction of *PMR2* transcription, calmodulin participates in a calcineurin-independent regulation of Na^+ tolerance which involves post-transcriptional activation of the Pmr2 ion pumps.

Results

Most strains of S.cerevisiae contain multiple PMR2 repeats

Figure 1A outlines the *PMR2* locus of *S.cerevisiae* strain S288C as deduced from DNA sequencing, extensive restriction mapping of cloned DNA and analysis of genomic DNA by Southern blots. For this strain a total number of five chromosomal *PMR2* repeats was determined by the following experiment. First, we measured the size of the single, very large fragments encompassing the complete *PMR2* locus released from the chromosome by digestion of S288C genomic DNA with the restriction enzymes *Bgl*II or *Cla*I. These fragments were measured by Southern analysis to be ~19 and 21 kb respectively (Figure 1B). Then the same enzymes were used to cut

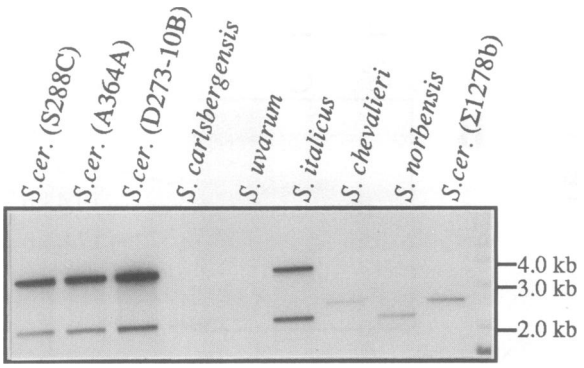

Fig. 2. Determination of the number of *PMR2* repeats in different yeast strains. Genomic DNA from each strain was cut with *Acc*I, separated by gel electrophoresis and transferred onto nylon membrane. After hybridization with a ^{32}P-labeled *PMR2A* probe (*Acc*I–*Mlu*I fragment) the radioactivity present in the fragments at 3.9 and 2.3 kb was quantitated using an imaging plate scanner (Fuji). For the strains indicated each value obtained for the 3.9 kb signal was divided by the value obtained from the 2.3 kb signal to produce the following numbers: S288C, set to 4.00 as internal standard; A364A, 3.99, 4.18; D273-10B, 3.86, 4.16; *S.italicus*, 1.35, 1.08. The second numbers given result from a similar quantitative analysis using *Sac*I-digested DNAs.

genomic DNA from a S288C derivative harboring a *pmr2-1*::*URA3* deletion/substitution allele. In this allele 1.1 kb of *URA3* sequences replace the entire region between the first *Sac*I site in *PMR2A* and the second *Sac*I site within *PMR2E*. Digestion of genomic DNA from this strain with *Bgl*II or *Cla*I released a single, very short *pmr2-1*::*URA3* fragment. The fragment lengths were estimated on Southern analysis to be 4.2 and 4.5 kb respectively (data not shown), revealing the total length of sequences flanking the *URA3* insertion in *pmr2-1*::*URA3*. Precise positions of *Sac*I and *Bgl*II sites within *PMR2A* and *PMR2E* coding sequences, as well as the length of a *PMR2* repeat unit, 3.89 kb, are all known from DNA sequencing (Rudolph *et al.*, 1989; Martinez *et al.* 1991, Garciadeblas *et al.*, 1993). With this information and the size determined for the flanking regions, we calculated that the theoretical length of the *Bgl*II and *Cla*I fragments for a strain with five *PMR2* repeats would be 19.2 and 21.6 kb. These results are in excellent agreement with the experimental values of 19 and 21 kb. We conclude that the *PMR2* locus in strain S288C is a tandem array of five *PMR2* repeats.

Next, we examined the *PMR2* tandem array in several laboratory strains of *S.cerevisiae* and phylogenetically related yeasts. In Southern blots two of the nine strains (*Saccharomyces carlsbergensis* and *Saccharomyces uvarum*) tested produced very weak hybridization signals with a *PMR2* probe and were excluded from further analysis. All other strains showed at least with some enzymes the *PMR2–KRS1* 3′-junction fragments predicted from the DNA sequence (Martinez *et al.*, 1991) and allowed us to determine the number of *PMR2* repeats. Genomic DNAs were digested with *Acc*I or *Sac*I, which cut once in every repeat, thereby producing multiple repeat length fragments plus two unique junction fragments. A *PMR2* probe annealing equally well to a unit length fragment and to one junction yielded two hybridization signals suitable for quantitative comparison. Figure 2 presents this analysis performed with *Acc*I. Four strains (lanes 1–3 and lane 6) show the expected 2.3 kb 3′-junction fragment and possess an additional strong signal at 3.9 kb, the size of a *PMR2* repeat. Quantitation reveals a total of five *PMR2* repeats in three common laboratory strains of *S.cerevisiae* (lanes 1–3). The strain of *Saccharomyces italicus* (lane 6) contains two copies of *PMR2*. Identical results were obtained in a similar quantitation experiment using *Sac*I (data not shown). Interestingly, one *S.cerevisiae* strain (Σ1278b, lane 9) looks quite different and appears to harbor only a single *PMR2* gene, like the strains of *Saccharomyces chevalieri* and *Saccharomyces norbensis* (lanes 7 and 8). Subsequent analysis using *Kpn*I and *Bgl*II confirmed the occurrence of single *PMR2* genes in these strains (data not shown).

Mutants lacking the entire PMR2 locus are viable, but salt-sensitive

Using plasmids p202-1 and p2-48 harboring the known 5′- and 3′-flanking regions of the *PMR2* locus (see Materials and methods), we constructed several *pmr2* mutant alleles which lack the entire *PMR2* locus. First, a null allele (*pmr2-1*::*URA3*) was constructed using the *URA3* gene to replace all *PMR2* DNA between the two *Sac*I sites located 374 bp downstream of the *PMR2A* translational start and 110 bp upstream of the *PMR2E* translational stop. One of the *PMR2* alleles in a wild-type diploid was replaced with *pmr2-1*::*URA3* by integrative transformation. Tetrads derived from such heterozygous transformants exhibited normal spore viability on YPD medium. Furthermore, the *pmr2-1*::*URA3* allele segrated 2:2, demonstrating that loss of the entire *PMR2* locus does not lead to lethality (data not shown). A second null allele (*pmr2-4*::*HIS3*) was constructed in which *HIS3* DNA replaces the entire *PMR2* coding region (*Sna*BI–*Sna*BI fragment starting 55 bp upstream of the *PMR2A* ORF and ending 58 bp downstream of the *PMR2E* translational stop). As haploids harboring *pmr2-1*::*URA3* (YR86) or *pmr2-4*::*HIS3* (YW11) were viable, we subsequently used direct transformation of haploid strains to generate congenic strains for further comparisons.

All *pmr2* null mutants grew poorly in media containing high concentrations of NaCl. In comparitive growth assays *pmr2-4*::*HIS3* cells (YW11) showed half-maximal inhibition at NaCl concentrations of ~100 mM (Figure 3, top), whereas wild-type cells (AA308) tolerated ~350 mM NaCl under the same conditions. Growth of *pmr2-4*::*HIS3* and *PMR2* strains in the presence of KCl was indistinguishable. Similarly, changing the osmolarity of the medium by the addition of sucrose had no effect (Figure 3, bottom). These data demonstrate that the *PMR2* gene products are not involved in osmotolerance, but rather play a specific role in Na^+ tolerance.

Using Northern blot analysis, we tested *PMR2* mRNA induction by Na^+ in three *S.cerevisae* strains and a *S.italicus* strain. All four strains show a single band of *PMR2*-specific mRNA migrating with the expected size of ~3500 nt (Figure 4B). In all cases the level of *PMR2* mRNA could be induced by the addition of NaCl to the medium. In fact, strain Σ1278b (Figure 4B, lanes 7 and 8), harboring only a single *PMR2* repeat, exhibits a >10-fold induction. To find strong induction of *PMR2* mRNA even in a strain with a single *PMR2* gene suggests that the transcriptional response of the *PMR2* cluster to Na^+ is primarily produced by the unique 5′-region of *PMR2A*.

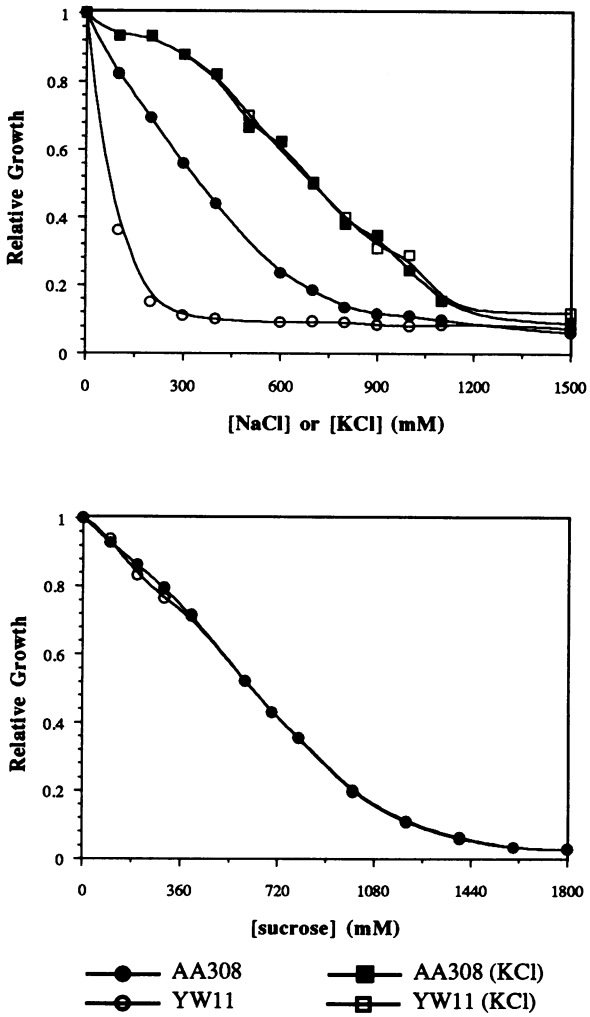

Fig. 3. *pmr2* null mutants show a specific defect in Na^+ tolerance. Equal amounts of wild-type (AA308) or *pmr2-4::HIS3* cells (YW11) pre-grown overnight in YPD (pH 5.5) were inoculated into YPD (pH 6.85, KOH) containing various amounts of NaCl, LiCl, KCl or sucrose as indicated. After 10 h at 30°C, OD_{578} of all cultures was measured. Relative growth was calculated as the ratio between the OD_{578} obtained for a given culture and the OD_{578} of a control culture in YPD (pH 5.5).

Interestingly, the Na^+ tolerance of these strains in YPD medium does not correlate with *PMR2* mRNA induction; the Σ1278b strain showed NaCl sensitivity similar to a *pmr2* null mutant of S288C. The occurrence of a canonical Ste12 binding site motif (reviewed in Sprague and Thorner, 1992) in the *PMR2B* promoter region, ATGAAACA at positions –397 to –390 relative to the ATG, prompted us to test whether *PMR2* mRNA was inducible by α-factor. However, poly(A)$^+$ RNA samples isolated from S288C cells before and after pheromone treatment contained essentially the same amount of *PMR2*-specific mRNA (Figure 4A). Induction of *FUS1* mRNA (Truehart *et al.*, 1987) served as a control to monitor pheromone response (data not shown).

Calmodulin enhances PMR2 function by a calcineurin-independent mechanism

Work from different groups has identified the protein phosphatase calcineurin, which requires Ca^{2+} and calmodulin (*CMD1*, Davis *et al.*, 1986) for full activity, as an

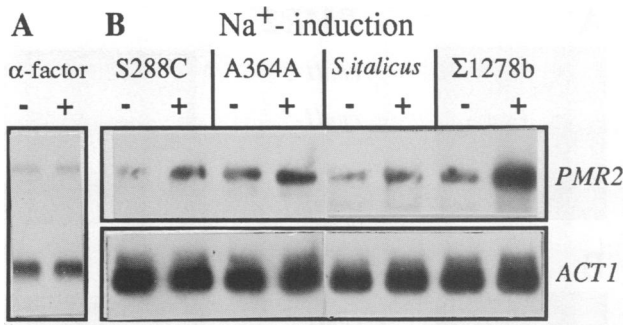

Fig. 4. Northern analysis of poly(A)$^+$ RNA isolated from various strains after treatment with α-factor or NaCl. RNA samples were run in agarose–formaldehyde gels, transferred to nylon membranes and hybridized to ^{32}P-labeled *PMR2* and *ACT1* probes. *ACT1* mRNA served as an internal loading control. (**A**) Poly(A)$^+$ RNA isolated from logarithmically grown S288C cells (YPD, 30°C, OD_{600} = 0.62) incubated further (2.5 h) without (–) or after addition (+) of α-factor (5 μM final). (**B**) Poly(A)$^+$ RNA isolated from logarithmically grown *S.cerevisiae* strains S288C, A364A and Σ1278b and *S.italicus* cells (YPD, pH 5.5, 30°C, OD_{600} ~0.8) incubated further (2 h) without (–) or after addition (+) of NaCl (400 mM).

important regulator of salt tolerance in yeast. Calcineurin mutants are sensitive to Na^+ or Li^+ (Nakamura *et al.*, 1993) and exhibit decreased induction of *PMR2* by salt-containing media (Mendoza *et al.*, 1994). To examine the role of calmodulin in Na^+ tolerance, we used various *cmd1* strains constructed by T.Davis and co-workers. In one mutant (*cmd1-1*), two amino acid exchanges are located in the third EF hand of calmodulin (Davis, 1992). A triple mutant (*cmd1-5*) contains a single exchange in each Ca^{2+} binding loop. Binding of Ca^{2+} to this mutant calmodulin is reduced ~100-fold (Geiser *et al.*, 1991). Finally, a sextuple mutant (*cmd1-3*) carries two exchanges in each EF hand and lacks any detectable Ca^{2+} binding. Cells containing any of these *cmd1* alleles as their sole source of calmodulin are viable in normal media at room temperature. Both *cmd1-1* and *cmd1-3* confer temperature sensitivity to mutant strains, while *cmd1-5* is viable at 37°C. As shown in Figure 5A, all three mutant calmodulins confer Na^+ sensitivity which increases with the number of mutations present in the Ca^{2+} binding loops.

Next, we wanted to determine the relationship between calmodulin and calcineurin in controlling *PMR2* expression. If the sole function of calmodulin in Na^+ tolerance is to control *PMR2* expression through calcineurin (*CNB1*), *cmd1* mutations should have no effect on Na^+ tolerance in *cnb1* mutants. This simple prediction, however, is not supported by the data presented in Figure 5. First, *cnb1* single mutants are more Na^+ tolerant than *cnb1 cmd1-1*, *cnb1 cmd1-5* or *cnb1 cmd1-3* double mutants (Figure 5C). This suggests that *CMD1* plays a role in Na^+ tolerance that is independent of calcineurin. Moreover, the mutations in calmodulin appear to have different effects on Na^+ tolerance when calcineurin is functional (Figure 5A), but these differences disappear upon introduction of the *cnb1* null mutation. Therefore, calmodulin seems to have calcineurin-independent and calcineurin-dependent functions in Na^+ tolerance.

To resolve the complex functions of calmodulin in adaptation to salt stress, we decided to disrupt calcineurin regulation of *PMR2* transcription by exchanging the *PMR2A* promoter with the *PMA1* promoter. Constitutive

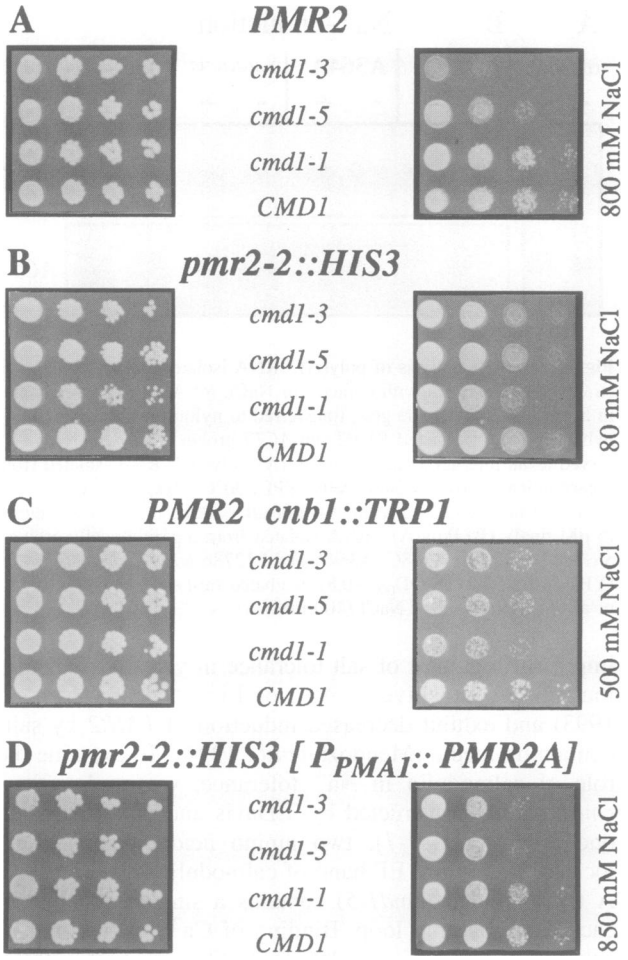

Fig. 5. Regulation of Na^+ tolerance by calmodulin includes a calcineurin-independent mechanism. Serial 10-fold (A, B and D) or 5-fold (C) dilutions of saturated cultures were spotted onto YPD (pH 7) plates without (left) or supplemented with (right) NaCl as indicated. The plates were photographed after 3 days incubation at 22°C. (**A**) Mutations in the Ca^{2+} binding sites of calmodulin reduce Na^+ tolerance of *PMR2* strains. Strains: CRY1 *CMD1*, JGY44-2A *cmd1-1*, JGY148 *cmd1-5* and JGY41 *cmd1-3*. (**B**) Mutations in the Ca^{2+} binding sites of calmodulin do not affect Na^+ tolerance of *pmr2* strains. Strains: YN10 *CMD1 pmr2-2::HIS3*, YN11 *cmd1-1 pmr2-2::HIS3*, YN12 *cmd1-5 pmr2-2::HIS3* and YN13 *cmd1-3 pmr2-2::HIS3*. (**C**) Mutations in the Ca^{2+} binding sites of calmodulin reduce Na^+ tolerance of calcineurin (*cnb1::TRP1*) mutants. Strains: YN20 *CMD1 cnb1::TRP1*, YN23 *cmd1-1 cbn1::TRP1*, YN24 *cmd1-5 cnb1::TRP1* and YN26 *cmd1-3 cnb1::TRP1*. (**D**) Mutations in the Ca^{2+} binding sites of calmodulin reduce Na^+ tolerance of *pmr2-2::HIS3* strains expressing *PMR2A* via the constitutive *PMA1* promoter on plasmid pJW11. Strains: YR552, YR553, YR554 and YR555.

expression of *PMR2* from a heterologous promoter should reveal whether calmodulin modulates *PMR2* by a post-transcriptional mechanism. Indeed, P_{PMA1}::*PMR2A* expression in *CMD1 pmr2* and *cmd1 pmr2* strains results in equal amounts of *PMR2A* mRNA (data not shown), but Na^+ tolerance is significantly decreased by the *cmd1* mutations (Figure 5D). Therefore, calmodulin appears to maintain an additional role in Na^+ tolerance that is independent of the calcineurin-regulated *PMR2A* promoter, but dependent on the *PMR2* gene product. In *pmr2* mutants (Figure 5B) *cmd1* mutations have no additive effects on Na^+ tolerance. We conclude that calmodulin is required for full *PMR2* function by a mechanism that is independent of the previously described effect of calcineurin on *PMR2* transcription.

Differential expression of repeats PMR2A and PMR2B in response to Na^+ and Li^+

As a first attempt to dissect the functional complexity of the *PMR2* locus, we concentrated our analysis on the first two genes in the cluster, *PMR2A* and *PMR2B*. To this end, four strains were constructed which express these genes under defined conditions in the absence of other *PMR2* repeats which could interfere with the analysis. Strains YW13 and YW15 (Figure 1C) harbor the alleles *PMR2A* and P_{PMR2A}::*PMR2B*, respectively, and express a solitary *PMR2A* or *PMR2B* gene via the same authentic *PMR2A* promoter. The strains depicted in Figure 1D were built from a cloned *PMR2A::PMR2B* tandem array as present in strains with multiple *PMR2* repeats. Here both genes are under the control of their own promoters and are thus expressed in strain YW17 (*PMR2A::PMR2B*). In strain YW19 (*pmr2A-1::PMR2B*) a frameshift mutation caused by a 4 bp deletion in *PMR2A* prevents synthesis of the Pmr2a protein. Consequently, this strain expresses only Pmr2b and reflects the functionality of a solitary *PMR2B* gene under its own promoter. The *LEU2* gene present in all alleles allowed the convenient transplacement of these constructs at the *PMR2* locus of a *pmr2-4::HIS3* strain.

We examined the growth of these strains in liquid YPD media containing NaCl or LiCl. Li^+ and Na^+ ions are thought to be carried by the same transport systems and Li^+ is often used as an analog of Na^+ in transport studies (Fleishman, 1991). Since internal Li^+ is much more toxic than Na^+, all strains were significantly more sensitive to LiCl than to NaCl (compare Figure 6, top and bottom). Wild-type cells with a total of five *PMR2* repeats (AA308) were most resistant to NaCl and LiCl, while the *pmr2-4::HIS3* null mutant (YW11) showed the greatest sensitivity. Interestingly, *pmr2A-1::PMR2B* cells (YW19) expressing only *PMR2B* were not more resistant to NaCl than YW11 (*pmr2-4::HIS3*), but exhibited some resistance to LiCl compared with YW11. This partial LiCl tolerance of *pmr2A-1::PMR2B* cells was also observed on plates (Figure 7C). Clearly a *PMR2B* repeat can confer LiCl tolerance *in vivo*, indicating that at least some Pmr2b protein is expressed and functional under these conditions. In contrast to the Li^+-specific tolerance seen in *pmr2A-1::PMR2B* cells, strain YW13, with a solitary *PMR2A* gene, showed a high tolerance to both Li^+ and Na^+ (Figure 6). Apparently the *PMR2A* 5′-region is primarily responsible for this disparity, as is evident from strain YW15, engineered to express *PMR2B* via the *PMR2A* 5′-region. Like YW13, strain YW15 grew well in the presence of Na^+ or Li^+ and the Pmr2a and Pmr2b proteins produced in these strains mediate quite similar levels of Li^+ and Na^+ tolerance (Figure 6). Obviously the *PMR2A* repeat functions well in the presence of Na^+ or Li^+, whereas expression of *PMR2B* appears to be specific to Li^+. This effect was also seen in *PMR2A::PMR2B* cells (YW17), which, due to the *PMR2B* gene, were more resistant to Li^+ (Figure 6, bottom) than the *PMR2A* strain (YW13). In the presence of Na^+, however, growth of both strains was virtually identical (Figure 6, top).

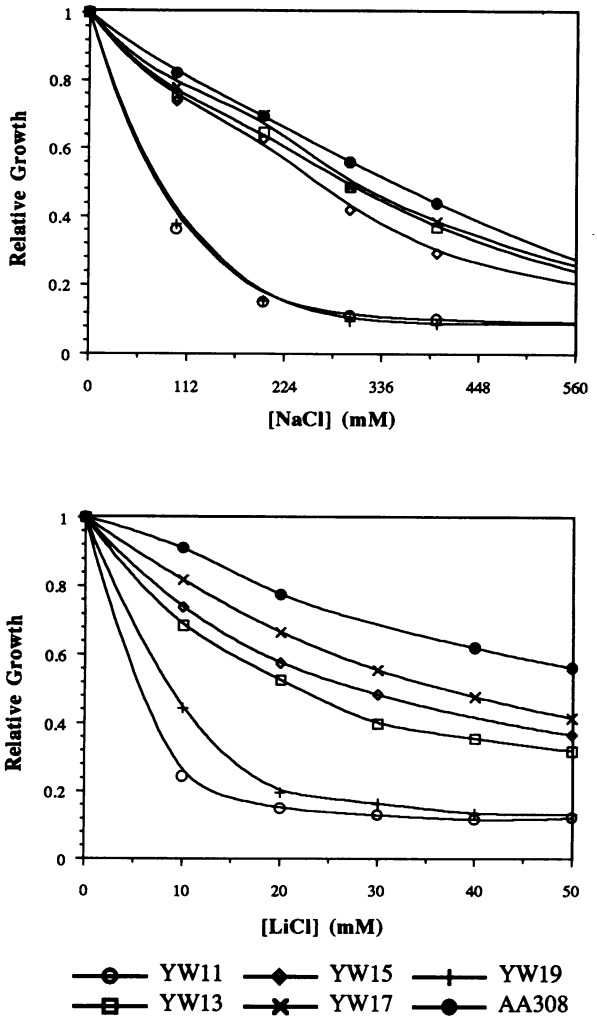

Fig. 6. Relative growth of strains expressing various *PMR2A* or *PMR2B* alleles. Equal amounts of cells pre-grown overnight in YPD (pH 5.5) were inoculated into YPD (pH 6.85, KOH) containing various amounts of NaCl (top) or LiCl (bottom) as indicated. After 10 h at 30°C, OD_{578} of all cultures was measured. Relative growth was calculated as the ratio between the OD_{578} obtained for a given culture and the OD_{578} of a control culture in YPD (pH 5.5). Strains: YW11 *pmr2-4::HIS3*, YW13 *PMR2A*, YW15 P_{PMR2A}::*PMR2B*, YW17 *PMR2A*::*PMR2B*, YW19 *pmr2A-1*::*PMR2B* and AA308 *PMR2*.

The ion pumps Pmr2a and Pmr2b confer distinct Na^+ and Li^+ tolerance

The apparent stability of *PMR2* tandem repetitions, in spite of the high level of homologous recombination, suggested to us that Pmr2a and Pmr2b might possess distinct properties. Hence we examined strains YW13 and YW15, which express *PMR2A* or *PMR2B* via the same *PMR2A* promoter and allow assay for differences in functions of the gene products. In liquid media (Figure 6) the *PMR2A* strain (YW13) was more resistant to Na^+ at all concentrations tested than the P_{PMR2A}::*PMR2B* strain (YW15). Conversely, YW15 cells were consistently more resistant to Li^+ than YW13 cells. Similar observations were made on solid media when serial dilutions of both strains were tested for growth in the presence of Na^+ or Li^+ (Figure 7). These data suggest a very attractive model: the small differences in the protein sequences might cause the two pumps to have distinct affinities towards monovalent cations. Consequently, Pmr2a may be the

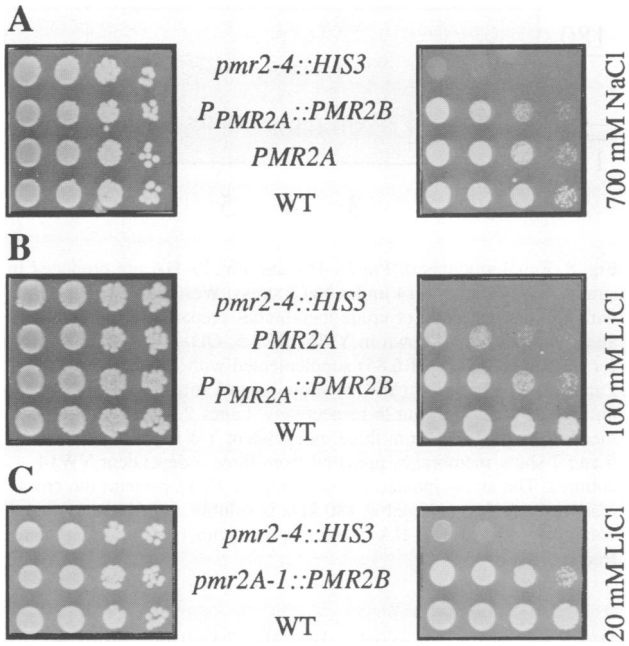

Fig. 7. Strains expressing *PMR2A* and *PMR2B* via the same promoter show differential Na^+ and Li^+ tolerance. Serial 10-fold (A) or 5-fold (B and C) dilutions of saturated cultures were spotted onto YPD (pH 5.5) plates without (left) or supplemented (right) with (**A**) NaCl or (**B** and **C**) LiCl as indicated. The plates were photographed after 3 days incubation at 30°C. Strains: YW11 *pmr2-4::HIS3*, YW13 *PMR2A*, YW15 P_{PMR2A}::*PMR2B*, YW19 *pmr2A-1*::*PMR2B* and AA308 *PMR2* (WT).

superior Na^+ pump, while Pmr2b is the better Li^+ pump. Alternatively, distinct subcellular localizations or different steady-state levels of the two proteins could account for these observations.

To monitor Pmr2a and Pmr2b protein levels we used cells expressing epitope-tagged Pmr2 derivatives which carry the nine amino acid HA epitope (Wilson *et al.*, 1984) at the C-terminus. Strains YW12 and YW14 express Pmr2a–HA or Pmr2b–HA under the *PMR2A* promoter. Both strains were tested under various conditions and found to be phenotypically indistinguishable from isogenic controls expressing untagged Pmr2 proteins. Addition of the HA epitope does not affect the apparent functionality of Pmr2; this was also found for all other Pmr2a–HA or Pmr2b–HA strains constructed (see Materials and methods). In Western blots probed with monoclonal anti-HA antibody crude membrane preparations from Pmr2a–HA and Pmr2b–HA strains showed a protein of ~120 kDa, in good agreement with the size expected for Pmr2 (mol. wt 120.260 kDa). This protein was absent from control strains expressing untagged Pmr2. A polyclonal antiserum directed against Pmr2 (a gift from A.Rodríguez-Navarro) recognized a single protein in Pmr2a–HA strains with the same apparent molecular weight as that detected by the anti-HA antibody (data not shown). To determine the amount of Pmr2 protein accumulated in *PMR2A* or P_{PMR2A}::*PMR2B* strains in response to high Na^+, we analyzed crude membranes prepared from three separate cultures of strains YW12 and YW14 exposed to the same salt stress conditions (Figure 8). In each case large amounts of protein recognized by the anti-HA antibody were found after incubation in salt-containing media. Importantly,

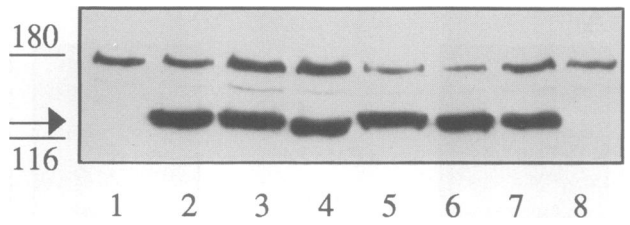

Fig. 8. Equal amounts of Pmr2a–HA and Pmr2b–HA are produced in strains YW12 and YW14 under NaCl stress. Western blot analysis with anti-HA antibody of crude membranes prepared from equal aliquots of cells pre-grown in YPD (pH 5.5; OD_{578} = 0.8) and shifted for 90 min to YPD (pH 6.85) supplemented with 700 mM NaCl. Lanes 1 (YW13) and 8 (YW15) show control strains expressing untagged Pmr2a and Pmr2b respectively. Lanes 2, 4 and 6 show membranes prepared from three independent YW12 cultures; lanes 3, 5 and 7 show membranes prepared from three independent YW14 cultures. The arrow indicates epitope-tagged Pmr2 protein; the cross-reacting material observed at 180 kDa is soluble and separates almost completely from Pmr2–HA on sucrose gradients (see Figure 9A and E, fractions 12–14).

Western blots showed virtually identical amounts of Pmr2a–HA and Pmr2b–HA in all preparations. We infer from these data that the distinct ion tolerance of strains which express *PMR2A* or *PMR2B* via the same *PMR2A* promoter is not caused by different steady-state levels of Pmr2a or Pmr2b.

Pmr2a and Pmr2b both localize to the plasma membrane

To determine the subcellular localization of Pmr2a and Pmr2b, extracts from strains YW12 and YW14 were fractionated by sucrose gradient centrifugation. All fractions collected from the gradients were tested by SDS–PAGE and Western blotting for the presence of marker proteins specific for plasma membrane (PM), endoplasmic reticulum (ER) and Golgi: H^+-ATPase (Pma1, PM; Serrano, 1978), dolichol phosphate mannose:protein *O*-mannosyltransferase I (Pmt1, ER; Strahl *et al.*, 1993) and dipeptidyl aminopeptidase A (DPAP A, Golgi; Roberts *et al.*, 1992). Vacuolar membranes were identified by monitoring α-mannosidase activity (Ams1; van der Wilden *et al.*, 1973). Figure 9 displays the data obtained from fractionation of an extract derived from a strain expressing Pmr2a–HA. Plasma membranes are enriched in fractions 1–4, which represent sucrose concentrations of 50–45% (Figure 9B and G). Clearly, Pmr2a–HA and Pmr2b–HA both co-localize with Pma1 in the plasma membrane fractions (Figure 9A and E). The other marker proteins show quite different patterns of distribution: Pmt1 (ER) is present in all fractions with sucrose concentrations >30%, but most abundant in fractions 9–11. The Golgi protein DPAP A is found in fractions 5–10 (41–30% sucrose). Nearly all the activity of the vacuolar α-mannosidase resides in fractions 8–12 (36–28% sucrose). All marker proteins are well separated from the bulk of soluble protein found in fractions 12–16 (28–20% sucrose).

The results obtained by cell fractionation were confirmed using indirect immunofluorescence microscopy. Here we used *pmr2* cells transformed with plasmids expressing either Pmr2a–HA or Pmr2b–HA via the *PMA1* promoter. Cells producing Pmr2a–HA exhibit a cell surface rim staining pattern consistent with localization of Pmr2a–HA in the plasma membrane (Figure 10). Pmr2b–HA showed virtually identical staining patterns (data not shown). Evidently subcellular fractionation and immunofluorescence microscopy identify Pmr2a–HA and Pmr2b–HA as plasma membrane proteins. The small differences present in the sequences of Pmr2a and Pmr2b do not alter the localization of the proteins.

Discussion

We have characterized two members of a multigene cluster at the *PMR2* locus in yeast. This unusual tandem array of nearly identical genes encodes putative ion pumps involved in salt tolerance. Mutants lacking the entire *PMR2* locus are viable, but sensitive to salt. We show that expression of two genes in the cluster, *PMR2A* and *PMR2B*, is differentially regulated in response to salt stress and involves a post-transcriptional regulation of the pumps by calmodulin. Furthermore, we demonstrate that the highly related proteins Pmr2a and Pmr2b both localize to the plasma membrane and confer salt tolerance. However, we find characteristic differences in adaptation to Na^+ or Li^+ stress between strains expressing Pmr2a or Pmr2b under identical conditions. Our studies suggest that individual Pmr2 pumps have distinct ion transport properties which may offer an advantage to cells maintaining the *PMR2* gene cluster.

Organization of the PMR2 locus

In our survey of phylogenetically related yeasts we found three *S.cerevisiae* strains with five *PMR2* genes repeated in tandem and a strain of *S.italicus* with two *PMR2* repeats, whereas strains of *S.norbensis* and *S.chevalieri* revealed the presence of single *PMR2* genes. Previous work identified four copies of *PMR2* in DBY746, another strain of *S.cerevisiae* (Garciadeblas *et al.*, 1993). Variations in the number of *PMR2* repeats could result from unequal crossing-over between tandem repeats on sister chromatids or homologous chromosomes (for review see Petes *et al.*, 1991). Evidently *S.cerevisiae* strains have acquired and maintain a *PMR2* multigene cluster despite a high level of homologous recombination known to make duplications unstable in this organism (Hicks *et al.*, 1979). The apparent stability of this gene cluster suggests that the individual genes may differ in some aspects to provide *PMR2* function under a variety of conditions.

Indeed, we found that *PMR2A* and *PMR2B*, the first two genes in the cluster, are expressed differently in response to salt stress. A single *PMR2A* gene on the chromosome functions well upon challenge with Na^+ or Li^+. However, a single *PMR2B* gene is unable to protect cells against Na^+, but leads to significant Li^+ tolerance. The different 5′-control regions of both genes are largely responsible for this effect, since a P_{PMR2A}::*PMR2B* gene obtained by precise sequence exchange responds very well to Na^+ and Li^+. Interestingly, the *PMR2B* coding sequence is preceded by a short open reading frame of 12 amino acids, which might constitute a translational control mechanism contributing to specific expression of *PMR2B*.

We found one strain of *S.cerevisiae* (Σ1278b) with a single *PMR2* gene. Upon treatment with NaCl, *PMR2* mRNA was induced >10-fold in this strain. Surprisingly, Σ1278b is much more sensitive to Na^+ than all other

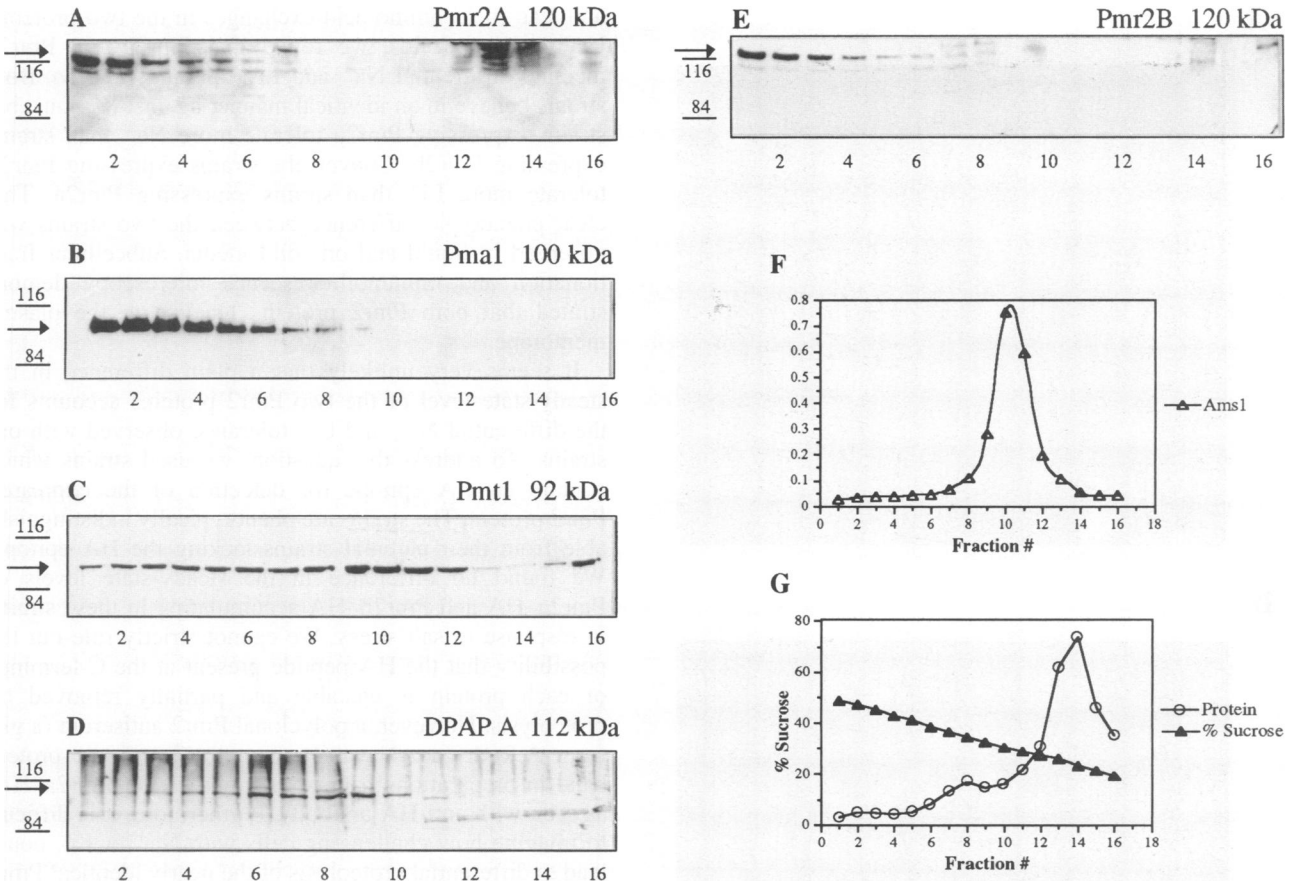

Fig. 9. Fractionation of Pmr2a–HA and Pmr2b–HA on sucrose gradients. Whole-cell extracts of YW12 (A–D, F and G) and YW14 (E) were fractionated by density gradient centrifugation. Aliquots of the gradient fractions were separated on 7.5% SDS–PAGE gels and analyzed by Western blotting with (**A**) anti-HA antibodies, (**B**) anti-Pma1 antibodies, (**C**) anti-Pmt1 antibodies and (**D**) anti-DPAP A antibodies. The sizes of marker proteins are given in kDa, their positions are marked by arrows and the fraction numbers are indicated. (**E**) For the YW14 fractionation only the blot with anti-HA antibodies is shown. All marker proteins distributed as in the YW12 fractionation. (**F**) α-Mannosidase activity (in arbitrary units), (**G**) density (% sucrose, w/w) and protein concentration (arbitrary units) are plotted against the fraction number. Cross-reacting material migrating above and below Pmr2–HA is frequently observed in control strains expressing untagged Pmr2; on sucrose gradients most of this material separates from Pmr2-HA.

S.cerevisiae strains tested, which exhibited only a moderate mRNA increase in response to Na^+. Perhaps the Pmr2 pump expressed in Σ1278b is poorly active or even inactive. We do not know whether the extreme sensitivity of Σ1278b to repression of nitrogen assimilation pathways by ammonia (Rytka, 1975; Wiame *et al.*, 1985) is connected to poor expression of *PMR2*, but Na^+ sensitivity seems to increase in ammonium-free media and *PMR2A* is induced under such conditions (Garciadeblas *et al.*, 1993).

Calmodulin regulates the Pmr2 ion pumps

Here we have shown that adaptation to high salt is also controlled by calmodulin. We analyzed a set of *cmd1* strains which express mutant calmodulins with altered Ca^{2+} binding properties and found differential effects on adaptation to salt stress: mutant calmodulins with a drastic reduction (*cmd1-5*) or complete loss (*cmd1-3*) of calcium binding activity cause Na^+ sensitivity compared with wild-type calmodulin (*CMD1*) or a conditional mutant (*cmd1-1*) with predominantly normal calcium binding sites. Most importantly, these observations may reflect a Ca^{2+}-specific role of calmodulin in Na^+ tolerance and implicate changes in cytosolic Ca^{2+} as a triggering factor for the adaptation process. In principle, the different levels of calmodulins in the strains (Geiser *et al.*, 1991) could also be responsible for the results, but in the absence of Na^+ all mutants except *cmd1-1* grow in YPD at 30°C indistinguishably from the wild-type.

The Ca^{2+}/calmodulin-dependent regulation we discovered is independent of calcineurin-mediated *PMR2* transcription (Mendoza *et al.*, 1994), but requires a functional *PMR2* gene product. We showed that mutant calmodulins still affect Na^+ tolerance in *cnb1::TRP1* strains, which are defective in calcineurin-dependent activation of *PMR2* transcription (Mendoza *et al.*, 1994). Second, we constructed a strain in which *PMR2* is expressed from the constitutive *PMA1* promoter and introduced the calmodulin mutations into this strain. The resulting strains showed differential Na^+ tolerance very similar to the original *cmd1* mutants with wild-type *PMR2*. Evidently the regulation of *PMR2*-mediated Na^+ tolerance by calmodulin does not depend on the *PMR2A* promoter. However, a functional *PMR2* gene product is required for the regulation, since calmodulin mutations have no effect in the *pmr2* null mutant.

An intriguing explanation for our findings is that

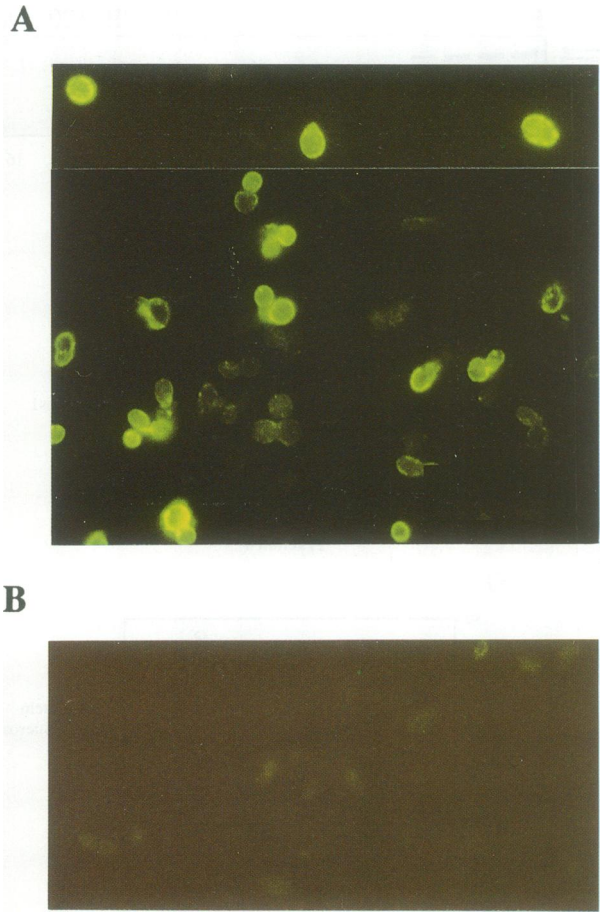

Fig. 10. Localization of Pmr2a–HA by immunofluorescence microscopy. Pmr2a–HA was visualized with anti-HA primary antibodies (12CA5) and FITC-conjugated anti-mouse secondary antibodies. (**A**) Strain YW4 *pmr2-2::HIS3* harboring plasmid P_{PMA1}::*PMR2A::HA* (pJW10). (**B**) Control strain YW5 *pmr2-2::HIS3* harboring plasmid P_{PMA1}::*PMR2A* (pJW11).

calmodulin activates the Pmr2 pumps post-translationally. In this respect it is noteworthy that the C-terminus of Pmr2e (encoded by the last repeat in the cluster) differs substantially from Pmr2a and Pmr2b (12 exchanges within 33 amino acids; Martinez *et al.*, 1991), but retains a cluster of serine/threonine residues. In preliminary experiments with a plasmid-borne *PMR2* gene, C-terminal truncations appear to cause loss of calmodulin regulation, but so far we have obtained no evidence for direct binding of calmodulin to Pmr2 (A.Nitsche and H.Rudolph, unpublished data). Together with calcineurin-activated transcription of *PMR2*, this presumably indirect regulation of the Pmr2 pumps by a Ca^{2+}/calmodulin-dependent post-translational mechanism would enable cells to use a Ca^{2+} signal to respond to high Na^+ by dual activation of the pumps.

Pmr2a and Pmr2b are functionally distinct isoforms

A main finding of our study is that Pmr2a and Pmr2b represent different isoforms of a putative plasma membrane ion pump. We constructed strains which express each pump individually from the same chromosomal 5′- and 3′-control regions. These strains are identical except for the 42 nt which differ within the *PMR2* coding regions and lead to 13 amino acid exchanges in the two proteins. Using these strains, we found that Pmr2a and Pmr2b mediate differential Na^+ and Li^+ tolerance *in vivo*. Both strains behave in an identical manner against K^+ ions, but strains expressing Pmr2a tolerate more Na^+ than strains expressing Pmr2b. Conversely, strains expressing Pmr2b tolerate more Li^+ than strains expressing Pmr2a. This clear phenotypic difference between the two strains was observed in liquid and on solid media. Subcellular fractionation and immunofluorescence microscopy demonstrated that both Pmr2 proteins localize to the plasma membrane.

It seems very unlikely that a plain difference in the steady-state level of the two Pmr2 proteins accounts for the differential Na^+ and Li^+ tolerance observed with our strains. To address this question, we used strains which contain an HA epitope for detection of the expressed Pmr2 protein. The strains are phenotypically indistinguishable from their parental strains lacking the HA epitope. We found no difference in the steady-state levels of Pmr2a–HA and Pmr2b–HA accumulating in these strains in response to salt stress. We cannot strictly rule out the possibility that the HA peptide present at the C-terminus of each protein is unstable and partially removed by proteolysis. However, a polyclonal Pmr2 antiserum (a gift from A.Rodríguez-Navarro) recognized a single protein band in preparations from strains expressing Pmr2a–HA, as seen with anti-HA antibody. Furthermore, it is difficult to imagine how challenging cells with Na^+ or Li^+ could lead to differential proteolysis of the nearly identical Pmr2 proteins to produce these results.

In our view, Pmr2a and Pmr2b are distinct isoforms of a plasma membrane ion pump customized to sustain ion homeostasis under a variety of conditions. Both pumps might differ in regulatory features affixed to an intrinsic monovalent ion pump to ensure appropriate control of pump activity under specific conditions. Alternatively, both isoforms represent distinct pumps with very similar, but nevertheless different, ion specificities or affinities. To resolve this issue, *in vitro* reconstitution of transport activity from purified proteins has to be carried out. Among the (Na^+, K^+)-ATPase isoforms found in different species, the hydrophilic N-terminal end on the cytoplasmic side of the membrane shows significant sequence diversity. This region appears to participate in a conformational shift which could control the passage of Na^+ and K^+ to ion binding sites transiently occupied during transport (Lingrel *et al.*, 1990). Interestingly, all but one of the 13 amino acid changes responsible for the characteristics of the Pmr2 isoforms lie within 90 amino acids at the N-terminus. Therefore, the amino acid exchanges in Pmr2a and Pmr2b could indeed directly affect the ion affinities of the pumps.

Our results indicate that the diversity found in the *PMR2* multigene cluster is not limited to differential regulation of individual *PMR2* genes, but includes distinct properties of the encoded gene products. In this respect, the *PMR2* cluster differs from rDNA (Schweizer *et al.*, 1969) and the *CUP1* locus (Fogel and Welch, 1982), which both seem to consist of functionally equivalent repeat units (reviewed by Olson, 1991). At present the prevalent cellular functions executed by the ensemble of Pmr2 isoforms are not well understood. They may protect

Table I. List of plasmids

Name	Markers	Source
p2-48	*PMR2E* plus 6 kb 3′-flanking DNA in YEp24	This work
p2-69	*PMR2A*::*PMR2B*::*PMR2C*::*PMR2D* in YEp24	This work
p202-1	*PMR2A* plus 4 kb 5′-flanking DNA in YIp5	This work
pHR112	*pmr2-ΔSac* in pBR322	This work
pHR113	*pmr2-1*::*URA3* in pYEp24	This work
pHR114	*pmr2-2*::*HIS3* in pYEp24	This work
pPS15-P_{PMA1}	*CEN6, LEU2, PMA1* promoter	Supply *et al.*, 1993
pJW10	*PMR2A*::*HA* under *PMA1* promoter in pRS316	This work
pJW11	*PMR2A* under *PMA1* promoter in pRS316	This work
pJW13	*PMR2B*::*HA* under *PMA1* promoter in pRS316	This work
pJW14	*PMR2B* under *PMA1* promoter in pRS316	This work
pJW20	*CEN6*, *Bst*XI deletion of pRS313	This work
pJW24	*pmr2-6*::*LEU2* in pJW20	This work
pJW25	*pmr2-3*::*URA3* in pJW20	This work
pJW28	*pmr2-4*::*HIS3* in pJW20	This work
pJW29	*PMR2A*::*HA*::*LEU2* in pJW20	This work
pJW30	*PMR2A*::*LEU2* in pJW20	This work
pJW32	P_{PMR2A}::*PMR2B*::*HA*::*LEU2* in pJW20	This work
pJW33	P_{PMR2A}::*PMR2B*::*LEU2* in pJW20	This work
pJW39	*PMR2A*::*PMR2B*::*LEU2* in pJW20	This work
pJW41	*pmr2A-1*::*PMR2B*::*LEU2* in pJ20	This work
pFF54	*CNB1* in pBluescript II KS (+)	Foor *et al.*, 1992
pAN25	*cnb1*::*TRP1* in pBluescript II KS (+)	This work

cells against high salt conditions and ensure removal of monovalent cations co-transported during uptake of nutrients and small solutes at the plasma membrane. Regulation of the Pmr2 pumps by Ca^{2+}/calmodulin could also indicate that cells possess mechanisms to lower cytosolic Ca^{2+} in response to increased Na^+ efflux. The characterization of mutants which require a functional *PMR2* gene for life under normal conditions should provide further insights into the functions of the Pmr2 ion pumps.

Materials and methods

Media and growth of yeast strains

YPD was prepared according to Sherman *et al.* (1986). In YPD (pH 5.5) the pH was adjusted using HCl without addition of buffers. YPD (pH 6.85) contained 50 mM MES and 50 mM MOPS and the pH was adjusted with KOH or NaOH. With NaOH the final concentration of Na^+ was ~100 mM higher than in YPD (pH 5.5) or in YPD (pH 6.85) adjusted with KOH. YPD (pH 7) was buffered with 50 mM MOPS and the pH was adjusted with KOH.

Growth of yeast strains in various liquid media was tested by inoculating equal amounts of cells pre-grown overnight in YPD (pH 5.5) into YPD (pH 6.85, KOH) containing various amounts of NaCl, LiCl, KCl or sucrose as indicated. After 10 h at 30°C, OD_{578} of all cultures was measured. Relative growth of a strain was expressed as the ratio between the OD_{578} obtained from this strain in a given medium and the OD_{578} of this strain in YPD (pH 5.5). For similar tests on solid media, YPD (pH 5.5) plates or YPD (pH 7) plates were supplemented with different amounts of NaCl or LiCl as indicated.

Analysis of cloned PMR2 repeats

We first noticed tandem repetitions of *PMR2* sequences in Southern blot experiments with genomic DNA. Final proof came from the analysis of plasmids identified in a yeast genomic library (Carlson and Botstein, 1982) by colony hybridization of *Escherichia coli* transformants using a 2.6 kb *Hin*dIII fragment of *PMR2* as a probe (Rudolph *et al.*, 1989). One plasmid (p2-69) contained the 5′-junction together with almost four complete *PMR2* repeats: *PMR2A*, *PMR2B*, *PMR2C* and part of *PMR2D*. Another plasmid (p2-48) carried the last repeat, *PMR2E*, and an additional 6 kb of DNA, including the *KRS1* gene next to *PMR2* on the 3′-side (Martinez *et al.*, 1991). Plasmid p202-1 carried ~4 kb of flanking DNA upstream of *PMR2A* and was evicted from strain AA202 by self-ligation of *Aat*II-cut genomic DNA harboring plasmid pL108-1 integrated at *PMR2A* (Rudolph *et al.*, 1989).

In total we sequenced 8369 bp of *PMR2* DNA from plasmid p2-69, starting 751 bp upstream of the AUG for *PMR2A* and ending 460 bp after the last codon of *PMR2B*. The DNA sequence of *PMR2A* has been published (Rudolph *et al.*, 1989) and is identical to the *ENA1* sequence (Haro *et al.*, 1991). Our sequence of *PMR2B* is identical to the reported *ENA2* sequence, but adds an additional 256 nt at the 3′-side (GenBank accession no. U24069). For details we refer the reader to the published sequence comparison (Garciadeblas *et al.*, 1993).

Plasmids and strains

All plasmids used in this study are listed in Table I. The *pmr2-ΔSac* allele in pHR112 was constructed by ligating the 11 kb *Aat*II–*Sac*I fragment of p2-48 with the 4 kb *Aat*II–*Sac*I fragment of p202-1. This deletes all *PMR2* DNA between the *Sac*I sites in *PMR2A* and *PMR2E*. Alleles *pmr2-1*::*URA3* and *pmr2-2*::*HIS3* were constructed from pHR112 by inserting appropriate *Sac*I cassettes into the unique *Sac*I site. The *URA3*–*Sac*I cassette in pHR113 was a gift from P.Ljungdahl, the *HIS3*–*Sac*I cassette in pHR114 was made by end filling of the 1.7 kb *HIS3*–*Bam*HI fragment (Struhl *et al.*, 1979) and subsequent addition of *Sac*I linkers. To build the null alleles *pmr2-3*::*URA3*, *pmr2-4*::*HIS3* and *pmr2-6*::*LEU2*, a 1.3 kB *Sal*I–*Sna*BI fragment with the *PMR2* 5′-junction (Garciadeblas *et al.*, 1993) was first cloned into a pRS313 vector (Sikorski and Hieter, 1989) lacking the *Bst*XI–*Bst*XI fragment (pJW20). Addition of a 1.7 kb *Sna*BI–*Bgl*II fragment carrying the 3′-junction of *PMR2* (Martinez *et al.*, 1991) to this plasmid allowed the subsequent insertion of *URA3* (pJW25), *LEU2* (pJW24) or *HIS3* (pJW28) into the unique *Sna*BI site. *URA3* insertion is antiparallel, while the two other insertions are oriented parallel to *PMR2* transcription. For transplacement into the chromosome these alleles and their derivatives were cut with either *Apa*I and *Spe*I or with *Bgl*II and *Spe*I (pJW25).

pJW24 contains a unique *Xho*I site restored upstream of *LEU2* (inserted as an end filled *Sal*I–*Xho*I fragment) which allowed addition of *PMR2A* or *PMR2B* DNA in several steps. The resulting plasmids express *PMR2A* (pJW30) or *PMR2B* (pJW33) under control of the chromosomal *PMR2A* promoter and differ only in the nucleotide changes present in the two coding regions. Plasmids pJW29 (*PMR2A*::*HA*) and pJW32 (*PMR2B*::*HA*) are identical to pJW30/33 except for the added HA epitope. Plasmid pJW39 (*PMR2A*::*PMR2B*) was obtained by the addition of a 4.2 kb *Sna*BI–*Nco*I *PMR2A* fragment from p2-69 into pJW33 and harbors an authentic *PMR2A*::*PMR2B* tandem. Digestion of pJW39 with *Pst*I, removal of the overhangs by T4 polymerase treatment and re-ligation produced pJW41. This 4 bp deletion in pJW41 causes a frameshift (*pmr2A-1*::*PMR2B*) 66 bp downstream of the ATG in *PMR2A*, followed by three in-frame stop codons within the next 50 bp.

Table II. List of yeast strains

Name	Genotype	Source/comment
AA308	MATa *ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 gal2*	Antebi and Fink, 1992
YR86	AA308 *pmr2-1::URA3*	This work
YR93	AA308 *pmr2-2::HIS3*	This work
YW4	YR93 [P_{PMA1}::*PMR2A::HA URA3 CEN6*]	This work
YW5	YR93 [P_{PMA1}::*PMR2A URA3 CEN6*]	This work
YW6	YR93 [P_{PMA1}::*PMR2B::HA URA3 CEN6*]	This work
YW7	YR93 [P_{PMA1}::*PMR2B URA3 CEN6*]	This work
YW10	YR93 *pmr2-3::URA3*	This work
YW11	YR86 *pmr2-4::HIS3*	This work
YW12	YW10 *PMR2A::HA::LEU2*	This work
YW13	YW10 *PMR2A::LEU2*	This work
YW14	YW10 P_{PMR2A}::*PMR2B::HA::LEU2*	This work
YW15	YW10 P_{PMR2A}::*PMR2B::LEU2*	This work
YW16	YW10 *PMR2A::PMR2B::HA::LEU2*	This work
YW17	YW10 *PMR2A::PMR2B::LEU2*	This work
YW18	YW10 *pmr2A-1::PMR2B::HA::LEU2*	This work
YW19	YW10 *pmr2A-1::PMR2B::LEU2*	This work
CRY1	MATa *ade2oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R.Fuller/T.Davis
JGY44-2A	CRY1 *cmd1-1*	Davis, 1992
JGY148	CRY1 *cmd1-5*	Geiser *et al.*, 1991
JGY41	CRY1 *cmd1-3*	Geiser *et al.*, 1991
YN10	CRY1 *pmr2-2::HIS3*	This work
YN11	JGY44-2A *pmr2-2::HIS3*	This work
YN12	JGY148 *pmr2-2::HIS3*	This work
YN13	JGY41 *pmr2-2::HIS3*	This work
YN20	CRY1 *cnb1::TRP1*	This work
YN23	JGY44-2A *cnb1::TRP1*	This work
YN24	JGY148 *cnb1::TRP1*	This work
YN26	JGY41 *cnb1::TRP1*	This work
YR552	YN10 [P_{PMA1}::*PMR2A URA3 CEN6*]	This work
YR553	YN11 [P_{PMA1}::*PMR2A URA3 CEN6*]	This work
YR554	YN12 [P_{PMA1}::*PMR2A URA3 CEN6*]	This work
YR555	YN13 [P_{PMA1}::*PMR2A URA3 CEN6*]	This work
S288C	*S.cerevisiae*	Fink collection
Σ1278b	*S.cerevisiae*	Fink collection
A364A	*S.cerevisiae*	Fink collection
D273–10B	*S.cerevisiae*	Fink collection
F603	*S.chevalieri*	Fink collection
F604	*S.italicus*	Fink collection
F607	*S.uvarum*	Fink collection
F839	*S.carlsbergensis*	Fink collection
GRF5	*S.norbensis*	Fink collection

To express *PMR2A* (pJW11) or *PMR2B* (pJW14) via the *PMA1* promoter, the corresponding coding regions were fused via their *Xmn*I sites to the *PMA1* promoter fragment present in pPS15-P_{PMA1} (Supply *et al.*, 1993) and transferred into pRS316 (Sikorski and Hieter, 1989). Identical constructions produced the epitope-tagged derivatives P_{PMA1}::*PMR2A::HA* (pJW10) and P_{PMA1}::*PMR2B::HA* (pJW13). The disruption allele *cnb1::TRP1* in pAN25 was constructed by digestion of pFF54 (Foor *et al.*, 1992) with *Sty*I, end filling with Klenow and ligation with a blunt-ended 827 bp *Eco*RI–*Pst*I fragment containing the *TRP1* gene (Struhl, 1979). For allele transplacement by transformation, pAN25 was digested with *Bss*HII. All yeast strains used in this study are listed in Table II.

DNA and RNA analysis

Genomic yeast DNA was prepared as described by Phillipsen *et al.* (1991). To resolve large DNA fragments in Southern blots, digests of genomic DNA were separated by gel electrophoresis (0.5 % agarose in TBE) for 18 h at 25 V followed by 8–12 h at 50 V. Transfer to nylon after brief acid treatment was as described (Sambrook *et al.*, 1989). Total RNA was isolated using hot acid phenol according to Ausubel *et al.* (1994). Poly(A)$^+$ RNA was prepared from total RNA using an Oligotex kit (Quiagen). For Northern analysis, electrophoresis of RNA samples in agarose (1.5%)–formaldehyde (6%)–MOPS gels, transfer to nylon and hybridization to *PMR2A* (4.1 kb *Dra*III–*Mlu*I fragment) and *ACT1* (900 bp *Bam*HI–*Hin*dIII fragment of pHD507, a gift from E.Schiebel) probes were performed essentially as in Ausubel *et al.* (1994). DNA sequencing of a 3.9 kb *Xho*I fragment carrying *PMR2B* (from p2-69) was performed after subcloning into pUC118 and generation of unidirectional deletions with exonuclease III as described (Rudolph *et al.*, 1989).

Preparation of crude membranes and subcellular fractionation

To prepare crude membranes, cells were pre-grown overnight in YPD (pH 5.5) and then transferred for 90 min into YPD (pH 6.85, NaOH) containing 600 mM NaCl. Subsequent steps were according to Serrano (1988). For subcellular fractionation, cells growing exponentially in YPD (pH 5.5; 100 ml cultures; OD_{600} = 0.5–1.0; 3.6×10^7 cells/ml) were harvested and transferred for 90 min to YPD (pH 6.85, NaOH) containing 200 mM NaCl . Fractionation was done according to Kölling and Hollenberg (1994). Briefly, lysis was achieved by vortexing the cell suspension in the presence of glass beads for 3 min. The cleared cell extract (500 *g*, 5 min) was loaded onto sucrose gradients prepared as follows. STED50, STED35 and STED20 (50, 35 or 20% sucrose, 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol) were layered on top of each other (4 ml each). The tubes (SW40) were slowly turned into a horizontal position. After 3–5 h the gradients were turned upright, loaded with extract and spun for 13–17 h at 30 000 r.p.m. at 4°C; 30-drop fractions (700–800 µl) were collected from the bottom of the gradients. Marker proteins were detected by Western blotting and treatment with suitable antibodies. α-Mannosidase was assayed as described (van der Wilden *et al.*, 1973).

Epitope tagging and immunofluorescence microscopy

Precise addition of the HA epitop YPYDVPDYA (Wilson *et al.*,1984) after the last sense codon in the *PMR2A* ORF was performed via site-

directed insertion mutagenesis (Kunkel *et al.*, 1987) and verified by DNA sequencing. Epitope-tagged versions of *PMR2B* were obtained by replacing appropriate *PMR2A* fragments with *PMR2B* DNA. For microscopy, cells growing logarithmically in YPD (pH 6.85, NaOH) were fixed by adding formaldehyde (3.7% w/v final) for 10 min at room temperature. Indirect immunofluorescence was carried out essentially according to Davis and Fink (1990), with the exception that protease inhibitors and methanol washes were omitted. Antisera were diluted as follows: 12CA5 from ascites fluid (Berkeley Antibody Co.) 1:250; fluorescein (DTAF)-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) 1:50.

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