Biochimica et Biophysica Acta 1798 (2010) 1841–1853

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Sodium or potassium efflux ATPase
A fungal, bryophyte, and protozoal ATPase

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ARTICLE INFO

Article history:
Received 26 May 2010
Received in revised form 6 July 2010
Accepted 13 July 2010
Available online 19 July 2010

Keywords:
ATPase
Sodium
Potassium
Cation efflux

ABSTRACT

The K^+ and Na^+ concentrations in living cells are strictly regulated at almost constant concentrations, high for K^+ and low for Na^+. Because these concentrations correspond to influx–efflux steady states, K^+ and Na^+ effluxes and the transporters involved play a central role in the physiology of cells, especially in environments with high Na^+ concentrations where a high Na^+ influx may be the rule. In eukaryotic cells two P-type ATPases are crucial in these homeostatic processes, the Na,K-ATPase of animal cells and the H^+-ATPase of fungi and plants. In fungi, a third P-type ATPase, the ENA ATPase, was discovered nineteen years ago. Although for many years it was considered to be exclusively a fungal enzyme, it is now known to be present in bryophytes and protozoa. Structurally, the ENA (from *exitus natru*: exit of sodium) ATPase is very similar to the sarco/endoplasmic reticulum Ca^{2+} (SERCA) ATPase, and it probably exchanges Na^+ (or K^+) for H^+. The same exchange is mediated by Na^+ (or K^+)/H^+ antiporters. However, in eukaryotic cells these antiporters are electroneutral and their function depends on a ΔpH across the plasma membrane. Therefore, the current notion is that the ENA ATPase is necessary at high external pH values, where the antiporters cannot mediate uphill Na^+ efflux. This occurs in some fungal environments and at some points of protozoa parasitic cycles, which makes the ENA ATPase a possible target for controlling fungal and protozoan parasites. Another technological application of the ENA ATPase is the improvement of salt tolerance in flowering plants.

Contents

1. Introduction

In the normal distribution of alkali cations across the plasma membrane of eukaryotic cells K^+ is accumulated and Na^+ is excluded, and an electrical potential, which is negative inside, is physiologically related to the K^+ and Na^+ distributions. The primary energy source of these processes is ATP and the transducers are two plasma membrane

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doi:10.1016/j.bbamem.2010.07.009

P-type ATPases, the Na,K-ATPase in the naked cells of animals and the H^+-ATPase in the walled cells of plant and fungi. The former pumps K^+ and Na^+ in opposite directions, creating the asymmetric distribution of these ions, which subsequently generates the electrical membrane potential with the mediation of K^+ and Na^+ channels. In walled cells, the H^+-pump creates the electrical membrane potential and the K^+ and Na^+ asymmetric distributions are built up subsequently by secondary transport systems that mediate K^+ uptake and Na^+ extrusion. These two bioenergetic models are the eukaryotic versions of the 'sodium and proton worlds' [1], where Na^+ or H^+ are used as the main coupling ions. The former model is adapted to environments that are quite constant in their composition with a high Na^+ concentration, such as the sea and the extracellular fluids of animals, and the latter to variable and usually dilute environments, such as the terrestrial environments where fungi and plants thrive. Simple energetic calculations [2] suggest that each model is adapted to the prevailing conditions in these two completely different media.

The aforementioned scheme for fungi and plants, based on the action of a single type of ATPase, changed when a P-type ATPase that was supposed to be a Na^+-ATPase was identified. This ATPase, which was named ENA (from *exitus natru*: exit of sodium), was identified in *Saccharomyces cerevisiae* [3], a species in which Li^+ and Na^+ tolerances depend on the extrusion of these cations [4,5] and in which Li^+ and Na^+ sensitive strains are common [6]. By backcrossing a wild Na^+ sensitive strain that showed a poor Na^+ efflux with a laboratory strain with a competent Na^+ efflux, the Na^+ sensitivity character was transferred to the laboratory strain. The *ENA1* gene was then identified by its capacity to suppress the Na^+ sensitivity of this strain [3]. Further studies demonstrated that the encoded ATPase exhibited very low discrimination among alkali cations [7]. Later, *ScENA1* homologue genes were identified in *Schwanniomyces occidentalis* [8], and subsequently in all fungi that were studied. These findings raised the possibility that the ENA ATPase was present in all fungi and that its function was not restricted to mediating Na^+ efflux [9].

After the identification in fungi, the ENA ATPase was identified in the moss *Physcomitrella patens* [10] and in the liverwort *Marchantia polymorpha* [11]. In contrast, *ENA* genes do not exist in any of the vascular plant genomes so far sequenced, including the lycophyte *Selaginella moellendorffii*.

In protozoa, biochemical studies on the Na^+-ATPase of *Trypanosoma cruzi* epimastigotes revealed the existence of an ATPase activity that was ouabain-insensitive, stimulated by Na^+, and inhibited by furosemide [12]. Later, fragments of genes that could encode ENA ATPases were cloned from *Trypanosoma brucei*, *T. cruzi*, and *Leishmania donovani* [13], and the ENA ATPase from *T. cruzi* and its encoding gene was characterized a few years later [14].

The existence of the ENA ATPase in almost all fungi, as well as in bryophytes and protozoa suggests that this enzyme is required for the adaptation to life conditions that prevail in organisms with very different lifestyles. This conclusion raises an interesting question regarding the biological consequences of its absence in flowering plants. Therefore, the possibility of using drug inhibitors of the ENA ATPase for controlling protozoan [15,16] and fungal [17] pathogens, and the use of the ENA ATPase for increasing the Na^+ tolerance of crop plants [18–20] open up a field of technological research on this enzyme.

This review describes current knowledge about the ENA ATPase and discusses the emerging functions that it may have especially in endomembranes.

2. The ENA ATPase across phyla

The phylogenetic study of P-type ATPases by Axelsen and Palmgren [21] classifies these ATPases into several types. ENA ATPases made up Type IID, which is closely related to endomembrane (Type IIA), plasma membrane (Type IIB), and Golgi or PMR1 Ca^{2+}-ATPases, as well as to Na^+/K^+ATPases (Type IIC). This phylogenetic analysis also suggests that the four subtypes appeared before the split of plants, fungi, and animals, and that each subtype evolved independently in these three groups of organisms (Fig. 1). Recently, other groups of Type II ATPases have been identified [22–25], including bacterial P-ATPases [10,25,26]. However, Type IID bacterial P-ATPases have not been described. A P-type Na^+ ATPase that has been described in *Exiguobacterium aurantiacum* [26] does not keep the conserved sequences that characterize ENA ATPases (see below).

For several years after the cloning of the *S. cerevisiae* ENA ATPase [3] only fungal enzymes were studied: in *S. occidentalis* [8], *Zygosaccharomyces rouxii* [27], *Neurospora crassa* [28], *Debaryomyces hansenii* [29], *Fusarium oxysporum* [30], *Hortaea wernecki* [31], *Torulaspora delbrueckii* [32], *Cryptococcus neoformans* [17], and *Ustilago maydis* [33]. The CTA3 ATPase of *Schyzosaccharomyces pombe* was initially described as a Ca^{2+}-ATPase [34,35] but a further study showed that by sequence and function CTA3 was an ENA ATPase [10]. A Blast search in the 65 fungal species in which their genomes have been sequenced (up to December, 2009) revealed that *ENA* genes have not been sequenced in nine species only.

Fungal ENA ATPases may be grouped into five phylogenetic clusters (Fig. 2), in which two ATPases are excluded, from *C. neoformans*, Basidiomycota, and *S. pombe*, Ascomycota; and four are in uncertain positions, ENA2 from *U. maydis*, Basidiomycota, ENA1 from *Spizellomyces punctatus*, Chytridiomycota, and ENA1 and ENA2 from *Glomus intraradices*, Glomeromycota (numbers 1, 41, 16, 2, 3, and 4, respectively, in Fig. 2). The species with ATPases in each cluster belong to the following phyla: 1, Chytridiomycota and Zygomycota; 2, Basidiomycota; 3 and 4, Ascomycota; and 5, Basidiomycota and Ascomycota. This cluster distribution cannot be expected from the consensus tree that explains the evolutionary radiations of fungi [36,37], especially in the dikarya clade, which includes the two phyla Ascomycota and Basidiomycota. As discussed below, it seems that several ENA ATPases existed before the divergence of fungal phyla.

In plants seven *ENA genes* have been identified in bryophytes, from the moss *P. patens* [10], and the liverworts *M. polymorpha* [11] and *Riccia fluitans* (Fig. 3). In addition, we have currently identified a partial sequence of an *ENA* cDNA from the moss *Bartramia pomiformis*. Taking into consideration that we have found ENA ATPases in all the bryophyte species that we have investigated, the ENA ATPase may exist in all or in most bryophyte species, as described for fungi. Among algae, only in *Tetraselmis viridis* has an ENA ATPase been identified. In contrast, *ENA* genes were not found in the seagrass *Cymodocea nodosa* and in barley (*Hordeum vulgare*) [38], and our Blast searches up to the end of 2009 did not find any *ENA* gene in the genomes of vascular plant sequenced so far, including *S. moellendorfii*. Genes encoding ENA ATPases are also absent in brown or green algae [22] and we could not identify genes encoding ENA ATPases in the Chlorophycea *Chlamydomonas* or Prasinophyceae *Ostreococcus* genome sequences. However, active Na^+ efflux in *Chara longifolia* at pH 9.0 strongly suggests the existence of a Na^+ ATPase [39], but the identity of this ATPase is not known.

Fragments of genes that could encode ENA ATPases have been cloned from the protozoa *T. brucei*, *T. cruzi*, and *L. donovani* [13], and the ENA ATPase from *T. cruzi* and its encoding gene have been characterized [14]. *Entamoeba histolytica* also expresses a Na^+-ATPase activity that is ouabain-insensitive, stimulated by Na^+ and K^+ similarly, and sensitive to furosemide, which might correspond to an ENA ATPase [15]. In fact, we identified a putative *ENA* gene in the genome of *E. histolytica* (accession number: XM_652464). We carried out Blast searches in protozoan genome databases using as query an ENA ATPase sequence, finding translated sequences that unequivocally corresponded to ENA ATPases in *T. brucei*, *Leishmania infantum*, *L. donovani*, *Leishmania major*, and *Leishmania braziliensis*. The *Leishmania* sequences are annotated as Ca^{2+}-ATPases but their amino acid

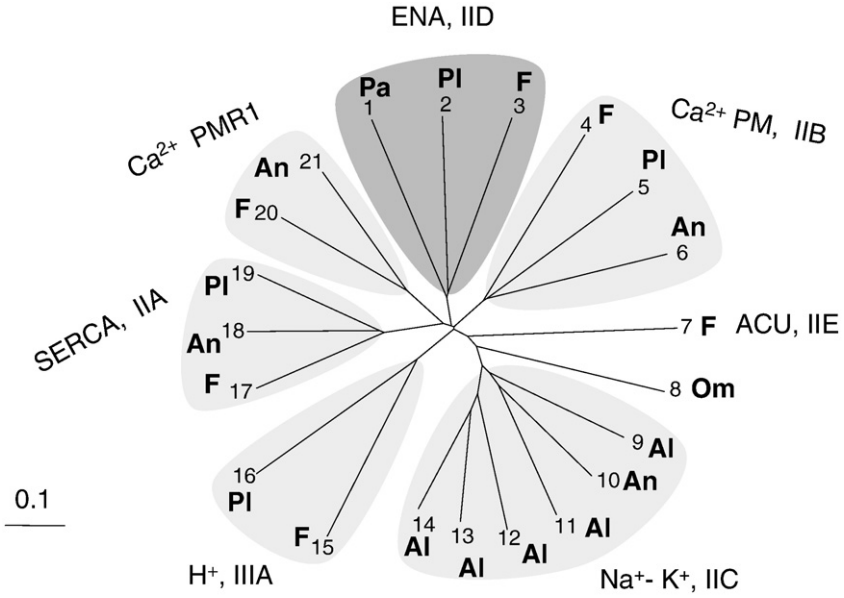


Fig. 1. Phylogenetic relationships of ENA, IID, with related P-type ATPases: IIA, sarcoendoplasmic reticulum Ca2+ (SERCA); IIB, plasma membrane (PM) Ca2+; IIC, Na+K+; IIE, ACU. Two H+-ATPases, IIIA, have been included as outgroup sequences. Subfamily names as given by Axelsen and Palmgren [122]. Abbreviations: Al, alga; An, animal; Om, oomycete; Pa, protozoa; Pl, plant; F, fungus. Number identification and accession numbers: 1, *Trypanosoma cruzi* ENA1, XP_817442; 2, *Physcomitrella patens* ENA1, CAD91917; 3, *Saccharomyces cerevisiae* ENA1, P13587; 4, *S. cerevisiae* PMC1, P38929; 5, *Arabidopsis thaliana* ACA1, Q37145; 6, *Rattus norvegicus* Atp2b1 PMCA1, P11505; 7, *Ustilago maydis* ACU1, CAF22245; 8, *Pythium aphanidermatum* KPA1, CAI99409; 9, *Porphyra yezoensis* KPA1, CAI99405; 10, *R. norvegicus* Atp1a1 Na+/K+, P06685; 11, *Heterosigma akashiwo* HANA, BAA82752; 12, *Chlamydomonas reinhardtii* Na+/K+, XP_001696293; 13, *Ostreococcus tauri* Na+/K+, CAL50001; 14, *Flabellia petiolata* KPA1, CAI99406; 15, *Neurospora crassa* PMA1 H+, P07038; 16, *A. thaliana* AHA1, P20649; 17, *N. crassa* NCA1, CAB65295; 18, *Oryctolagus cuniculus* SERCA1a, ABW96358; 19, *A. thaliana* ECA3, Q9SY55; 20, *S. cerevisiae* PMR1, P13586; 21, *Homo sapiens* ATP2C, P98194. The amino acid sequences were aligned with the CLUSTAL X program [123] with default settings and the tree was visualized with the TreeView program [124].

sequences identify them as typical ENA ATPases (Fig. 3). An ouabain-insensitive Na^+-ATPase activity studied in *Leishmania amazonensis* [40] has the characteristics of an ENA ATPase. We have also identified *ENA* genes in the genomes of the amoeba-flagellate *Naegleria gruberi* (accession number: gw1.5.10.1) and the Filasterea *Capsaspora owczarzaki* (accession number: CAOG_03039).

In summary, the phylogenetic relationships of the protein sequences encoded by the currently identified *ENA* genes or cDNAs suggest that a single ancestral gene gave rise to two or three genes before or immediately after the plant–animal–fungal splits. This conclusion is suggested by (i) the fact that the chlorophyte *Tetraselmis* enzyme might be closer to some protozoa ENA than to bryophyte ENA; (ii) two ENA ATPases of *Spizellomyces* (Chytridiomycota; Spizp1 and Spizp2), *Rhizopus* (Zygomycota; Ro1 and Ro2), and *Phycomyces* (Zygomycota; Pb2 and Pb4) are as divergent as protozoal, bryophyte, and fungal ENA ATPases; and (iii) the *Neurospora* (Ascomycota) NcENA1 is closer to the *Ustilago* (Basidiomycota) UmENA1 than to the *Neurospora* NcENA2 (Fig. 3). All this suggests that the ENA ATPase was important at a certain moment of the evolution of life. Interestingly, while extant fungi maintain the enzyme, with very few exceptions, in plants only bryophytes maintain it. Possibly, at a certain point of their evolution, some early terrestrial plants lost the ENA ATPase and vascular plants evolved from one lacking it. This loss could occur because at the prevailing ΔpH and ΔpNa across the plasma membrane, Na^+ extrusion mediated by a Na^+/H^+ antiporter did not have energetic restrictions (Section 4.3).

3. Sequence and structural characteristics

Type II, P-type ATPases have ten transmembrane fragments and two cytoplasmic loops that play a central role in the functional mechanism of the enzyme [15,41]. In the cytoplasmic loops three important domains have been characterized: actuator (A), between TM2 and TM3; phosphorylation (P), between TM4 and TM5, where an aspartic residue is phosphorylated; and nucleotide binding (N), where ATP is bound [42–45]. The ENA ATPase shows maximum sequence similarity to Ca^{2+}- and Na,K-ATPases in which the functional mechanisms have been extensively studied [45–49]. ENA ATPases maintain conserved sequences that are characteristic of P-type ATPases and others that are specific to ENA ATPases.

To investigate the specific structural features of the 3D structure and the position of the conserved fragment sequences of the ENA ATPase, a homology modeling of ScENA1 was constructed using the protein structure prediction Phyre server [50]. The highest scoring alignments were obtained with the SERCA (PDB accession number 3B9R), Na,K- (PDB accession number 3B8E), and H^+-ATPases (PDB accession number 3B8C). The overall sequence identities of ScENA1 with the abovementioned ATPases were 26, 27, and 21%, respectively, but in the P domain the sequence homology was much higher, 41% with the SERCA ATPase. The SERCA ATPase has 994 amino acid residues versus 1091 in ScENA1, which is 13 and 39 residues longer than the former in the N- and C-terminus, respectively. In the C-terminus of ScENA1 a potential calmodulin binding site has been proposed [51]. In all models the four domains, A, N, P, and transmembrane (blue, green, red, and yellow, respectively, in Fig. 4) were clearly recognized. Fig. 4 shows a cartoon of the ScENA ATPase constructed using the SERCA ATPase as a structural template.

Sequence alignments showed that ENA proteins possess the highly conserved sequences that correspond to the catalytic sites of all P-type ATPases, especially the signature sequences $DKTGT^{373}$, which includes the phosphorylable aspartate, and the TGD^{675} and $DPPR^{652}$ sequences, as well as the $DGVND^{761}$ sequence involved in Mg^{2+} binding. They also conserve the $TGES^{183}$ residues in the A domain, and the conserved residues F^{537}, K^{542}, K^{561} involved in nucleotide binding (red circle in Fig. 4; the residue numbers correspond to ScENA1; see also [44,52]).

All ENA ATPases have a conserved sequence singularity in the P domain close to the conserved DGVND loop. This conserved sequence,

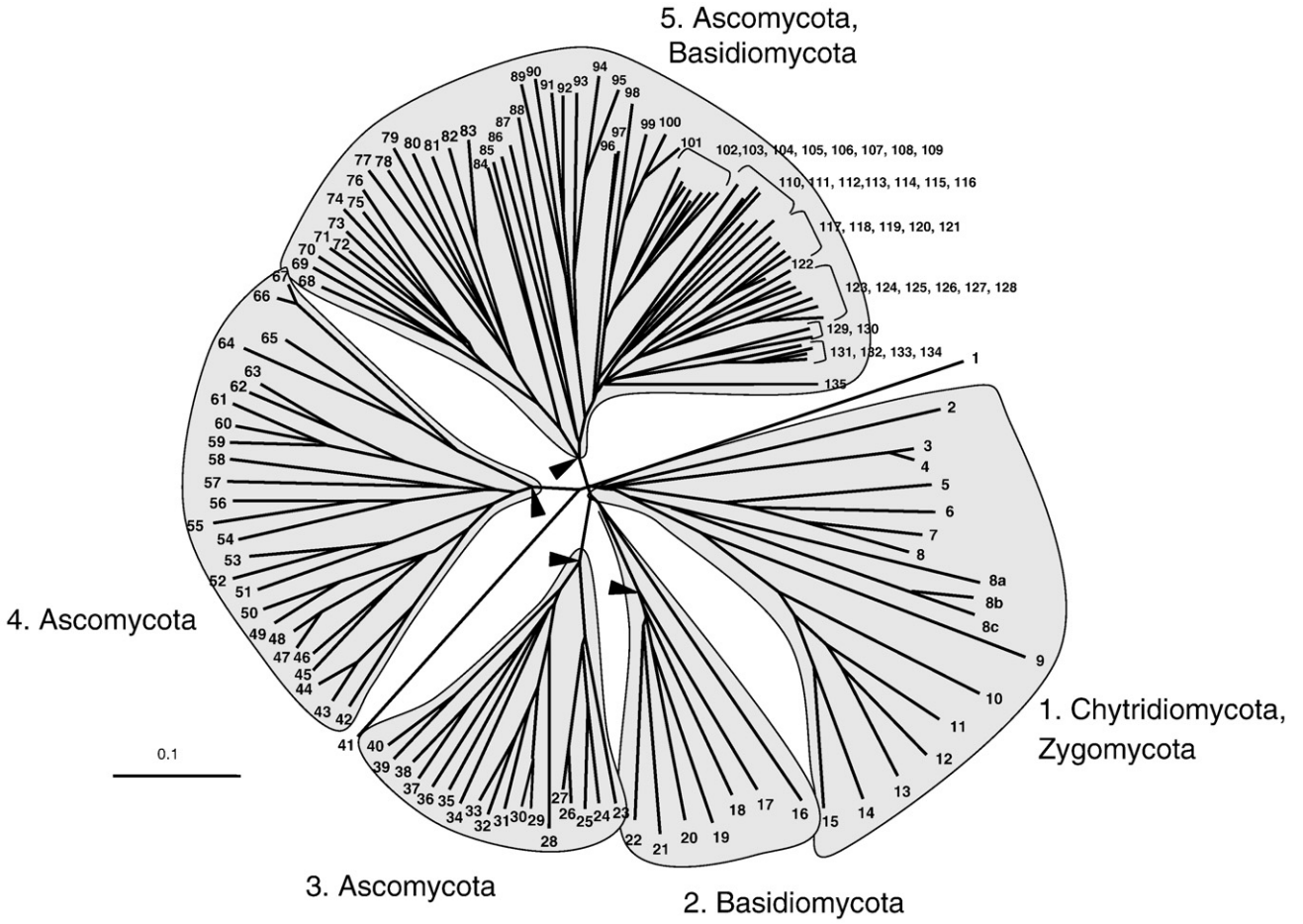


Fig. 2. Phylogenetic tree of fungal ENA ATPases. All ENA ATPases encoded within the 65 fungal genomes that have been sequenced are included. Number identifications: 1: *Cryptococcus neoformans* (B): XM_772704.1; 2: *Spizellomyces punctatus* (C): SPPG_08664; 3: *Glomus intraradices* (G): CAJ42021.1; 4: *G. intraradices* (G) CAJ42022.1; 5: *Mucor circinelloides* (Z): fgeneshMC_pm.1_#_404; 6: *Phycomyces blakesleanus* (Z): e_gw1.1.327.1; 7: *Rhizopus oryzae* (Z): RO3G_11514.3; 8: *M. circinelloides* (Z): e_gw1.6.60.1; 8a: *S. punctatus* (C): SPPG_06728; 8b: *Allomyces macrogynus* (C): AMAG_16513; 8c: *A. macrogynus* (C): AMAG_02549; 9: *S. punctatus* (C): SPPG_05612; 10: *Mucor circinelloides* (Z): stExt_fgeneshMC_pm.C_100102; 11: *P. blakesleanus* (Z): estExt_Genewise1Plus.C_310035; 12: *P. blakesleanus* (Z): e_gw1.4.635.1; 13: *P. blakesleanus* (Z): e_gw1.21.22.1; 14: *R. oryzae* (Z): RO3G_00014.3; 15: *M. circinelloides* (Z): e_gw1.1.1402.1; 16: *Ustilago maydis* (B): CAQ86601.1; 17: *Pleurotus ostreatus* (B): estExt_Genewise1Plus.C_20763; 18: *Schyzophylum commune* (B): e_gw1.3.1437.1; 19: *Coprinus cinereus* (B): CC1G_07450.2; 20: *C. cinereus* (B): CC1G_00228.2; 21: *P. ostreatus* (B): e_gw1.4.2196.1; 22: *P. ostreatus* (B): e_gw1.4.164.1; 23: *Histoplasma capsulatum* (A): HCBG_04114.2; 24: *Coccidioides posadasii* (A): EER24302.1; 25: *Uncinocarpus reseeii* (A): UREG_00592.1; 26: *Mycrosporum gypseum* (A): MGYG_06912.1; 27: *Trychophyton equinum* (A): TEQG_03832.1; 28: *Verticillium dahliae* (A): VDAG_09301.1; 29: *Gibberella zeae* (A): XP_384421.1; 30: *Nectria haematococca* (A): estExt_fgenesh1_pg.C_sca_20_chr6_4_00051; 31: *Trichoderma reesei* (A): estExt_GeneWisePlus.C_250195; 32: *Trichoderma atroviridae* (A): estExt_fgenesh1_pg.C_40579; 33: *Trichoderma virens* (A): e_gw1.4.635.1; 34: *Cryphonectria parasitica* (A): Crypa1.fgenesh1_pg.C_scaffold_3000418; 35: *Magnaporthe grisea* (A): MGG_05078.6; 36: *Neurospora crassa* (A): XP_962099.1; 37: *Chaetomium globosum* (A): XP_001224140.1; 38: *Sporotrichum thermophile* (A): estExt_fgenesh1_pm.C_10194; 39: *Botrytis cinerea* (A): BC1G_15342.1; 40: *Sclerotinia sclerotiorum* (A): SS1G_10477.1; 41: *Schyzosaccharomyces pombe* (A): CAB46699.1; 42: *Aspergillus clavatus* (A): XP_001273189.1; 43: *Aspergillus fumigatus* (A): XP_749268.1; 44: *Neosartorya fisheri* (A): XP_001265693.1; 45: *C. posadasii* (A): EER24612.1; 46:*U. reseeii* (A): UREG_00257.1; 47: *M. gypseum* (A): MGYG_00748.1; 48: *T. equinum* (A): TEQG_02574.1; 49: *Blastomyces dermatitidis* (A): BDBG_00747.1; 50: *Histoplasma capsulatum* (A): HCBG_04401.2; 51: *Stagonospora nodorum* (A): SNOG_11155.1; 52: *Cochliobolus heterotrophus* (A): estExt_fgenesh1_pg.C_70257; 53: *Pyrenophora tritici-repentis* (A): PTRG_09434.1; 54: *N. crassa* (A): CAB65297.1; 55: *C. globosum* (A): XP_001228662.1; 56: *S. thermophile* (A): estExt_Genewise1.C_53299; 57: *M. grisea* (A): MGG_13279.6; 58: *Verticillium dahliae* (A): VDAG_02935.1; 59: *T. virens* (A): fgenesh1_pm.C_scaffold_24000006; 60: *T. reesei* (A): estExt_GeneWisePlus.C_240293; 61: *T. atroviridae* (A): e_gw1.5.810.1; 62: *G. zeae* (A): XP_385171.1; 63: *N. haematococca* (A): estExt_Genewise1.C_sca_17_chr8_1_00470; 64: *Mycosphaerella fijiensis* (A): estExt_Genewise1Plus.C_150168; 65: *Mycosphaerella graminicola* (A): estExt_Genewise1Plus.C_chr_80432; 66: *Hortaea werneckii* (A): ABD64570.1; 67: *H. werneckii* (A): ABD64571.1; 68: *Pichia stipitis* (A): XP_001385604.2; 69: *Debaryomyces hansenii* (A): AAK28385.2; 70: *Debaryomyces occidentalis* (A): AAB86426.1; 71: *Lodderomyces* elongisporus (A): XP_001526650.1; 72: *Candida albicans SC5314* (A): XP_719032.1; 73: *C. albicans SC5314* (A): XP_716992.1; 74: *D. hansenii* (A): AAK52600.2; 75: *P. stipitis* (A): XP_001387351.1; 76: *D. occidentalis* (A): AAB86427.1; 77: *Zygosaccharomyces rouxii* (A): XP_002499224.1; 78: *Torulaspora delbrueckii* (A): AAZ04389.1; 79: *Saccharomyces cerevisiae* (A): P13587; 80: *Vanderwaltozyma polyspora* (A): XP_001644531.1; 81: *Ashbya possypii* (A): AAS54394.1; 82: *Kluyveromyces lactis* (A): XP_456007.1; 83: *K. lactis* (A): XP_454607.1; 84: *Yarrowia lipolytica* (A): XP_499639.1; 85: *Y. lipolytica* (A): XP_504141.1; 86: *Sporobolomyces roseus* (B): e_gw1.1.48.1; 87: *U. maydis* (B): CAQ86600.1; 88: *Mallasezia globosa* (B): XP_001729255.1; 89: *C. globosum* (A): XP_001222926.1; 90: *V. dahliae* (A): VDAG_03863.1; 91: *G. zeae* (A): XP_382853.1; 92: *N. haematococca* (A): e_gw1.20.512.1; 93: *N. haematococca* (A): e_gw1.2.25.1; 94: *T. reesei* (A): estExt_fgenesh5_pg.C_160247; 95: *T. virens* (A): fgenesh1_pm.C_scaffold_12000206; 96: *T. equinum* (A): TEQG_03743.1; 97: *M. gypseum* (A): MGYG_06822.1; 98: *Aspergillus niger* (A): fgenesh1_pg.C_scaffold_6000163; 99: *A. clavatus* (A): XP_001270181.1; 100: *A. fumigatus* (A): XP_747708.1; 101: *N. fisheri* (A): XP_001257574.1; 102: *A. niger* (A): fgenesh1_pg.C_scaffold_11000476; 103: *A. fumigatus* (A): XP_751881.1; 104: *N. fisheri* (A): XP_001267067.1; 105: *M. gypseum* (A): MGYG_07172.1; 106: *U. reseeii* (A): UREG_02849.1; 107: *C. posadasii* (A): EER26862.1; 108: *H. capsulatum* (A): HCBG_01348.2; 109: *B. dermatitidis* (A): BDBG_07515.1; 110: *B. cinerea* (A): BC1G_11540.1; 111: *B. cinerea* (A): BC1G_04830.1; 112: *S. sclerotiorum* (A): SS1G_06551.1; 113: *M. grisea* (A): MGG_02074.6; 114: *N. haematococca* (A): estExt_Genewise1Plus.C_sca_17_chr8_1_00310; 115: *M. grisea* (A): MGG_10730.6; 116: *V. dahliae* (A): VDAG_05014.1; 117: *V. dahliae* (A): VDAG_09836.1; 118: *S. nodorum* (A): SNOG_15715.1; 119: *C. heterotrophus* (A): fgenesh1_pm.C_scaffold_4000168; 120: *Alternaria brassicicola* (A): AB06515.1; 121: *P. tritici-repentis* (A): PTRG_07283.1; 122: *N. crassa* (A): CAB65298.1; 123: *S. thermophile* (A): estExt_Genewise1Plus.C_60137; 124: *C. globosum* (A): XP_001219310.1; 125: *T. virens* (A): fgenesh1_pg.C_scaffold_14000220; 126: *N. haematococca* (A): estExt_Genewise1Plus.C_sca_1_chr1_3_01487; 127: *Fusarium oxysporum* (A): AAR01872.1; 128: *G. zeae* (A): XP_385095.1; 129: *M. fijiensis* (A): estExt_fgenesh1_pg.C_11227; 130: *M. graminicola* (A): estExt_fgenesh1_pm.C_chr_50123; 131: *S. nodorum* (A): SNOG_09636.1; 132: *P. tritici-repentis* (A): PTRG_11526.1; 133: *A. brassicicola* (A): AB01088.1; 134: *C. heterotrophus* (A): estExt_fgenesh1_pg.C_10064; 135: *M. graminicola* (A): fgenesh1_pm.C_chr_2000108. The amino acid sequences were aligned with the CLUSTAL X program [123] with default settings. The data sets were bootstrapped (1000 resampling events) and the tree was visualized with TreeView program [124]. Arrowheads indicate selected nodes with bootstrap values of 1000 in order to show the statistical support of the existence of more than one cluster for ENA ATPases in Ascomycota and Basidiomycota. The letter in brackets indicates the phyla: A, Ascomycota; B, Basidiomycota; C, Chytridiomycota; G, Glomeromycota; Z, Zygomycota.

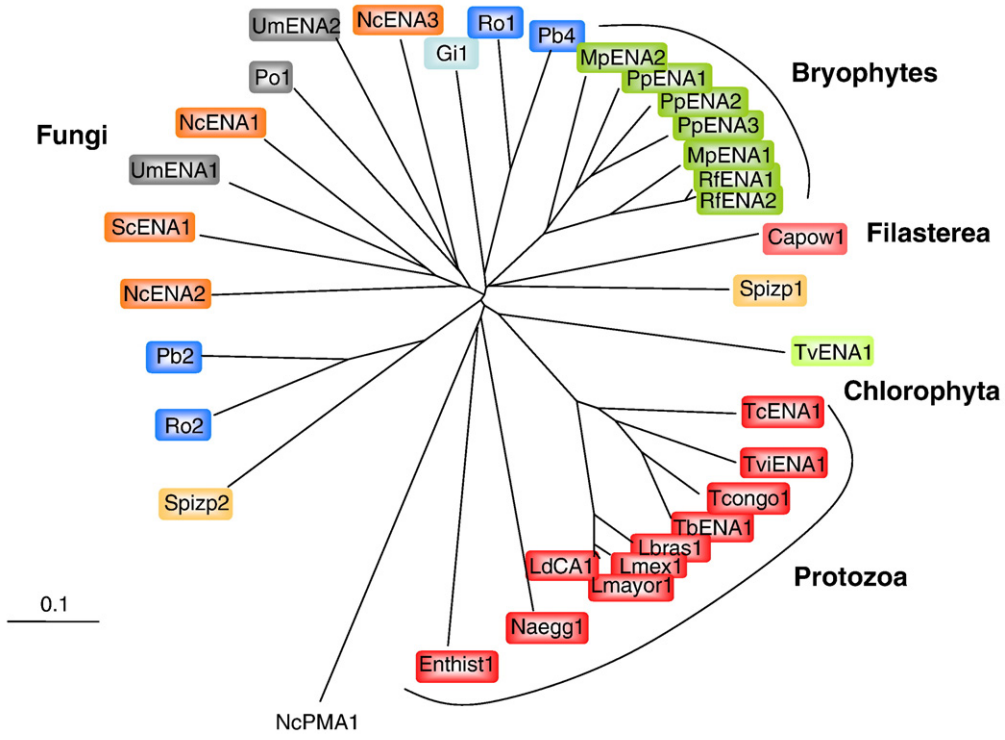


Fig. 3. Phylogenetic relationships among the ENA ATPases of different taxonomic groups. The PMA1 H^+-ATPase of *Neurospora crassa* has been included as an outgroup sequence. Fungal sequences have been selected to show the characteristic divergences described in text and Fig. 2. Species abbreviations and accession numbers: NcPMA1, *N. crassa*: XP_957691.2; Spizp2, *Spizellomyces punctatus*: SPPG_05612; Ro2, *Rhizopus oryzae*: RO3G_00014.3; Pb2, *Phycomyces blakesleanus*: e_gw1.4.635.1; NcENA2, *N. crassa*: CAB65297.1; ScENA1, *Saccharomyces cerevisiae:* P13587; UmENA1, *Ustilago maydis*, CAQ86600.1; NcENA1, *N. crassa*: CAB65298.1; Po1, *Pleurotus ostreatus*, e_gw1.4.164.1; UmENA2, *U. maydis*, CAQ86601.1; NcENA3, *N. crassa*: XP_962099.1; Gi1, *Glomus intraradices*: CAJ42021.1; Ro1, *R. oryzae*: RO3G_11514.3; Pb4, *P. blakesleanus*: e_gw1.1.327.1; MpENA2, *Marchantia polymorpha*: CAX27440; PpENA1, *Physcomitrella patens*: CAD91917; PpENA2, *P. patens*: CAD91924; PpENA3, *P. patens*: CAX20544; MpENA1, *M. polymorpha*: CAX27437; RfENA1, *Riccia fluitans:* FN691478; RfENA2, *R. fluitans:* FN691479; Capow1, *Capsaspora owczarzaki*: CAOG_03039; Spizp1, *S. punctatus*: SPPG_08664; TvENA1, *Tetraselmis viridis*: FN691482; TcENA1, *Trypanosoma cruzi:* XP_817442.1; TviENA1, *Trypanosoma vivax:* TVIV.0.112366; Tcongo1, *Trypanosoma congolense*: congo1013f03; TbENA1, XP_827683; Lbras1, *Leishmania braziliensis*: XP_001568308.1; Lmex1, Lmayor1, *Leishmania mayor*: XP_843313.1; LdCA1, *Leishmania donovani*: AAC19126; Naegg1, *Naegleria gruberi*: gw1.5.10.1; Enthist1, *Entamoeba hystolytica*: XM_652464. The tree was constructed as described in Fig. 1.

MIEALHRR in ScENA1, shows certain variability in ENA ATPases but all keep a high similarity. The modeled P domain shows a typical Rossmann fold with a central six-stranded beta-sheet flanked by alpha-helices one of which is where the conserved MIEALHRR^{748} sequence of ENA proteins locates (Fig. 5). The spatial disposition of this conserved sequence and the measurement of the atomic distances between the basic residues RR^{748} and the residues that are involved in the catalytic cycle of the P-type ATPases (DKTGT^{373}, TGD^{675}, DPPR^{652}, DGVND^{761}) suggest that the MIEALHRR fragment does not have a critical role on catalytic activation. However, mutation in this region of the HRRGR^{746} residues of the *N. crassa* ENA1 ATPase into the corresponding QSYDE sequence of the rabbit SERCA ATPase abolished the Na^+ efflux function of NcENA1 in yeast cells without conferring the capacity of transporting Ca^{2+}.

The TM domain, which consists of ten transmembrane spanning helices as already explained, has a low sequence homology with the SERCA ATPase, 19%, but a very similar overall structure. Alignments of the sequences of these fragments and those of ScENA1 showed putative residues that can be involved in Na^+ or K^+ binding site of ENA proteins (Fig. 4). Seven residues that contribute to the cation binding sites of Ca^{2+}- and Na,K-ATPases locate in TM4, TM5, TM6, and TM8 [48,53,54], and only two out of these seven residues, N in TM5 and T in TM6, are present in most ENA ATPases. In TM4 there is another sequence peculiarity of ENA ATPases. The unwound region IPEGLP^{312}, where one cation binding pocket is formed [55] in Ca^{2+} and Na,K-ATPases, changes into IPSSLV^{330} in ScENA1. In most fungal ENA ATPases the SS residues are not conserved but a Ser residue occurs in any of the two positions. Interestingly, bryophyte ENA ATPases keep the IPEGLP sequence, but no functional differences between fungal and plant ENA ATPases have been reported.

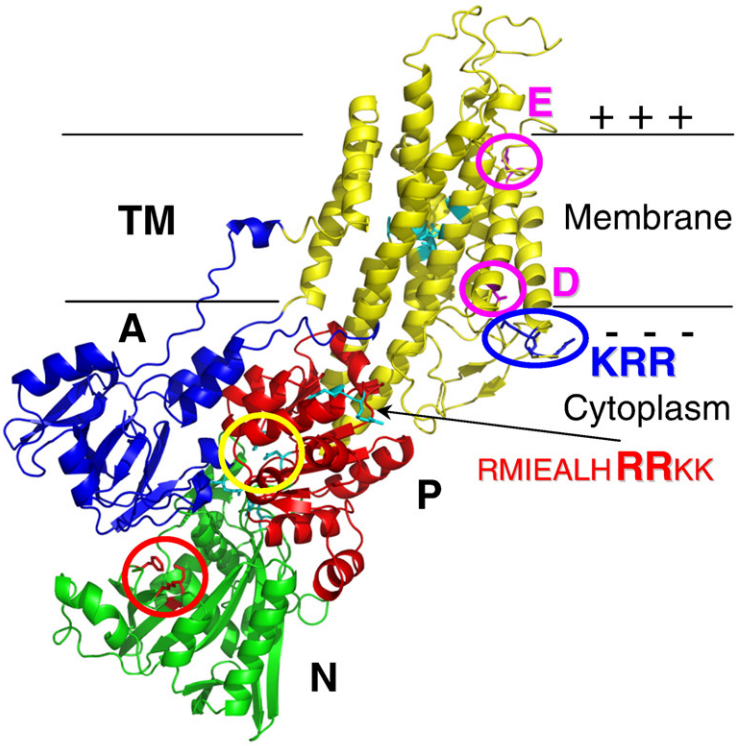
At the end of the TM10 helix most ENA ATPases and the Na,K-ATPase have three basic residues (KRR^{1049} in ScENA1, RRR^{1005} in pig renal Na,K-ATPase; dark blue circle in Fig. 4). This arginine cluster at the membrane edge region has been proposed as a putative voltage sensor of the membrane potential [54].

A mutational study on the ENA1 ATPase of *Z. rouxii* has been published [56]. In this study nine Asp and Glu residues in the P domain, and two in TM7 and TM10 were mutated. Two of these residues, D852A and E981A, in TM7 and TM10, respectively (Fig. 4, magenta circled in ScENA1), are conserved in all ENA proteins and absent in Ca^{2+}, Na,K-, and H^+-ATPases. Their mutations substantially reduced the function of ScENA1, which suggests that these residues have been conserved for functional reasons.

4. Functional characteristics

Two experimental approaches have been used in functional studies of ENA ATPases: biochemical studies of the ATPase in plasma membranes and determination of the Na^+ and K^+ effluxes mediated by different ENA ATPases in null *ena* mutants of *S. cerevisiae*.

Biochemical studies of P-type ATPases have been routinely based on the rate of ATP hydrolysis by crude or purified membrane preparations. However, by unknown reasons, ENA1-dependent ATP hydrolysis by plasma membranes of yeast cells is very low and extensive biochemical studies of ScENA1 have not been carried out. In contrast with the difficulties of biochemical studies, functional



TM4

SERCA	289	IRGAIYYfkiaVALAVAAIPEGLPAVI	315
Na/K	318	LEAVIFL----IGIIVANVPEGLLATV	340
ScENA1	307	FDVDKRVaiyaICVALSMIPSSLVVVL	333

TM5

SERCA	757	MKQFIRYLISSNVGEVVCIFLTA	779
Na/K	772	LKKSIAYTLTSNIPEITPFLIFI	794
ScENA1	812	IQKFVLQLLAENVAQALYLIIGL	834

TM6

SERCA	788	IPVQLLWVNLVTDGLPATALG	808
Na/K	803	GTVTILCIDLGTDMVPAISLA	823
ScENA1	848	SPVEVLWIIVVTSCFPAMGLG	868

TM8

SERCA	893	APEPMTMALSVLVTIEMCNALNSL	916
Na/K	915	EFTCHTPFFVTIVVVQWADLVICK	938
ScENA1	939	VYRSRSAAFATMTWCALILAWEVV	962

Fig. 4. Structural and sequence characteristics of ENA ATPases. Top: overall structural model of the ENA1 ATPase of *S. cerevisiae*. Bottom: alignments of the TM4, TM5, TM6, and TM8 sequences in SERCA, Na/K, and ScENA1 ATPases. The structural model was constructed using the protein structure prediction Phyre server [50], www.sbg.bio.ic.ac.uk/phyre/, based on the model of the SERCA ATPase and visualized with the standard molecular viewer PyMOL 2002 (DeLano, W.L. The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, USA; http://pymol.sourceforge.net/); the ten transmembrane helices are shown in yellow, the nucleotide-binding domain (N) in green, the actuator domain (A) in blue, and the phosphorylation domain (P) in red; key residues mentioned in the text are highlighted in a red circle (F^{537}, K^{542}, K^{561} conserved residues belonging to the N domain) or in a yellow circle (DKTGT^{373}, DGVND^{761}, DPPR^{652} and TGD^{675} in P domain); red letters depicted the ENA conserved sequence. The dark blue circle shows the three basic residues of KRR^{1049} in ScENA1 conserved in the Na,K-ATPase that has been proposed as a putative voltage sensor of the membrane potential [54]; magenta circles indicate the negative charged residues D^{896} and E^{1025} of ScENA1 that correspond to the mutated D^{852} and E^{981} of *Zygosaccharomyces rouxii* ENA1 that are conserved in all ENA proteins and seem to be functionally important. In the alignment the amino acid residues that are involved in cation binding are highlighted. SERCA and Na/K sequences correspond to the crystallized ATPases, PDB ID: 3B9R and 3B8E, respectively.

studies based on the determination of Na^+ and K^+ effluxes that either disappear in null *ena* mutants or that appear when *ENA* genes or cDNAs are expressed Na^+-K^+ efflux mutants of *S. cerevisiae* have been extensively carried out with many ENA ATPases. Although a *Δena1-4* mutant could be used for these tests a double *Δena1-4 Δnha1* mutant is normally used (NHA1 is the Na^+ or K^+/H^+ antiporter described below). The induction of ENA-mediated effluxes in these strains may be deduced from the increases in their Na^+ or K^+ tolerances.

4.1. Na^+ and K^+ effluxes

Although the first report on the ENA1 ATPase of *S. cerevisiae* demonstrated that it mediated Li^+ and Na^+ effluxes and suggested

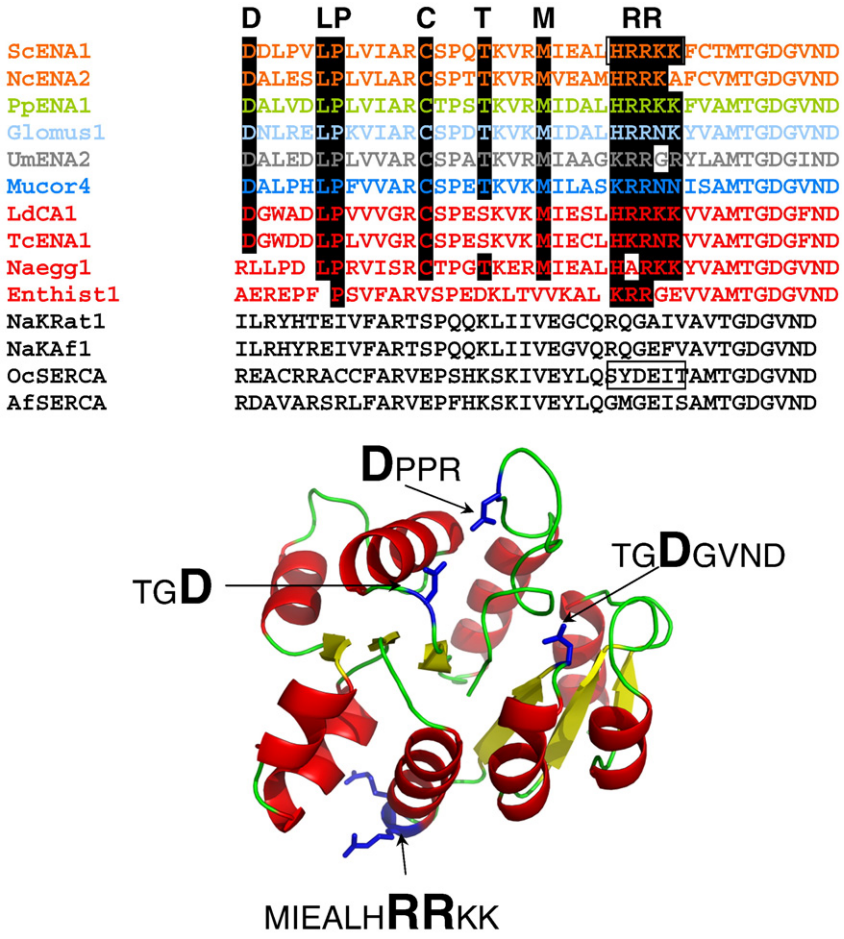


Fig. 5. Amino acid residues that are specifically conserved in the P domain of ENA ATPases, as shown in Fig. 4. Top: alignment of representative sequences of ENA and other P-type ATPases. The boxed sequence in OcSERCA shows the amino acids residues which substituted for the HKRRG residues in an NcENA1 mutant (see text). Bottom: Rossman fold of the P domain in ScENA1 amplified from Fig. 4. Side chains of conserved Asp residues and the ENA conserved RR side chains are shown as sticks. Accession numbers: ScENA1: *Saccharomyces cerevisiae*, P13587; NcENA2: *Neurospora crassa*, CAB65297.1; PpENA1: *Physcomitrella patens*: CAD91917; Glomus1: *Glomus intraradices*, CAJ42021.1; UmENA2: *Ustilago maydis,* CAQ86601.1; Mucor4: *Mucor circinelloides,* stExt_fgeneshMC_pm.C_100102; LdCA1: *Leishmania donovani*: AAC19126; TcENA1: *Trypanosoma cruzi:* XP_817442.1; Naegg1: *Naegleria gruberi*: gw1.5.10.1; Enthist1, *Entamoeba hystolytica*: XM_652464; NaKRat1: *Rattus norvegicus*, P06685.1; NaKAs1: *Artemia franciscana*, P17326.1; OcSERCA: *Oryctolagus cuniculus*, ABW96358; AsSERCA: *A. franciscana*, P35316.1.

that it could mediate K^+ efflux [3], the notion that the ENA ATPase is not specific for Na^+ was not established until the conditions for the phosphorylation of the enzyme were studied [7]. In these experiments, ScENA1 is phosphorylated from ATP in the presence of alkali cations and from Pi in the absence of alkali cations [7]. In the first assay all alkali cations were effective and in the second all of them inhibited, which indicates that the specificity of the enzyme is low. Similarly, an ENA specific ATP hydrolytic activity has been determined in the membrane fraction of mammalian cells expressing the ENA ATPase of *T. cruzi*. This activity is insensitive to ouabain and stimulated by both Na^+ and K^+ [14].

Later, the cloning of two ENA ATPases from *S. occidentalis* with different specificities for Na^+ and K^+ in flux experiments further support the notion of the double physiological function of ENA ATPases as Na^+ and K^+ pumps [8]. Finally, the identification of the CTA3 ATPase of *S. pombe* helped to establish the current notion that some ENA ATPases are specific for Na^+ efflux while others show a poor discrimination between Na^+ and K^+ or even that some might be K^+ specific [9]. Systematic studies of the specificity of ENA ATPases can be carried out in transformants of an *ena1-4 nha1* null mutant of *S. cerevisiae*, which lack Na^+ and K^+ effluxes. The tests measure the proportion of Na^+ and K^+ effluxes mediated by different ATPases from cells loaded with similar amounts of Na^+ and K^+ in a medium in which the only alkali cation is Rb^+. In this medium, Rb^+ is taken up in exchange for Na^+ and K^+, and the effluxes of these cations are maintained even when a large proportion of them are lost to the external medium [9]. Fig. 6 shows the results of parallel experiments carried out with three ENA ATPases that have been proposed to have different cation specificity: NcENA1, Na^+ specific; CTA3, K^+ specific; and ScENA1, similarly effective for Na^+ and K^+. The results confirmed that the ENA1 ATPase of *N. crassa* is Na^+ specific while the other two mediate both Na^+ and K^+ effluxes. However, although in these efflux experiments CTA3 behaves similarly to ScENA1 and is not K^+ specific, previous [9] and present (Fig. 6B) growth experiments clearly support that CTA3 and ScENA1 exhibit different cation specificities. Similarly, the ENA ATPases of *S. cerevisiae* ScENA1, ScENA2, and ScENA4 (ScENA3 is identical to ScENA2) are Na^+ and K^+ unspecific in flux experiments when expressed independently but in wild type cells with the four ATPases, Na^+ efflux is much more important than K^+ efflux [9]. These contradictions suggest that selectivity does not depend exclusively from the sequence of the protein and is regulated by unknown factors. Selectivity may depend on the amount of the expressed protein or may be modulated by the C-terminal region of the protein, as proposed for the NHX1 *Arabidopsis* antiporter [57]. As regards the latter possibility, very little is known about the regulation of the activity of the ENA ATPase by the C-terminal region. Interestingly, fungal enzymes have much longer C-termini than bryophyte enzymes, which suggests that the C-terminus of fungal

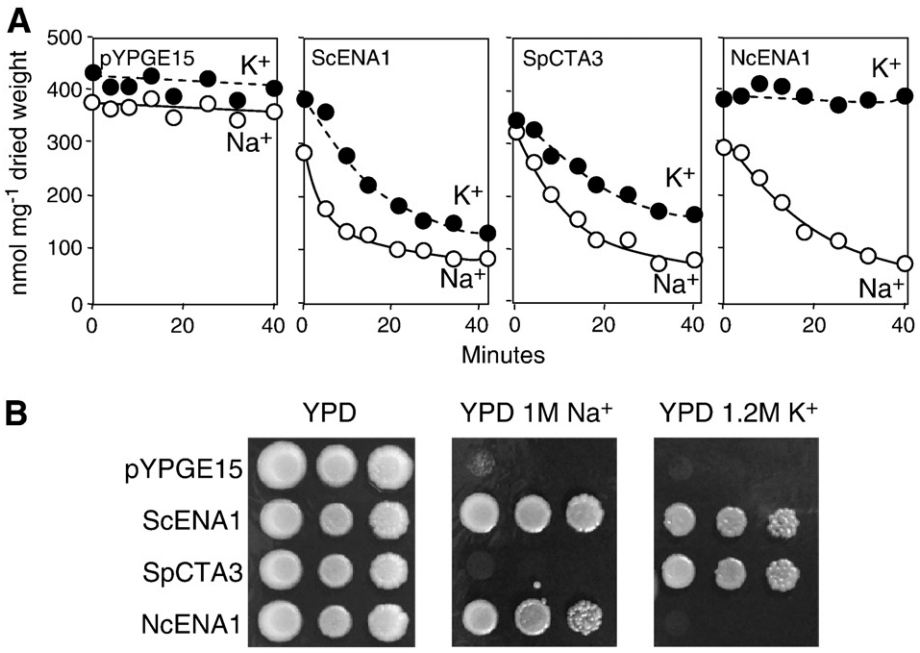


Fig. 6. Variability of the Na^+/K^+ discrimination of ENA ATPases. A, Time courses of K^+ and Na^+ losses from *Saccharomyces cerevisiae* B31 (*Δena1-4 Δnha1*) cells expressing the NcENA1 (*Neurospora crassa*), SpCTA3 (*Schyzosaccharomyces pombe*), or ScENA1 (*S. cerevisiae*). Yeast cells with similar contents of K^+ and Na^+ were prepared by growing the cells in the arginine phosphate medium described in [9] with 3 mM K^+ and then incubating these cells in 10 mM trisodium citrate pH 8.5, 50 mM NaCl, 2% glucose for 20 to 60 min depending on the expressed ATPase. K^+ and Na^+ losses were determined by transferring the Na^+ loaded-cells to 10 mM TAPS buffer pH 8.0 containing 2% glucose, 0.1 mM $MgCl_2$, 50 mM RbCl, and 20 mM NH_4Cl. B, Growth of serial dilutions of the same strains in plain YPD (1% yeast extract, 2% peptone, 2% glucose) or supplemented with the indicated concentrations of NaCl or KCl. In both panels, pYPGE15 indicates B31 cells transformed with the empty vector.

enzymes has a functional role. In fact, we have already mentioned a putative regulation of the ENA1 ATPase of *S. cerevisiae* by calmodulin [51]. Interestingly, two mutants that eliminate the last thirteen or sixteen amino acid residues of the C-terminus of the ScENA1 protein have been studied, but neither of them produces apparent changes in the activity of the ATPase [7]. Although, these mutations eliminate most of the C-terminus of the protein they probably do not affect the aforementioned calmodulin binding domain [51], which is coincident with the end of TM10.

In summary, the biochemical basis of the cation promiscuity of the ENA ATPase and the biological reasons for which some enzymes are cation specific while others are unspecific are unknown. In contrast,

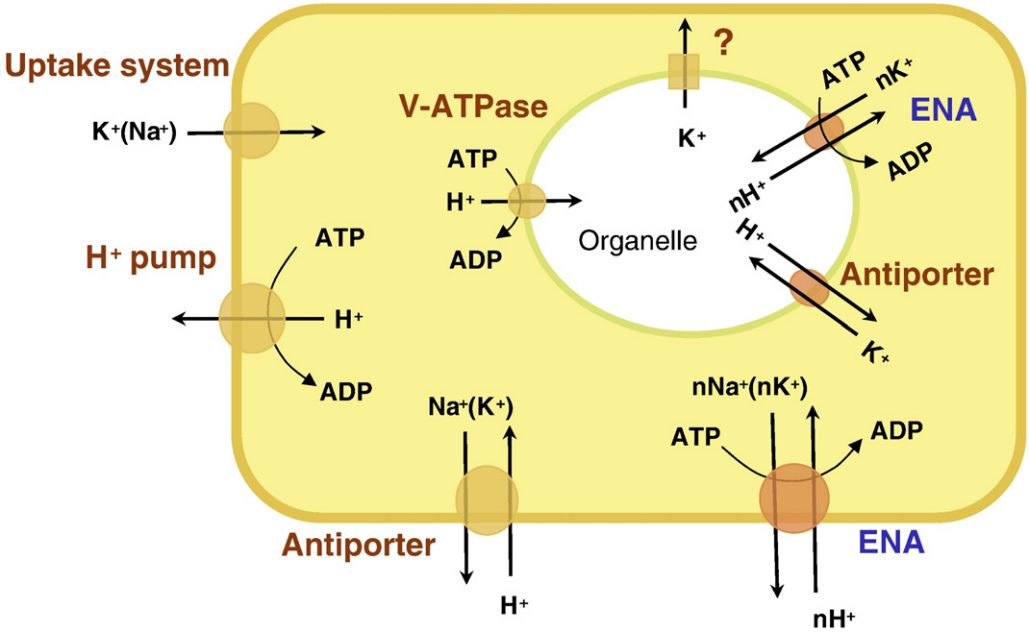


Fig. 7. Putative mechanism and function of the ENA ATPase in the plasma membrane and in organelles when external and lumenal pHs are above the cytosolic pH. In the plasma membrane the ENA ATPase mediates the effluxes of Na^+ or K^+ in exchange for H^+ in order to control cation contents and cytosolic pH. In organelles the ENA ATPase would mediate the entrance of K^+ and the return of H^+ to the cytosol. Electroneutral antiporters cannot fulfill these functions when external or organelle pHs are above the cytosolic pH. The ENA ATPase stoichiometry might be higher than one cation per ATP.

the function of the promiscuity is clear because the ENA ATPase maintains the cytosolic levels of any alkali cation when the antiporters are not functional (Fig. 7 and Section 4.3).

4.2. Induction of ENA gene expressions

Soon after the cloning of the *ScENA1* gene it was found that the expression of this gene in the normal growth conditions of *S. cerevisiae*, a complex medium with a low pH (≈4.5), was almost undetectable. Even when increasing the pH to 6.5 the expression of the gene in complex medium was still very low. High expressions were found at high pH, high Na^+, or in mineral medium, but high pH showed the strongest inductions [58]. It was also observed early on that the regulation of the promoter was complex and that the effects of calcineurin [59], HAL3 [60], and HAL1 [61] on the salt tolerance of yeast cells took place through the regulation of the expression of the *ScENA1* gene. Since these early reports the regulation of the expression of *ScENA1* has been studied extensively and there are two recent reviews on the subject [62,63].

Cloning of *ENA* genes in other fungal species generalized the notion that the ENA ATPase is weakly expressed in normal growth conditions: low pH (4–6) and low K^+ and Na^+ concentrations (<10 mM). As in *S. cerevisiae* high pH or high Na^+ concentrations trigger the expression of the enzyme in *S. occidentalis* [8], *N. crassa* [28], *D. hansenii* [29], *F. oxysporum* [30], *H. wernecki* [31], *Aspergillus nidulans* [64], and *U. maydis* [33]. In *P. patens* the three *ENA* genes also respond to alkali cations and pH, although in this species the combination of high pH and high Na^+ elicits an expression that is approximately ten times higher than with any of the two stimuli separately [11].

All these results strongly suggest that although the ENA ATPase is an enzyme that mediates Na^+ efflux its function is related or especially necessary in high pH media.

4.3. ENA ATPase and Na^+ or K^+/H^+ antiporters

The mechanism whereby the ENA ATPase pumps alkali cations out of the cell is unknown. However, through its similarity with other P-type ATPases a Na^+ (or K^+)/H^+ exchange can be anticipated [65]. Furthermore, considering the very negative membrane potentials that have been measured in *N. crassa* [66,67], which may be the norm in fungal species, the most likely possibility is that this Na^+ (or K^+)/H^+ exchange is electroneutral. The basis for this prediction is that if the membrane potential is very negative and the ENA ATPase mediated an electrogenic exchange, e.g. two Na^+ moving outward and one H^+ inward, Na^+ efflux would be restrained in high Na^+, high pH media, owing to thermodynamic reasons. In contradiction with this restriction, ENA ATPases are expressed to function in high pH media, as above described. The restriction does not apply if the exchange is electroneutral, and this mechanism does not violate any functional principle of P-type ATPases. In fact, although some P-type ATPases mediate electrogenic exchanges [48,68], electroneutral exchanges have been proposed for others [65,69].

If the ENA ATPase mediates Na^+/H^+ exchange its function results identical to that of Na^+/H^+ antiporters, which are universally present in fungi and plants [70]. However, this functional identity is only apparent because most fungal and plant Na^+/H^+ antiporters are electroneutral [70], in which Na^+ efflux is driven by the transmembrane ΔpH that is created by an external acidic pH. This limitation does not apply to an ATP fueled ENA ATPase. Therefore, the ENA ATPase and the electroneutral Na^+/H^+ antiporters are more complementary than redundant systems (Fig. 7). The same considerations apply to K^+ efflux. The only difference between K^+ and Na^+ effluxes is that the cytoplasmic K^+ concentration is high and K^+ efflux may be thermodynamically possible through an electroneutral K^+/H^+ antiporter even when the external pH is high if external K^+ is low. These principles of ionic homeostasis and pH dependence of antiporters are universal and apply also to bacteria [71,72], the only difference between bacteria and fungi is that many bacterial antiporters are electrogenic and function at high external pH [71].

Taking into consideration the multiplicity of environments regarding the pH range, and the Na^+ or K^+ concentrations where fungi [9] and bryophytes [11] can grow, the complementariness of electroneutral Na^+ or K^+/H^+ antiporters and ENA ATPases is evident. This complementariness was initially demonstrated in *S. cerevisiae* where it was shown that the NHA1 antiporter conferred protection against high Na^+ or K^+ concentrations at low pH but that at pH 7.0 ScENA1 was required [73]. However, *S. cerevisiae* is not adequate for this type of study because it grows poorly or does not grow when the external pH is higher than the cytosolic pH. In species that grow at pH 9.0 or even higher the requirement of the ENA ATPase can be shown more clearly. In fact, in *U. maydis* the disruption of the *ENA* genes abolishes growth of at high pH in the absence of Na^+ even when the external K^+ is as low as 1 mM [33]. The same occurs in *C. neoformans* although at slightly higher K^+ concentrations. In this species the *ENA1* gene behaves as a virulence gene, suggesting that the fungus may have to survive at elevated pH during infection of the mammalian host [17].

Interestingly, as in ENA ATPases, there are two types of plasma membrane Na^+/H^+ antiporters, one type has broad alkali cation specificity and the other is specific for Na^+ or Li^+ but not for K^+ [74,75]. A representative of the former type is NHA1 from *S. cerevisiae* [8,76] while a representative of the second type is Sod2 from *S. pombe* [77,78]. Both types of antiporters coexist in some fungi [79–81] but only the antiporters of broad substrate specificity seem to be involved in the regulation of the K^+ content and cellular pH [75] and in the cell cycle [82]. Interestingly, the transmembrane domains of SpSod2 and ScNHA1 are very similar in sequence while the hydrophilic carboxy termini are very different [79]. Sequence characteristics to identify the specificity of Na^+ (or K^+)/H^+ antiporters has not been established [75].

The general principle that ENA ATPases and electroneutral antiporters control the cellular Na^+ and K^+ contents in parallel, dominating the effluxes through either the ATPases or the antiporters depending on the external pH, has variants. In *S. cerevisiae* ScENA1 is much more important than ScNHA1 for Na^+ tolerance and *ena* mutant strains are Na^+ sensitive [3], even at low pH values. The opposite occurs in *S. pombe* where *sod2* mutants are Na^+ sensitive [77]. *S. pombe* has two antiporters, SpSod2, which is Na^+ specific, and SpSod22, of broad substrate specificity [80], but a single ENA ATPase, CTA3, which might be of low relevance regarding Na^+ tolerance [9]. It is worth observing that both *S. cerevisiae* and *S. pombe* are acidophilic organisms, which grow poorly at the high pH values where the ENA ATPase is essential.

An exception to the abovementioned complementation of ENA ATPases and Na^+/H^+ antiporters is the proposal that the NHA1 antiporter of *S. cerevisiae* is electrogenic [76]. If this proposal were correct the requirement of the ENA ATPase for high pH Na^+ and K^+ effluxes would not exist. However, there is no report of a fungal *ena* mutant that is competent for extruding Na^+ or K^+ against high Na^+ or K^+ concentrations at high pH. The exchange mechanism of ScNHA1 was studied in a *sec4-2* yeast mutant, which accumulates secretory vesicles at restrictive temperatures [83], and the electrogenicity was established by the conventional use of CCCP, K^+, and valinomycin (see [71]) in these vesicles. Although the results are not questioned, the aforementioned contradiction between them and the physiological findings raise the caveat that the function of the antiporter in the vesicles may not match the function in the plasma membrane. Therefore, the functional electrogenicity of ScNHA1, i.e. that the $\Delta\Psi$ component of the proton motive force contributes to the energization of antiporter, needs further study.

Similar principles to those described so far regarding the requirement of the ENA ATPase at high external pH may apply to some parasite protozoa. In these protozoa the molecular mechanisms of the processes that maintain the intracellular Na^+ and K^+ concentrations remains unclear. Studies aimed at clarifying the transporters involved in these processes, originally focused on the Na,K-ATPase that is sensitive to ouabain in *T. cruzi* epimastigotes [12], revealed the existence of an ENA ATPase [13,14]. The existence of ENA ATPases that control the Na^+ or K^+ contents in any part of the cycle of parasitic protozoa would explain how the ENA ATPase inhibitors furosemide [12,14] and miltefosine [16] help to control trypanosomal infections.

In *P. patens* the ENA ATPase coexists with an electroneutral antiporter, PpSOS1 [10,11], which is the only Na+ efflux system of vascular plants [84,85]. Assuming that SOS1 is electroneutral [86,87] the absence of the ENA ATPase in vascular plants suggests a defective Na^+ efflux when the soil pH is high [87]. Although the apoplast of plants is acidic [88,89] and plant roots acidify the rhizosphere [90], the absence of the ENA ATPase in vascular plants might have imposed a restriction on their growth in high Na^+ media when the soil pH is high, unless another active Na^+ efflux system replaces for the ENA ATPase. In fact, it has been proposed that the observed cytosolic Na^+ concentrations cannot be explained by the action of an electroneutral Na^+/H^+ antiporter [91].

4.4. Endomembrane functions

The genomes of many fungi contain two or more *ENA* genes (Fig. 2). In some species these genes have similar or very similar sequences, and the most evident example is the tandem of *ENA* genes in *S. cerevisiae* [51,63,92]. In this species and in two more that have been investigated, *S. occidentalis* [8] and *D. hansenii* [29], the encoded ATPases have similar sequences and apparently similar functions, except for different cation specificity. In contrast, in other fungi the *ENA* genes are considerably divergent and probably originated from gene duplications that occurred before the Basidiomycota/Ascomycota split. In these cases, e.g. ENA1 and ENA2 in *N. crassa* and in *U. maydis*, the divergence of two ENA proteins in the same species is very high (Figs. 2 and 3) and only one of the ENA ATPases suppresses the Na^+ or K^+ sensitivity of *ena1-4 nha1* null mutants of *S. cerevisiae* while the other shows a weak effect [33]. Similarly, the two bryophytes species that have been studied, *P. patens* and *M. polymorpha*, also have three and two ENA ATPases, respectively, and again only one of the ATPases in each species shows a strong functional expression in *ena1-4 nha1* null mutants of *S. cerevisiae* [10,11].

The functional diversity of ENA ATPases has been studied in *U. maydis*. By using GFP fusions to the two UmEna proteins it was found that while UmEna1p localizes to the plasma membrane, UmEna2p localizes to the ER and other endomembranes. A low suppression of the Na^+ sensitivity of the *S. cerevisiae* Na^+ efflux mutant and endomembranes localization is also observed with the NcENA2 ATPase of *N. crassa* [33], which suggests that UmENA2 and NcENA2 might have similar functions. However, parallel expressions of UmENA2-GFP and NcENA2-GFP fusions in *S. cerevisiae* from similar constructs show different cellular locations. While the overexpression of UmENA2-GFP apparently induces the assembly of karmellae structures [93], NcENA2-GFP localizes to discrete organelles (Fig. 8) suggesting that NcENA2 and UmENA2 may have different locations in the endomembranes of their original organisms. Disruption of the *ena2* gene of *U. maydis* does not produce any growth defect that has been so far detected but the double *Δena1 Δena2* strain shows sensitivity to a component of bacteriological peptones at low external pH. Currently, it is unknown whether the defect is related to an abnormal cytoplasmic pH regulation or to the dysfunction of an organelle, which has not been identified so far. However, the first possibility seems unlikely because at low pH, Na^+/H^+ antiporters can substitute for ENA ATPases in the regulation of the cellular pH.

We have already mentioned a certain parallelism between fungal and bryophyte ENA ATPases regarding the number of genes, and lack of functional expression of some of these genes in *S. cerevisiae*. Again, it seems likely that in both *P. patens* and *M. polymorpha* one ENA ATPase localizes mainly to the plasma membrane [11] and others mainly to internal membranes (Fig. 8 shows the yeast expression of MpENA2). As regards to putative representatives of endomembranes ENA ATPases, there are similarities but also remarkable differences between the fungal, UmENA2, and the bryophyte, PpENA2. They are similar in that their transcript expressions are very low in normal conditions and highly expressed at high pH with Na^+. However, they are different because the expression of *UmENA2* transcripts is not induced by K^+ starvation [33] while *PpENA2* transcripts are highly induced by K^+ starvation.

Although a comprehensive investigation of the function of ENA ATPases in endomembranes is still pending, by similarity with plasma

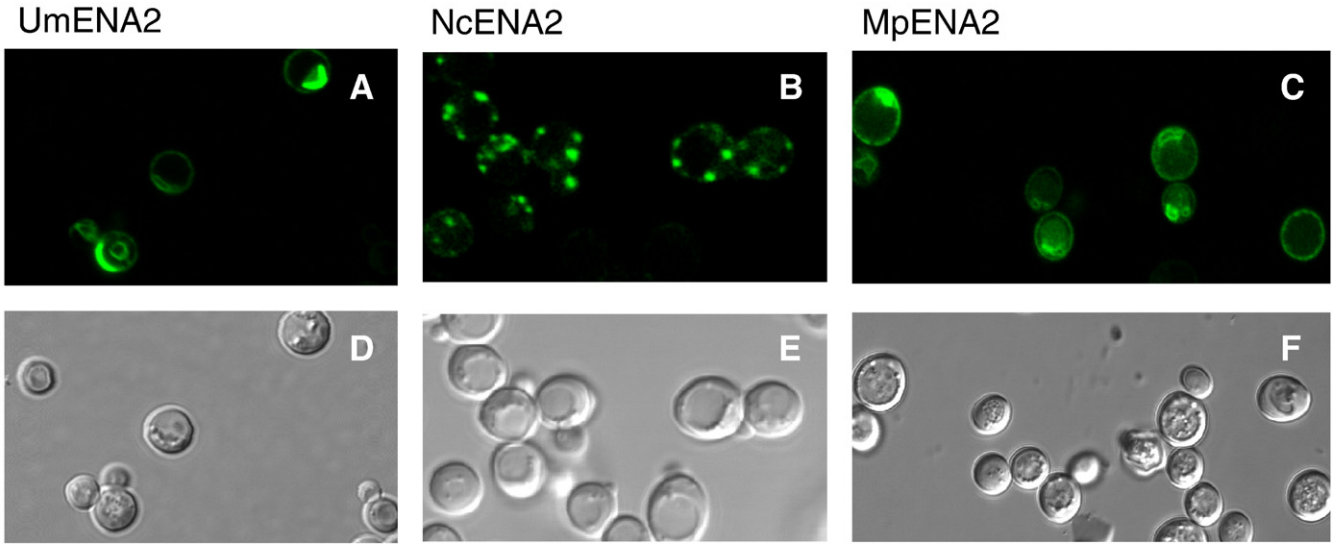


Fig. 8. Localization of the UmENA2-, NcENA2-, and MpENA2-GFP fusion proteins in *Saccharomyces cerevisiae* B31 (*Δena1-4 Δnha1*) cells. A, B, and C, GFP fluorescence. D, E, and F, DIC images. Experimental conditions as described in [11]. The images of UmENA2-GFP and MpENA2-GFP are compatible with endoplasmic reticulum expressions. NcENA2-GFP seems to be expressed in a spherical organelle, which we did not identify.

membrane ENA ATPases a likely function of endomembrane ENA ATPases is the regulation of Na^+ or K^+ contents and consequently of the organelle pH. When the organelle pH is lower than the cytosolic pH, H^+ pumping mediated by the V-ATPase or V-PPase [94–96] in cooperation with passive transporters [97] can regulate the pH. Among these cooperative transporters, K^+ (or Na^+)/H^+ antiporters seem crucial in fungi and plants as a pathway for the return of H^+ to the cytosol [70,98–101]. In contrast with this, when the pH of the organelle is more alkaline than the cytosol, e.g. peroxisomes [102], K^+ (or Na^+)/H^+ antiporters cannot mediate the effective return of H^+ to the cytosol and a different system is necessary. An ENA ATPase that exchanges lumenal H^+ with cytoplasmic K^+ is an obvious candidate to fulfill the function of the antiporter when the ΔpH would drive K^+ out of the organelle's lumen instead of into the organelle (Fig. 7). Although in fungal species with two or more *ENA* genes distinct functions may be performed by specific isoforms, species with a single *ENA* gene might express an ENA ATPase that at least transitorily localizes in endomembranes, as proposed for the Na^+,K^+-ATPase [103]. The metabolomic changes produced by the overexpression of PpENA1 in rice and barley [19] might be explained by this mechanism [11].

While there are reasonable grounds for assuming that of the ENA ATPase plays a role in the control of the pH of some organelle it has to be mentioned that the disruptions of the *ena2* gene in *U. maydis* [33] and *PpENA2* in *P. patens* [11] did not produce any detectable effect, except the abovementioned defective growth in peptone of *U. maydis*.

5. Technological and pharmacological interest

Research progress made on the ENA ATPases has two prospective applications: as a drug target in protozoan and fungal pathogenesis, and for improving Na^+ tolerance in crop plants.

In certain regions and risk populations, protozoan [104,105] and fungal [106,107] infections produce serious diseases, for which the number of chemotherapeutics is limited. The ENA ATPase does not have a functional homologue in animal cells and, therefore, is a potential target for antiparasitic and antifungal drugs. This possibility has been reviewed for other P-type ATPases [108]. To be a convenient drug target, the ENA ATPase has to fulfill two conditions: (i) that its inhibition results in the growth inhibition of the pathogen and (ii) that specific drugs can be designed against it; the ENA ATPase fulfills both. The first condition is fulfilled in *C. neoformans*, in which the mutation of the *ENA1* gene produces avirulence [17]. Information for the second condition comes from two drugs, furosemide and miltefosine. Furosemide is a well-known loop diuretic, which inhibits the Na–K–Cl cotransporter [109] and that also inhibits the ouabain-insensitive Na-ATPase of *E. histolytica* [15], *T. cruzi* [12,16] and *L. amazonensis* [40]. Miltefosine inhibits the growth of *Leishmania* and *T. cruzi* probably through the inhibition of the Na^+-ATPase [16]. In *T. cruzi* the inhibited Na^+-ATPase is likely to be the ENA ATPase [14].

All this information has been obtained recently and very little is known about the exact point of the pathogenic cycle in which the inhibition of the ENA ATPase is lethal for the pathogen. The important aspect of this line of research is that it opens up new perspectives in the control of two types of diseases for which the number of available drugs is limited [104,110].

The use of the ENA ATPase for improving Na^+ tolerance has substantial empiric support. Salinity is a detrimental problem of agriculture, in part due to the toxicity of Na^+ in plant cells [111,112]. This toxicity is limited by a very important Na^+ extrusion process in the roots of probably all flowering plants [113–115] and the question is whether a Na^+/H^+ antiporter can mediate Na^+ extrusion in all conditions and in which ones, if any, the ENA ATPase would be beneficial. According to our previous discussion about antiporters and the ENA ATPase, the determinant factor is soil pH but another factor has to be considered, the extensive Na^+ exchange that takes place in plant roots [113–115] in which a high proportion of the Na^+ taken up is rapidly extruded to the external medium. For example, in durum wheat Na^+ efflux may amount to as much as 99% of the Na^+ influx [116]. Considering this high Na^+ exchange the mechanism of the Na^+ efflux is an important detail because in conditions of salinity the resulting futile cycle of Na^+ exchange may represent an unbearable energetic price for the plant [117]. The energetic price of an electroneutral antiporter coupled to the H^+-ATPase is one ATP per Na^+ extruded assuming that the stoichiometry of the H^+-ATPase is one H^+ pumped out per cycle of the enzyme [118]. In contrast, although the stoichiometry of the ENA ATPase is unknown, considering the stoichiometry of the Na,K-ATPase [119], it may pump more than one Na^+ per ATP hydrolyzed (Fig. 7). In summary, the independence from a ΔpH driving force and a possible high energetic efficiency in relation to Na^+/H^+ antiporters make the ENA ATPase an attractive enzyme for improving Na^+ tolerance in flowering plants.

The abovementioned uncertainties regarding the use of the ENA ATPase for improving the Na^+ tolerance of flowering plants need to be solved empirically, but, so far, the number of experiments is limited to very few cases [18–20]. Furthermore, the ENA ATPases used in these experiments were of the type that does not discriminate between Na^+ and K^+, which may be inappropriate for increasing the Na^+ tolerance in cells that only tolerate low Na^+ concentrations. This reason could explain the better performance of the Na^+ specific SpSod2 antiporter [120,121] than the ENA ATPase [19,20] for improving plant Na^+ tolerance.

6. Concluding remarks

Although the ENA ATPase was discovered nineteen years ago current knowledge about this ATPase is still limited. The notion that this pump was a dispensable enzyme in *S. cerevisiae* without a clear biotechnological use has probably contributed to the low interest that this ATPase has attracted. However, its universal presence in fungi and extensive presence in bryophytes and protozoa as well as possible biotechnological applications are having a direct impact on the biological attractiveness of the ENA ATPase. Furthermore, the function of the ENA ATPase in endomembranes opens up a new field of research on this ATPase.

Acknowledgements

The data presented in Figs. 6 and 8 are unpublished results of Blanca Garciadeblás. We thank her for allowing us to include these figures in this review. This work was supported by the Spanish Ministerio de Ciencia e Innovación and by the ERDF European program through grant no. AGL2007-61075. Additional financial support was provided by DGUI-UPM Research Group Program.

References

[1] V.P. Skulachev, Membrane Bioenergetics, Springer-Verlag, Berlin Heidelberg, 1988.
[2] A. Rodríguez-Navarro, Potassium transport in fungi and plants, Biochim. Biophys. Acta 1469 (2000) 1–30.
[3] R. Haro, B. Garciadeblas, A. Rodríguez-Navarro, A novel P-type ATPase from yeast involved in sodium transport, FEBS Lett. 291 (1991) 189–191.
[4] A. Rodríguez-Navarro, J. Asensio, An efflux mechanism determines the low net entry of lithium in yeast, FEBS Lett. 75 (1977) 169–172.
[5] A. Rodríguez-Navarro, M.D. Ortega, The mechanism of sodium efflux in yeast, FEBS Lett. 138 (1982) 205–208.
[6] E.D. Sancho, E. Hernández, A. Rodríguez-Navarro, Presumed sexual isolation in yeast populations during production of sherrylike wine, Appl. Env. Microbiol. 51 (1986) 395–397.
[7] B. Benito, F.J. Quintero, A. Rodríguez-Navarro, Overexpression of the sodium ATPase of *Saccharomyces cerevisiae*. Conditions for phosphorylation from ATP and Pi, Biochim. Biophys. Acta 1328 (1997) 214–225.
[8] M.A. Bañuelos, A. Rodríguez-Navarro, P-type ATPases mediate sodium and potassium effluxes in *Schwanniomyces occidentalis*, J. Biol. Chem. 273 (1998) 1640–1646.

[9] B. Benito, B. Garciadeblás, A. Rodríguez-Navarro, Potassium- or sodium-efflux ATPase, a key enzyme in the evolution of fungi, Microbiology 148 (2002) 933–941.

[10] B. Benito, A. Rodríguez-Navarro, Molecular cloning and characterization of a sodium-pump ATPase of the moss *Physcomitrella patens*, Plant J. 36 (2003) 382–389.

[11] A. Fraile-Escanciano, B. Garciadeblás, A. Rodríguez-Navarro, B. Benito, Role of ENA ATPase in Na^+ efflux at high pH in bryophytes, Plant Mol. Biol. 71 (2009) 599–608.

[12] C. Caruso-Neves, M. Einicker-Lamas, C. Changas, M.M. Oliveira, A. Vieyra, A.G. Lopes, Ouabain-insensitive Na^+-ATPase activity in *Trypanosoma cruzi* epimastigotes, Z. Naturforch. 54c (1999) 100–104.

[13] J.K. Stiles, Z. Kucerova, B. Sarfo, C.A. Meade, W. Thompson, P. Shah, L. Xue, J.C. Meade, Identification of surface-membrane P-type ATPases resembling fungal K $^+$- and Na^+-ATPases, in *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania donovani*, Ann. Trop. Med. Parasit. 97 (2003) 351–366.

[14] K. Iizumi, Y. Mikami, M. Hashimoto, T. Nara, Y. Hara, T. Aoki, Molecular cloning and characterization of ouabain-insensitive Na^+-ATPase in the parasitic protist *Trypanosoma cruzi*, Biochim. Biophys. Acta 1758 (2006) 738–746.

[15] A.M. De-Souza, E.J.O. Batista, A.A.d.S. Pinheiro, M. Carvalhaes, A.G. Lopes, W. De-Souza, C. Caruso-Neves, *Entamoeba histolytica*: ouabain-insensitive Na^+-ATPase activity, Exp. Parasitol. 117 (2007) 195–200.

[16] V.B. Saraiva, M. Wengert, E. Gomes-Quintana, N. Heise, C. Caruso-Neves, Na^+-ATPase and protein kinase C are targets to 1-*O*-hexadecylphosphocoline (miltefosine) in *Trypanosoma cruzi*, Arch. Biochem. Biophys. 481 (2009) 65–71.

[17] A. Idnurm, F.J. Walton, A. Floyd, J.L. Reedy, J. Heitman, Identification of *ENA1* as a virulence gene of the human pathogenic fungus *Cryptococcus neoformans* through signature-tagged insertional mutagenesis, Eukaryot. Cell 8 (2009) 315–326.

[18] H. Nakayama, K. Yoshida, A. Shinmyo, Yeast plasma membrane Ena1p ATPase alters alkali-cation homeostasis and confers increased salt tolerance in tobacco cultured cells, Biotechnol. Bioeng. 85 (2004) 776–789.

[19] A. Jacobs, C. Lunde, A. Bacic, M. Tester, U. Roessner, The impact of constitutive heterologous expression of a moss Na^+ transporter on the metabolomes of rice and barley, Metabolomics 3 (2007) 307–317.

[20] X. Kong, X. Gao, W. Li, J. Zhao, Y. Zhao, H. Zhang, Overexpression of *ENA1* from yeast increases salt tolerance in *Arabidopsis*, J. Plant Biol. 51 (2008) 159–165.

[21] K.B. Axelsen, M.G. Palmgren, Evolution of substrate specificities in the P-type ATPase superfamily, J. Mol. Evol. 46 (1998) 84–101.

[22] J. Barrero-Gil, B. Garciadeblás, B. Benito, Sodium, potassium-ATPases in algae and oomycetes, J. Bioenerg. Biomembr. 37 (2005) 269–278.

[23] N. Corradi, I.R. Sanders, Evolution of the P-type II ATPase gene family in the fungi and presence of structural genomic changes among isolates of *Glomus intraradices*, BMC Evol. Biol. 6 (2006) 21.

[24] A.G. Sáez, E. Lozano, A. Zaldívar-Riverón, Evolutionary history of Na, K-ATPase and their osmoregulatory role, Genetica 136 (2009) 479–490.

[25] M.D. Thever, M.H.J. Saier, Bioinformatic characterization of P-type ATPases encoded within fully sequenced genomes of 26 eukaryotes, J. Membrane Biol. 229 (2009) 115–130.

[26] Y. Suzuki, S. Ueno, R. Ohnuma, N. Koyama, Cloning, sequencing and functional expression in *Escherichia coli* of the gene for a P-type Na^+-ATPase of a facultative anaerobic alkaliphile, *Exiguobacterium aurantiacum*, Biochim. Biophys. Acta 1727 (2005) 162–168.

[27] Y. Watanabe, T. Iwaki, Y. Shimomno, A. Ichimiya, Y. Nagaoka, Y. Tamai, Characterization of the Na^+-ATPase gene (ZENA1) from the salt-tolerant yeast *Zygosaccharomyces rouxii*, J. Biosci. Bioeng. 88 (1999) 136–142.

[28] B. Benito, B. Garciadeblas, A. Rodríguez-Navarro, Molecular cloning of the calcium and sodium ATPases in *Neurospora crassa*, Mol. Microbiol. 35 (2000) 1079–1088.

[29] A. Almagro, C. Prista, B. Benito, M.C. Lourero-Dias, J. Ramos, Cloning and expression of two genes coding for sodium pumps in the salt-tolerant yeast *Debaryomyces hansenii*, J. Bacteriol. 183 (2001) 3251–3255.

[30] Z. Caracuel, C. Casanova, M.I.G. Roncero, A. Di-Pietro, J. Ramos, pH response transcription factor PacC controls salt stress tolerance and expression of the Na $^+$-ATPase Ena1 in *Fusarium oxysporum*, Eukaryot. Cell 2 (2003) 1246–1252.

[31] A. Gorjan, A. Plemenitas, Identification and characterization of ENA ATPases HwENA1 and HwENA2 from the halophilic black yeast *Hortaea werneckii*, FEMS Microbiol. Lett. 265 (2006) 41–50.

[32] M.J. Hernandez-Lopez, J. Panadero, J.A. Prieto, F. Randez-Gil, Regulation of salt tolerance by *Torulaspora delbrueckii* calcineurin target Crz1p, Eukaryot. Cell 5 (2006) 469–479.

[33] B. Benito, B. Garciadeblás, J. Pérez-Martín, A. Rodríguez-Navarro, Growth at high pH and sodium and potassium tolerance in media above the cytoplasmic pH depend on ENA ATPases in *Ustilago maydis*, Eukaryot. Cell 8 (2009) 821–829.

[34] M. Ghislain, A. Goffeau, D. Halachmi, Y. Eilan, Calcium homeostasis and transport are affected by disruption of *cta3*, a novel gene encoding Ca^{2+}-ATPase in *Schizosaccharomyces pombe*, J. Biol. Chem. 265 (1990) 18400–18407.

[35] D. Halachmi, M. Ghislain, Y. Eilam, An intracellular ATP-dependent calcium pump within the yeast *Schizosaccharomyces pombe*, encoded by the gene *cta3*, Eur. J. Biochem. 207 (1992) 1003–1008.

[36] M.L. Berbee, J.W. Taylor, Dating the evolutionary radiations of the true fungi, Can. J. Bot. 71 (1993) 1114–1127.

[37] H. Wang, Z. Xu, L. Gao, B. Hao, A fungal phylogeny based on 82 complete genomes using the composition vector method, BMC Evol. Biol. 9 (2009) 195.

[38] B. Garciadeblas, B. Benito, A. Rodríguez-Navarro, Plant cells express several stress calcium ATPases but apparently no sodium ATPase, Plant Soil 235 (2001) 181–192.

[39] E.A. Kiegle, M.A. Bisson, Plasma membrane Na^+ transport in a salt-tolerant Charophyte. Isotopic fluxes, electrophysiology, and thermodynamics in plant adapted to saltwater and freshwater, Plant Physiol. 111 (1996) 1191–1197.

[40] E.E. Almeida-Amaral, C. Caruso-Neves, V.M.P. Pires, J.R. Meyer-Fernandes, *Leishmania amazonensis*: characterization of an ouabain-insensitive Na^+-ATPase activity, Exp. Parasitol. 118 (2008) 165–171.

[41] A.G. Lee, Ca2+-ATPase structure in the E1 and E2 conformations: mechanism, helix–helix and helix–lipid interactions, Biochim. Biophys. Acta 1565 (2002) 246–266.

[42] P.A. Pedersen, J.R. Jorgensen, P.L. Jorgensen, Importance of conserved ∝-subunit segment 709GDGVND for Mg2+ binding, phosphorylation, and energy transduction in Na, K-ATPase, J. Biol. Chem. 275 (2000) 37588–37595.

[43] P.L. Jorgensen, P.A. Pedersen, Structure–function relationships of Na+, K+, ATP, or Mg2+ binding and energy transduction in Na, K-ATPase, Biochim. Biophys. Acta 1505 (2001) 57–74.

[44] D.L. Stokes, N.M. Green, Structure and function of the calcium pump, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 445–468.

[45] F. Di-Leva, T. Domi, L. Fedrizzi, D. Lim, E. Carafoli, The plasma membrane Ca^{2+} ATPase of animal cells: structure, function and regulation, Arch. Biochem. Biophys. 476 (2008) 65–74.

[46] K.J. Sweadner, C. Donnet, Structural similarities of Na, K-ATPase and SERCA, the Ca2+ ATPase of the sarcoplasmic reticulum, Biochem. J. 356 (2001) 685–704.

[47] L.D. Faller, Mechanistic studies of sodium pump, Arch. Biochem. Biophys. 476 (2008) 12–21.

[48] C. Toyoshima, Structural aspects of ion pumping by Ca^{2+}-ATPase of sarcoplasmic reticulum, Arch. Biochem. Biophys. 476 (2008) 3–11.

[49] C. Toyoshima, How Ca^{2+}-ATPase pumps ions across the sarcoplasmic reticulum membrane, Biochim. Biophys. Acta 1793 (2009) 941–946.

[50] L.A. Kelley, M.J.E. Sternberg, Protein structure prediction on the Web: a case study using the Phyre server, Nat. Protoc. 4 (2009) 363–371.

[51] J. Wieland, A.M. Nitsche, J. Strayle, H. Steiner, H.K. Rudolph, The PMR2 gene cluster encodes functionally distinct isoforms of a putative Na+ pump in the yeast plasma membrane, EMBO J. 14 (1995) 3870–3882.

[52] J.V. Møller, B. Juul Test, M.l. Maire, Structural organization, ion transport, and energy transduction of P-type ATPases, Biochim. Biophys. Acta 1286 (1996) 1–51.

[53] D.M. Clarke, T.W. Loo, G. Inesi, D.H. MacLennan, Location of high affinity Ca^{2+}-binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum Ca^{2+}-ATPase, Nature 339 (1989) 476–478.

[54] J.P. Morth, B.P. Pedersen, M.S. Toustrup-Jensen, T.L.M. Sørensen, J. Petersen, J.P. Andersen, B. Vilsen, P. Nissen, Crystal structure of sodium–potassium pump, Nature 450 (2007) 1043–1050.

[55] C. Toyoshima, M. Nakasako, H. Nomura, H. Ogawa, Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution, Nature 405 (2000) 647–655.

[56] Y. Watanabe, Y. Shimono, H. Tsuji, Y. Tamai, Role of the glutamic and aspartic residues in Na^+-ATPase function in the ZrENA1 gene of *Zygosaccharomyces rouxii*, FEMS Microbiol. Lett. 209 (2002) 39–43.

[57] T. Yamaguchi, G.S. Aharon, J.B. Sottosanto, E. Blumwald, Vacuolar Na^+/H^+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca^{2+}- and pH-dependent manner, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 16107–16112.

[58] B. Garciadeblas, F. Rubio, F.J. Quintero, M.A. Bañuelos, A. Rodríguez-Navarro, Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Saccharomyces cerevisiae*, Mol. Gen. Genet. 236 (1993) 363–368.

[59] I. Mendoza, F. Rubio, A. Rodríguez-Navarro, J.M. Pardo, The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*, J. Biol. Chem. 269 (1994) 8792–8796.

[60] A. Ferrando, S.J. Kron, G. Ríos, G.R. Fink, R. Serrano, Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene *HAL3*, Mol. Cell. Biol. 15 (1995) 5470–5481.

[61] G. Ríos, A. Ferrando, R. Serrano, Mechanisms of salt tolerance conferred by overexpression of the *HAL1* gene in *Saccharomyces cerevisiae*, Yeast 13 (1997) 515–528.

[62] A. Ruiz, J. Ariño, Function and regulation of the *Saccharomyces cerevisiae ENA* sodium ATPase system, Eukaryot. Cell 6 (2007) 2175–2183.

[63] J. Ariño, J. Ramos, H. Synchrová, Alkali metal cation transport and homeostasis in yeast, Microbiol. Mol. Biol. Rev. 74 (2010) 95–120.

[64] A. Spielvogel, H. Findon, H.N.J. Arst, L. Araúzo-Bazán, P. Hernández-Ortiz, U. Stahl, V. Meyer, E.A. Espeso, Two zinc finger transcription factors, CrzA and SltA, are involved in cation homoeostasis and detoxification in *Aspergillus nidulans*, Biochem. J. 414 (2008) 419–429.

[65] V. Niggli, E. Sigel, Anticipating antiport in P-type ATPases, Trends Biochem. Sci. 33 (2008) 156–160.

[66] C.L. Slayman, Electrical properties of *Neurospora crassa*. Effects of external cations on the intracellular potential, J. Gen. Physiol. 49 (1965) 69–92.

[67] A. Rodríguez-Navarro, M.R. Blatt, C.L. Slayman, A potassium-proton symport in *Neurospora crassa*, J. Gen. Physiol. 87 (1986) 649–674.

[68] J.H. Kaplan, Biochemistry of Na, K-ATPase, Annu. Rev. Biochem. 71 (2002) 511–535.

[69] R.C. Thomas, The plasma membrane calcium ATPase (PMCA) of neurones is electroneutral and exchanges 2 H^+ for each Ca^{2+} or Ba^{2+} ion extruded, J. Physiol. 587 (2009) 315–327.

[70] C.L. Brett, M. Donowitz, R. Rao, Evolutionary origins of eukaryotic sodium/proton exchangers, Am. J. Physiol. Cell Physiol. 288 (2005) C223–C239.
[71] E. Padan, E. Bibi, M. Ito, T.A. Krulwich, Alkaline pH homeostasis in bacteria: new insights, Biochim. Biophys. Acta 1717 (2005) 67–88.
[72] M.V. Radchenko, K. Tanaka, R. Waditee, S. Oshimi, Y. Matsuzaki, M. Fukuhara, H. Kobayashi, T. Takabe, T. Nakamura, Potassium/proton antiport system of *Escherichia coli*, J. Biol. Chem. 281 (2006) 19822–19829.
[73] M.A. Bañuelos, H. Synchrová, C. Bleykasten-Grosshans, J.-L. Souciet, S. Potier, The Nha1 antiporter of *Saccharomyces cerevisiae* mediates sodium and potassium efflux, Microbiology 144 (1998) 2749–2758.
[74] M.A. Bañuelos, J. Ramos, F. Calero, V. Braun, S. Potier, Cation/H^+ antiporters mediate potassium and sodium fluxes in *Pichia sorbitophila*. Cloning of the *PsHHA1* and *PsNHA2* genes and expression in *Saccharomyces cerevisiae*, Yeast 19 (2002) 1365–1372.
[75] O. Kinclová, S. Potier, H. Synchrová, Difference in substrate specificity divides the yeast alkali-metal-cation/H+ antiporters into two subfamilies, Microbiology 148 (2002) 1225–1232.
[76] R. Ohgaki, N. Nakamura, K. Mitsui, H. Kanazawa, Characterization of the ion transport activity of budding yeast Na^+/H^+ antiporter, Nha1p, using isolated secretory vesicles, Biochim. Biophys. Acta 1712 (2005) 185–196.
[77] Z.P. Jia, N. McCullough, R. Martel, S. Hemminngsens, P.G. Young, Gene amplification at a locus encoding a putative Na^+/H^+ antiporter confers sodium and lithium tolerance in fission yeast, EMBO J. 11 (1992) 1631–1640.
[78] K.M. Hahnenberger, Z. Jia, P.G. Young, Functional expression of the *Schizosaccharomyces pombe* Na^+/H^+ antiporter gene, *sod2*, in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5031–5036.
[79] K. Papouskova, H. Synchrova, *Yarrowia lipolytica possesses* two plasma membrane alkali metal cation/H^+ antiporters with different functions in cell physiology, FEBS Lett. 580 (2006) 1971–1976.
[80] K. Papouskova, H. Synchrova, *Schizosaccharomyces pombe* possesses two plasma membrane alkali metal cation/H^+ antiporters differing in their substrate specificity, FEMS Microbiol. Lett. 7 (2007) 188–195.
[81] L. Pribylova, K. Papouskova, H. Synchrova, The salt tolerant yeast *Zygosaccharomyces rouxii* possesses two plasma-membrane Na^+/H^+-antiporters (ZrHha1p and ZrSod2-22p) playing different roles in cation homeostasis and cell physiology, Fungal Genet. Biol. 45 (2008) 1439–1447.
[82] E. Simón, A. Barceló, J. Ariño, Mutagenesis analysis of the yeast Nha1 Na^+/H^+ antiporter carboxy-terminal tail reveals residues required for function in cell cycle, FEBS Lett. 545 (2003) 239–245.
[83] P. Novick, C. Field, R. Schekman, Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway, Cell 21 (1980) 205–215.
[84] H. Shi, M. Ishitani, C. Kim, J.-K. Zhu, The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 6896–6901.
[85] H. Shi, B. Lee, S.-J. Wu, J.-K. Zhu, Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*, Nat. Biotechnol. 21 (2003) 81–85.
[86] Q.S. Qiu, B.J. Barkla, R. Vera-Estrella, J.K. Zhu, K.S. Schumaker, Na^+/H^+ exchange activity in the plasma membrane of *Arabidopsis*, Plant Physiol. 132 (2003) 1041–1052.
[87] A. Fraile-Escanciano, Y. Kamisugi, A.C. Cuming, A. Rodríguez-Navarro and B. Benito, The SOS1 transporter of *Physcomitrella patens* mediates sodium efflux *in planta*, New Phytologist (in press).
[88] H.H. Felle, The apoplastic pH of the *Zea mays* root cortex as measured with pH-sensitive microelectrodes: aspects of regulation, J. Exp. Bot. 323 (1998) 987–995.
[89] Q. Yu, J. Kuo, C. Tang, Using confocal laser scanning microscopy to measure apoplastic pH change in roots of *Lupinus angustifolius* L. in response to high pH, Ann. Botany 87 (2001) 47–52.
[90] P. Hinsinger, C. Plassard, C. Tang, B. Jaillard, Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: a review, Plant Soil 248 (2003) 43–59.
[91] D.E. Carden, D.J. Walker, T.J. Flowers, A.J. Miller, Single-cell measurements of the contribution of cytosolic Na^+ and K^+ to salt tolerance, Plant Physiol. 131 (2003) 676–683.
[92] A. Rodríguez-Navarro, F.J. Quintero, B. Garciadeblás, Na^+-ATPases and Na^+/H^+ antiporters in fungi, Biochim. Biophys. Acta 1187 (1994) 203–205.
[93] R. Wright, M. Basson, L. D'Ari, J. Rine, Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus, J. Cell Biol. 107 (1988) 101–114.
[94] K. Schumacher, Endomembrane proton pumps: connecting membrane and vesicle transport, Curr. Opin. Plant Biol. 9 (2006) 595–600.
[95] G.A. Martínez-Muñoz, P. Kane, Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast, J. Biol. Chem. 283 (2008) 20309–20319.
[96] S.S. Samarão, C.E.S. Teodoro, F.E. Silva, C.C. Ribeiro, T.M. Granato, N.R. Bernardes, C.A. Retamal, A.R. Façanha, A.L. Okorokova-Façanha, L.A. Okorokov, V H+-ATPase along the yeast secretory pathway: energization of the ER and Golgi membranes, Biochim. Biophys. Acta 1788 (2009) 303–313.
[97] M. Grabe, G. Oster, Regulation of organelle acidity, J. Gen. Physiol. 117 (2001) 329–343.
[98] K. Venema, A. Belver, M.C. Marín-Manzano, M.P. Rodríguez-Rosales, J.P. Donaire, A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants, J. Biol. Chem. 278 (2003) 22453–22459.
[99] S. Mukherjee, L. Kallay, C.L. Brett, R. Rao, Mutational analysis of the intramembranous H10 loop of yeast Nhx1 reveals a critical role in ion homoeostasis and vesicle trafficking, Biochem. J. 398 (2006) 97–105.
[100] J.M. Pardo, B. Cubero, E.O. Leidi, F.J. Quintero, Alkali cation exchangers: roles in cellular homeostasis and stress tolerance, J. Exp. Bot. 57 (2006) 1181–1199.
[101] O. Cagnac, M. Leterrier, M. Yeager, E. Blumwald, Identification and characterization of Vnx1p, a novel type of vacuolar monovalent cation/H^+ antiporter of *Saccharomyces cerevisiae*, J. Biol. Chem. 282 (2007) 24284–24293.
[102] C.W.T. van-Roermund, M. de-Jong, L. Ijist, J. van-Marle, T.B. Dansen, R.J.A. Wanders, H.R. Waterham, The peroxisomal lumen in *Saccharomyces cerevisiae* is alkaline, J. Cell Sci. 117 (2004) 4231–4237.
[103] T. Feldmann, V. Glukmann, E. Medvenev, U. Shpolansky, D. Galili, D. Lichtstein, H. Rosen, Role of endosomal Na+, K+-ATPase and cardiac steroids in the regulation of endocytosis, Am. J. Physiol. Cell Physiol. 293 (2007) C885–C896.
[104] P. Borst, M. Ouellette, New mechanisms of drug-resistance in parasitic protozoa, Annu. Rev. Microbiol. 49 (1995) 427–460.
[105] J.A. Freason, P.G. Wyatt, I.H. Gilbert, A.H. Fairlamb, Target assessment for antiparasitic drug discovery, Trends Parasitol. 23 (2007) 589–595.
[106] F.C. Odds, A.J.P. Brown, N.A.R. Gow, Antifungal agents: mechanisms of action, Trends Microbiol. 11 (2003) 272–279.
[107] P.C. Ting, S.S. Walker, New agents to treat life-threatening fungal infections, Curr. Top. Med. Chem. 8 (2008) 592–602.
[108] L. Yatime, M.J. Buch-Pedersen, M. Musgaard, J.P. Morth, A.M.L. Winther, B.P. Pedersen, C. Olesen, J.P. Andersen, B. Vilsen, B. Schiøtt, M.G. Palmgren, J.V. Møller, P. Nissen, N. Fedosova, P-type ATPases as drug targets: tools for medicine and science, Biochim. Biophys. Acta 1787 (2009) 207–220.
[109] M. Haas, B. Forbush-III, The Na–K–Cl cotransporters, J. Bioenerg. Biomembr. 30 (1998) 161–172.
[110] P.K. Mukherjee, D.J. Sheehan, C.A. Hitchcock, M.A. Ghannoum, Combination treatment of invasive fungal infections, Clin. Microbiol. Rev. 18 (2005) 163–194.
[111] T.J. Flowers, T.D. Colmer, Salinity tolerance in halophytes, New Phytol. 179 (2008) 945–963.
[112] R. Munns, M. Tester, Mechanisms of salinity tolerance, Annu. Rev. Plant Biol. 59 (2008) 651–681.
[113] H.J. Kronzucker, M.W. Szczerba, M. Moazami-Goudarzi, D.T. Britto, The cytosolic Na^+:K^+ ratio does not explain salinity-induced growth impairment in barley: a dual-tracer study using $^{42}K^+$ and $^{24}Na^+$, Plant Cell Environ. 29 (2006) 2228–2237.
[114] B. Wang, R.J. Davenport, V. Volkov, A. Amtmann, Low unidirectional sodium influx into root cells restricts net sodium accumulation in *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thalian*a, J. Exp. Bot. 57 (2006) 1161–1170.
[115] C.M. Wang, J.L. Zhang, X.S. Liu, Z. Li, G.Q. Wu, J.Y. Cai, T.J. Flowers, S.M. Wang, *Puccinellia tenuiflora* maintains a low Na^+ level under salinity by limiting unidirectional Na^+ influx resulting in a high selectivity for K^+ over Na^+, Plant Cell Environ. 32 (2009) 486–496.
[116] R. Davenport, R.A. James, A. Zakrisson-Plogander, M. Tester, R. Munns, Control of sodium transport in durum wheat, Plant Physiol. 137 (2005) 807–818.
[117] D.T. Britto, H.J. Kronzucker, Ussing's conundrum and the search for transport mechanisms in plants, New Phytol. 183 (2009) 243–246.
[118] M.G. Palmgren, Plant plasma membrane H+-ATPases: powerhouses for nutrient uptake, Annu. Rev. Plant Mol. Biol. 52 (2001) 817–845.
[119] P.L. Jorgensen, K.O. Hakansson, S.J.D. Karlish, Structure and mechanism of Na, K-ATPase: functional sites and their interactions, Annu. Rev. Physiol. 65 (2003) 817–849.
[120] X.H. Gao, Z.H. Ren, Y.X. Zhao, H. Zang, Overexpression of *SOD2* increases salt tolerance of *Arabidopsis*, Plant Physiol. 133 (2003) 1873–1881.
[121] F. Zhao, S.L. Guo, H. Zang, Y.X. Zhao, Expression of yeast SOD2 in transgenic rice results in increased salt tolerance, Plant Sci. 170 (2006) 216–224.
[122] M.G. Palmgren, K.B. Axelsen, Evolution of P-type ATPases, Biochim. Biophys. Acta 1365 (1998) 37–45.
[123] J.D. Thompson, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (1997) 4876–4882.
[124] R.D.M. Page, TreeView: an application to display phylogenetic trees on personal computers, Comput. Appl. Biosci. 12 (1996) 357–358.