Sodium, Potassium-ATPases in Algae and Oomycetes

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We have investigated the presence of K^+-transporting ATPases that belong to the phylogenetic group of animal Na^+,K^+-ATPases in the *Pythium aphanidermatum* Stramenopile oomycete, the *Porphyra yezoensis* red alga, and the *Udotea petiolata* green alga, by molecular cloning and expression in heterologous systems. PCR amplification and search in EST databases allowed one gene to be identified in each species that could encode ATPases of this type. Phylogenetic analysis of the sequences of these ATPases revealed that they cluster with ATPases of animal origin, and that the algal ATPases are closer to animal ATPases than the oomycete ATPase is. The *P. yezoensis* and *P. aphanidermatum* ATPases were functionally expressed in *Saccharomyces cerevisiae* and *Escherichia coli* alkali cation transport mutants. The aforementioned cloning and complementary searches in silico for H^+- and Na^+,K^+-ATPases revealed a great diversity of strategies for plasma membrane energization in eukaryotic cells different from typical animal, plant, and fungal cells.

INTRODUCTION

Potassium is a major element in all living cells, where it undertakes crucial functions (Rodríguez-Navarro, 2000). To fulfill the K^+ demand, cells are furnished with different types of plasma membrane transporters that are adapted to mediate K^+ uptake even when the cation is extremely scarce or in environments with high Na^+ concentrations. In the latter case, the entrance of Na^+ cannot be completely prevented and Na^+ has to be extruded from the cells while K^+ is taken up. Due to this, when Na^+ is present, the Na^+ extruding systems become as important for the cell as the systems for K^+ uptake.

Eukaryotic cells energize their plasma membranes using P-type ATPases that may or may not be involved in K^+ and Na^+ transport simultaneously. Attending to the mechanisms involved in these processes, eukaryotic cells have traditionally been grouped in two models: (i) the naked cells of animals, in which the asymmetric distribution of K^+ and Na^+ across the plasma membrane is accomplished by a Na^+,K^+-ATPase, and the membrane potential is dominated by the permeability of the membrane to K^+ and Na^+; and (ii) the walled cells of plants and fungi, in which the membrane potential is dominated by a H^+-pump ATPase, and K^+ and Na^+ transports are energized by the membrane potential or proton motive force across the plasma membrane (Rodríguez-Navarro, 2000). These two models are related to two different lifestyles, the former to the constant environment and Na^+ abundance that prevail in the sea and in the extracellular fluids of animal cells, and the latter to the changeable but normally dilute medium of the terrestrial soil solutions where fungi and plants thrive. Very simple energetic calculations suggest that each model is specifically adapted to the prevailing conditions in these two completely different media (Rodríguez-Navarro, 2000).

In this simplified two-model scheme, only animal, fungi, and plants have been considered, although phylogenetic studies have demonstrated that many groups of eukaryotic organisms that are adapted to live either in the sea or on the land are very distant from typical animals, fungi, and plants. Many of these organisms are of economic and ecologic importance, and an increasing number of reports devoted to them show a significant variability

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in the modes in which K^+ and Na^+ are transported. In all these cases, new P-type ATPases that are phylogenetically related to animal Na^+,K^+-ATPases seem to be the key enzymes. These ATPases have been described in the chytridiomycete *Blastocladiella emersonii* (Brunt *et al*., 1982; Fietto *et al*., 2002; Stump *et al*., 1980), in the Stramenopile alga *Heterosigma akashiwo* (Shono *et al*., 1995, 1996, 1998, 2001), and in the myxomycete *Dictyostelium discoideum* (accession number, AC116977.2). In fungi a recently described ATPase mediates the uptake of K^+ or Na^+, working in parallel with the H^+-ATPase and secondary K^+ transporters (Benito *et al*., 2004).

In order to increase our understanding of K^+ transport and energization of the plasma membrane in eukaryotic cells, we have studied the presence of Na^+ or K^+ ATPases in the *Pythium aphanidermatum* Stramenopile oomycete, the *Porphyra yezoensis* red marine alga, and the *Udotea petiolata* green marine alga. The group of oomycetes is made up of a large collection of eukaryotic species that belong to the group of Stramenopiles, together with brown algae and diatoms. Many oomycetes are terrestrial organisms that live associated to plants and resemble fungi, although oomycetes lack taxonomic affinity with true fungi (i.e., ascomycetes and basidiomycetes) (Latijnhouwers *et al*., 2003). The red algae are probably monophyletic with their close relatives green algae and land plants (Burger *et al*., 1999) but are phylogenetically distant from these two groups and more distant from Stramenopiles (Turmel *et al*., 2002; Van-de-Peer and Wachter, 1997). From the point of view of their lifestyles, oomycetes thrive in dilute medium, resembling fungi, while marine red and green algae thrive in Na^+ abundant environments, resembling animal cells.

We here report that *P. aphanidermatum*, *P. yezoensis*, and *U. petiolata* have P-type ATPases that cluster in the phylogenetic group of the animal H^+,K^+- and Na^+,K^+-ATPases although all of them are considerably divergent from the animal enzymes. We describe a preliminary functional characterization of IIC-type ATPases of *P. aphanidermatum* and *P. yezoensis* and discuss the function of these enzymes and plasma membrane energization in eukaryotic cells different from typical animal, plant, and fungal cells.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

Pythium aphanidermatum, strain 356128, was obtained from the CABI Bioscience UK Centre (Egham, UK) collection and was grown routinely in MYP medium containing 1% maltose, 1% yeast extract, 2% peptone. *Udotea petiolata* and *Porphyra leucosticta* were collected from the Mediterranean Sea, and immediately frozen in liquid nitrogen. *Escherichia coli* strain DH5α was routinely used for plasmidic DNA propagation. Tests of functional expression of the cloned genes or cDNAs were carried out with two transport mutants derived from the *Saccharomyces cerevisiae* wild-type strain W303.1B (*Matα ura3 his3 leu2 ade2 trp1*), WΔ3 (*Mat **a** ade2 ura3 trp1 trk1Δ::LEU2 trk2Δ::HIS3*) deficient in the TRK1 and TRK2 K^+ uptake systems (Haro *et al*., 1999), and B31 (*ena1Δ::HIS3::ena4 nha1Δ::LEU2*) deficient in the two Na^+ efflux systems (Bañuelos *et al*., 1998); and with the *E. coli* strain TKW4205 (*thi rha lacZ nagA recA Sr::Tn10 ΔkdpABC5 trkA405 Kup1*), deficient in the three K^+ uptake systems, Kdp1, TrkA, and Kup, as described previously (Senn *et al*., 2001). Yeast strains were routinely grown in YPD or YPD supplemented with 30 mM K^+ (for strain WΔ3). Strain TKW4205 was routinely grown in LB medium supplemented with 50 mM K^+. For functional expression tests, the genes or cDNAs were cloned into plasmids pYPGE15 (Brunelli and Pall, 1993) for yeast tests and pBAD24 (Guzman *et al*., 1995) for bacterial tests. The yeast vector has the phospho glycerate kinase constitutive expression promoter, and in the bacterial vector the promoter is induced by arabinose. The tests were performed at several K^+ and arabinose concentrations. The procedures and composition of the yeast (Rodríguez-Navarro and Ramos, 1984; Santa-María *et al*., 1997) and bacterial (Senn *et al*., 2001) media that were used for testing the capacity of the cloned cDNAs to suppress the defective growth of the mutants at low K^+ have been described previously. These media contain arginine and phosphoric acid but do not contain K^+ or Na^+, which are added as required in each experiment.

Recombinant DNA Techniques

Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer's instructions. PCRs were performed in a Perkin-Elmer thermocycler, using the Expand-High-Fidelity PCR System (Roche Molecular Biochemicals). Some of the PCR fragments were first cloned into the PCR2.1-Topo vector using the TOPO TA Cloning Kit (Invitrogen). DNA sequencing was performed in an automated ABI PRISM 377 DNA sequencer (Perkin-Elmer). Total RNA and DNA were obtained using the RNeasy Plant kit and DNeasy Plant kit (Qiagen). PCR amplification of *PaKPA1* and *UpKPA1* cDNA fragments was carried out on doubled-stranded DNA synthesized from total RNA by using the cDNA Synthesis System kit (Amersham

Pharmacia Biotech) and degenerated primers described in Benito *et al.* (2000). Full-length cDNAs were obtained by using the 5′/3′ Race Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. PCR amplification of *PlSOS1* fragment was carried out on doubled-stranded DNA synthesized from total RNA of *P. leucosticta* using the specific primers designed from *Porphyra yezoensis* EST sequence obtained from Kazusa DNA Research Institute (http://www.kazusa.or.jp/) (accession number AU192232). Nucleotide sequence for the sense primer was 5′-GTCATCTGCAACGTCA CATCTG and for the antisense primer was 5′-CAGCCCATTGACAAGGAGTGAC. For cloning of *PaKPA1* and *PyKPA1* cDNAs into the expression vectors, DNA fragments corresponding to the ORFs encoding the transporters were amplified from the gene or full-length cDNA by PCR using specific primers that included the start and stop codons. Southern hybridization analyses were performed with a digoxygenin (DIG)-labelled probe (Roche Molecular Biochemicals) according to the manufacturer's instructions, at 42°C in the presence of 50% formamide.

Rb$^+$ Transport Assay

Rb$^+$ transport assays in bacteria were carried out as described elsewhere (Senn *et al.*, 2001). Cells were grown at 37°C in LB medium supplemented with $100 \mu g/mL$ ampicillin and 30 mM K$^+$ up to an absorbance of 1.0. Arabinose was then added to a final concentration of 13 mM and the incubation of the cells was continued for an additional period of 15 min. Then the cells were centrifuged, transferred, and kept for 30 min in K$^+$-free minimal medium (Senn *et al.*, 2001) supplemented with 13 mM arabinose. After this K$^+$ starving period, the cells were transferred to fresh K$^+$-free minimal medium to which Rb$^+$ was added to assay the initial rate uptake. Cell samples were taken at intervals, filtered through 0.45 μm pore membrane filters (Millipore), and washed with the same minimal medium supplemented with 50 mM MgCl$_2$. Cells were acid extracted overnight in a 0.1 M HCl solution and the Rb$^+$ concentration in the supernatant was determined by atomic emission spectrophotometry. All experiments were repeated at least three times.

RESULTS

Cloning of the PaKPA1 Gene

By using standard PCR methods and degenerate oligonucleotides that correspond to conserved sequences of Ca^{2+}-, Na$^+$-, and Na$^+$,K$^+$- ATPases (Benito *et al.*, 2002; Garciadeblas *et al.*, 2001), we amplified DNA frag-

ments of three genes of *P. aphanidermatum* that could encode P-type ATPases (*KPA1*, *Pyt1*, and *Pyt2*). The cloned fragments could encode polypeptides of approximately 1200 amino acids that started in the conserved sequence CSDK and included the TGDGV sequence (these sequences delimitate a large part of the intracellular fragment located between the fourth and fifth transmembrane fragments of P-type ATPases, in which the site for ATP binding and hydrolysis is located). According to previous studies (Axelsen and Palmgren, 1998), a phylogenetic analysis of these translated sequences indicated that one of these putative ATPases belonged to the cluster of Na$^+$,K$^+$-ATPases (IIC), another to the SERCA-type Ca^{2+}-ATPases (IIA), and a third sequence was close to several prokaryotic ATPases that have been conventionally classified as Ca^{2+}-ATPases (see Fig. 2 in Benito and Rodríguez-Navarro, 2003). Related to these prokaryotic ATPases, which are distant from the eukaryotic ATPases of groups IIA, IIB, IIC, or IID, there are three eukaryotic ATPases, from *Plasmodium falciparum*, *Plasmodium yoeli*, and *Trichomonas vaginalis*. The *P. falciparum* ATPase, which is named PfATP4, has been characterized and defines a novel subclass of Ca^{2+}-ATPases (Krishna *et al.*, 2001). The ATPase of *P. aphanidermatum* in this group is close to the *P. falciparum* and *P. yoeli* ATPases and distant from the *T. vaginalis* ATPase (Fig. 1). These findings suggested that two of the ATPases identified from *P. aphanidermatum* were Ca^{2+}-ATPases, which were not within the scope of this study. Therefore, we did not continue to research them and concentrated on the gene that could encode the Na$^+$,K$^+$-ATPase, which we named KPA1 (K$^+$ P-type ATPase), according to the function described below.

To obtain the complete sequence of the *PaKPA1* gene, the DNA fragment that was amplified with the degenerate primers had to be extended toward the 5′ and 3′ ends of the gene because the fragment was located approximately toward the middle of a gene encoding a typical P-type ATPase. For this purpose we used primers that corresponded to this fragment and carried out PCR amplifications on cyclic DNA fragments generated from the *Xba*I digestion of the genomic DNA following the technique described previously (Bañuelos *et al.*, 2002). Using this procedure we amplified a complete ORF of 3351 bp that comprised the original fragment. The translated polypeptide had 1117 amino acids and a calculated molecular mass of 122.2 kDa. By Southern Blot analysis (Fig. 2), it was demonstrated that the cloned gene belonged to *P. aphanidermatum* and that it probably occurred as a single copy (the fragments revealed by the probe in Fig. 2 correspond to those expected from the sequence of *PaKPA1* gene). Alignment of PaKPA1 with other P-type ATPases revealed the presence of the conserved fragments (not

Cation-transporting ATPases
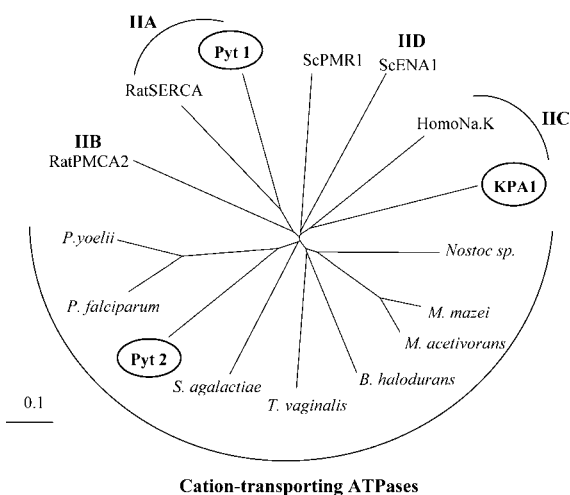
Fig. 1. Phylogenetic relationships of a putative Ca^{2+}-ATPase of *Pythium aphanidermatum* with prokaryotic and parasite P-type ATPases. Eukaryotic ATPases of other types are presented as references. Alignments of the sequences were performed with the ClustalX program, aligning the fragments comprised between the conserved sequences CSDK and TGDGV of P-type ATPases. The scale bar corresponds to a distance of 10 changes per 100 amino acid positions. Sequence identifications and accession numbers: Human-Na,K, P05023; KPA1, AJ972678; *Nostoc sp*, NP_487415; *Methanosarcina mazei*, NP_632859; *Methanosarcina acetivorans*, NP_618950; *Bacillus halodurans*, NP_243381; *Trichomonas vaginalis*, AAD37688; *Streptococcus agalactiae*, NP_687925; Pyt2, AJ973121; *Plasmodium falciparum*, AAF17245; *Plasmodium yoelii*, EAA15769; RatPMCA2, P11506; RatSERCA, A34307; Pyt1, AJ973120; ScPMR1, P13586; ScENA1, P13587.

shown) and some amino acid residues (Fig. 3) that are important for the function of H^+,K^+- and Na^+,K^+-ATPases (group IIC) (Kaplan, 2002; Pedersen *et al.*, 1998). Interestingly, the conserved sequences of these ATPases **NVPEG** in M4 and **DLGTD** in M6 (in bold the amino acids that are essential for the function) were not entirely maintained in PaKPA1, Gln substituted for Glu in M4 and Glu for the second Asp in M6. In M5 a Ser residue that is conserved in Na^+,K^+-ATPases is changed into a Lys residue in H^+,K^+-ATPases and into an His residue in PaKPA1. In M5 a single positively charged residue seems to determine electroneutrality in H^+,K^+- and Na^+,K^+-ATPases (Burnay *et al.*, 2003), a Ser residue is conserved in Na^+,K^+-ATPases (which exchange 3 Na^+ for 2 K^+), and a Lys residue in H^+,K^+-ATPases (which probably exchange 2 H^+ for 2 K^+). Instead of these residues, PaKPA1 has a His residue, which suggests that PaKPA1 may be electroneutral.

Cloning of the PyKPA1 cDNA

P. yezoensis has been cultivated for many years in countries of East and South Asia. The biology and ge-
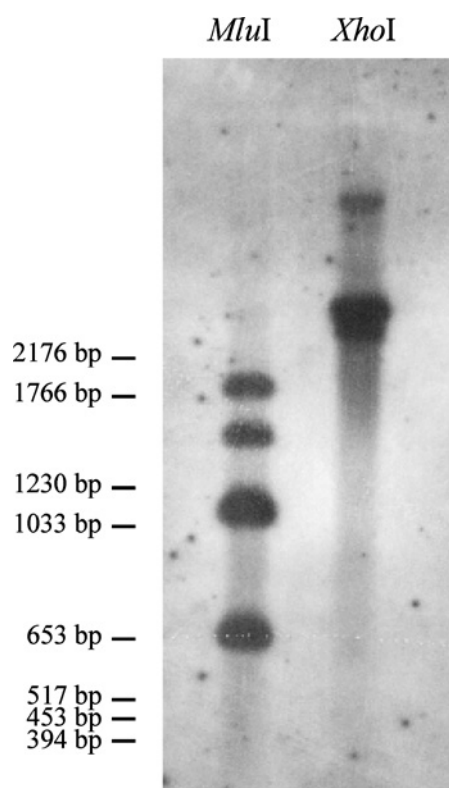
Fig. 2. Southern Blot analysis of total DNA from *P. aphanidermatum* probed with a DNA fragment of the *Kpa1* gene between positions +1 and +1644. The DNA ($10 \mu g$) was digested with *Mlu*I or *Xho*I and, after electrophoresis, it was transferred to a nylon membrane, and hybridized at $42°C$ in the presence of 50% formamide. Identical results were obtained at 20% formamide.

netics of this and related species have been studied, and a large database of ESTs has been generated. A Blast search using as query the *H. akashiwo* Na^+,K^+-ATPase (accession number, BAA82752) in http://www.ncbi.nlm.nih.gov allowed us to identify 116 ESTs that could correspond to Na^+,K^+-ATPases. One of these ESTs (accession number, AV429924) clearly belonged to a full-length cDNA clone that was subsequently obtained from Kazusa DNA Research Institute (http://www.kazusa.or.jp/). The sequencing of the clone revealed an ORF of 3507 bp that could encode a protein of 1169 amino acids with a molecular mass of 128.43 kDa, (we named PyKPA1 so far). A Blast search using as query the *PyKPA1* cDNA sequence revealed that 111 ESTs out of the 116 that we had originally identified corresponded to *PyKPA1*. Alignment of PyKPA1 with other P-type ATPases revealed the presence of the conserved fragments (not shown) and the amino acid residues (Fig. 3) that are necessary for the function of Na^+,K^+-ATPases (Pedersen *et al.*, 1998).

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                         M4                        M5                        M6

IIC
BufoNaK          FLIGIIVANVPEGLLATVTV     AYTLTSNIPEITPFLIFIIAD     TVTILCIDLGTDMVPAISLAY
RatNaK           FLIGIIVANVPEGLLATVTV     AYTLTSNIPEITPFLIFIIAN     TVTILCIDLGTDMVPAISLAY
HeterosigmaNaK   FLISIIVANVPEGLLATVTV     CYTLTSNIPEISPFLCFIVIG     TVLILGIDLGTDMVPAISMAY
UdoteaKPA1       FTIGIIVANVPEGLLATVTV     AYTLTSNIPEIAPFLLYITIN     TVLILCVDLGTDMIPAISLAY
PorphyraKPA1     FVIGIIVANVPEGLLATVTV     AYTLSSNIPEISPFLAFILTG     TVLILCIDLGTDMLPAISLAY
RatHK            FLISIIVANVPEGLLATVTV     AYTLTKNIAELCPFLIYIVAG     TITILFIDLGTDIIPSIALAY
BufoHK           FLIGIIVANVPEGLLATVTV     GYTLTKNVAELCPFLIYIIAD     TITILFIDLGTDIIPSVSFAY
PythiumKPA1      GFLVIIVANVPQGLPATVTS     AYTLTHLWPEIAPVAINLAFG     SLQVLSIDLGTELGPAISLAH

IIA
RatSERCA         IAVALAVAAIPEGLPAVITT     RYLISSNVGEVVCIFLTAALG     PVQLLWVNLVTDGLPATALGF

IID
ScENA1           YAICVALSMIPSSLVVVLTI     LQLLAENVAQALYLIIGLVFR     PVEVLWIIVVTSCFPAMGLGL
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Fig. 3. Amino acid sequence alignment of the M4, M5, and M6 fragments of the *Pythium, Porphyra*, and *Udotea* KPA1 ATPases together with selected ATPases of groups IIC, IIA, and IID. The amino acid residues with a black background are functional determinants according to Jorgensen and Pedersen (2001). The boxes highlight amino acid residues that determine the electrogenicity and therefore the stoichiometry of cation exchange in Na^+, K^+- and H^+,K^+-ATPases according to Burnay *et al.* (2003).

PCR Amplification of the UpKPA1 Gene

By using standard reverse transcription-PCR methods and RNA preparations of *U. petiolata* collected in the Mediterranean sea we cloned a DNA fragment of 1210 nucleotides that could encode an Na^+,K^+-ATPase fragment that included the CSDK and TGDGV sequences, as described above for the cloning of the *PaKPA1* gene. The full-length cDNA was then obtained by making use of the 5′/3′ Race Kit (Boehringer) as described in the Materials and Methods section. An analysis of the full-length cDNA sequence suggested the presence of at least three introns that included STOP codons and which made it impossible to use the clone that we had obtained for expression analyses in heterologous systems. In order to overcome this problem, we obtained additional full-length cDNA clones that included the polyT tail, but in all cases the introns were present. Amplification of the gene, which was named *UpKPA1*, from *U. petiolata* DNA preparations, proved that the gene and cDNA sequences were identical, which suggested that the dominant mRNA molecules in our preparations were unprocessed. The reason for this is unknown but may be related to the sampling time and the oscillating characteristics of K^+ uptake in some algae (Escassi *et al.*, 2002). In any case, this problem prevented further expression studies.

Alignment of possible translated sequences of the *UpKPA1* gene with group IIC ATPases allowed us to delimit the putative introns and obtain the most probable sequence of the encoded ATPase. The predicted protein has 1178 amino acids and a molecular mass of 129.5 kDa. Alignment of the putative UpKPA1 with other P-type ATPases revealed the presence of the conserved fragments (not shown) and the amino acid residues (Fig. 3) that are necessary for the function of Na^+,K^+-ATPases.

Functional Expression of PaKPA1 and PyKPA1 in Yeast and Bacteria

In order to investigate the functions of the PaKPA1 and PyKPA1 ATPases, we inserted the *PaKPA1* gene and the *PyKPA1* cDNA in the yeast expression vector pYPGE15 (Brunelli and Pall, 1993) and the constructs were then transformed into the *Saccharomyces cerevisiae* mutants W△3 (*trk1△ trk2△*), which is deficient for K^+ transport (Madrid *et al.*, 1998), and B31 (*ena1-4△ nha1△*), which is deficient in Na^+ efflux (Bañuelos *et al.*, 1998; Haro *et al.*, 1991). The *PaKPA1* and *PyKPA1* clones transformed into W△3 (*trk1△ trk2△*) failed to suppress the defective growth of this strain at low K^+. In B31 (*ena1-4△ nha1△*), *PyKPA1* showed a weak suppression of the Na^+ sensitivity (Fig. 4), allowing growth at 1 mM K^+, 20 mM Na^+, but we could not detect any effect of the *PaKPA1* gene. These results suggested that the putative PyKPA1 ATPase could mediate Na^+ extrusion but no further information could be obtained from the yeast expression experiments.

In our search for a clear demonstration of the functions of the PaKPA1 and PyKPA1 proteins, we cloned the corresponding gene and cDNA into the bacterial expression vector pBAD24 (Guzman *et al.*, 1995) under the control of the arabinose-responsive promoter P_{BAD}, and the constructs were transformed into the *E. coli* TKW4205

SD- Ura

B3.1 (pYPGE15)

B3.1 (PyKPA1)

1 mM K$^+$/20mM Na$^+$

B3.1 (pYPGE15)

B3.1 (PyKPA1)
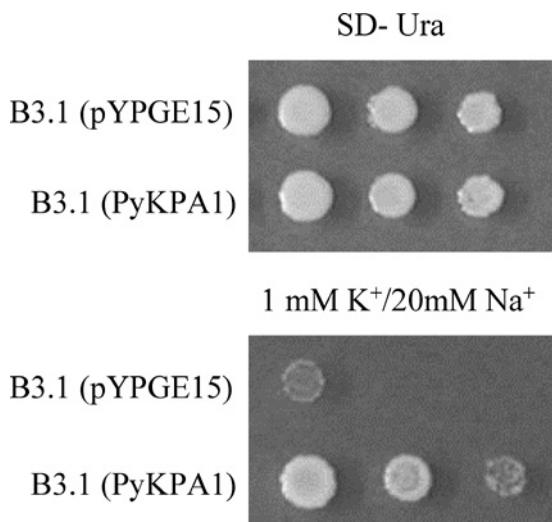
Fig. 4. PyKPA1 partially suppressed the defective growth of the *S. cerevisiae* strain B31 (*ena1-4Δ nha1Δ*) in the presence of Na$^+$. Drops of three serial dilutions of the B31 mutant transformed with the empty pYPGE15 plasmid (negative control) and the plasmid pYPGE15 containing the *PyKPA1* cDNA were inoculated in the arginine phosphate minimal medium supplemented with 1 mM K$^+$ and 20 mM Na$^+$.

strain, deficient in the endogenous K$^+$ uptake systems. Growth tests were then carried out at low K$^+$ concentrations and at several concentrations of arabinose, in order to modulate the expressions of the ATPases. This approach has been successfully used for the functional expression analysis of plant K$^+$ transporters (Garciadeblas *et al.*, 2002; Senn *et al.*, 2001). Strain TKW4205 transformed with the pBAD24 constructs that included the *PaKPA1* gene and the *PyKPA1* cDNA grew better than the recipient strain transformed with the empty plasmid at low K$^+$ and pH 5.5, in a medium containing 13 mM arabinose. Interestingly, the effect of *PaKPA1* was less clear in the presence of Na$^+$ whereas the effect of *PyKPA1* was clearer in the presence of 5 mM Na$^+$ (Fig. 5(A)). *PaKPA1* also improved the growth of the recipient strain at low K$^+$ and pH 7.5. These results suggested that the putative PaKPA1 and PyKPA1 ATPases were K$^+$ transporters, and that Na$^+$ activated the K$^+$ uptake mediated by PyKPA1 but not that mediated by PaKPA1. To further investigate the transport function of PaKPA1 and PyKPA1, we carried out Rb$^+$ uptake experiments with the bacterial transformants, because Rb$^+$ is transported by animal Na$^+$,K$^+$-ATPases (Jorgensen and Pedersen, 2001). The *PyKPA1* transformant showed more rapid Rb$^+$ uptake than the mutant strain transformed with the empty plasmid (Fig. 5(B)) but the *PaKPA1*-transformed strain failed to show a similar effect (not shown).

K$^+$-, Na$^+$-, or H$^+$-ATPases in Nonanimal Eukaryotic Cells

The study of K$^+$ transport and its relationship with the membrane potential in the water mold *Blastocladiella* (Brunt *et al.*, 1982; Stump *et al.*, 1980) suggests that plasma membrane energization in this organism does not belong to any of the two models described for animal, fungal, and plant cells (Rodríguez-Navarro, 2000). Cloning of the *Blastocladiella* BePAT1 and BePAT2 ATPases (Fietto *et al.*, 2002) and the PaKPA1 ATPase described in this study further supports this notion and that it applies to other organisms. This prompted us to carry out a search for sequences that could encode K$^+$-, Na$^+$-, or H$^+$-ATPases in EST databases of algae, oomycetes, and other selected eukaryotic nonmetazoan organisms (Table I).

In *Porphyra* the search produced a clear model, ESTs encoding Na$^+$,K$^+$-ATPase were abundant, as described above, whereas ESTs encoding H$^+$-ATPase were absent. This suggests that *Porphyra* belongs to the animal model. Interestingly, we detected the presence of an *SOS1* gene (EST accession number AU192232), which is a typical plant gene that encodes an Na$^+$/H$^+$ antiporter (Zhu, 2003). The existence of only one SOS1 EST raised the possibility that the corresponding cDNA was a contamination. Therefore, in order to rule this out, we investigated the presence of an SOS1 cDNA in *Porphyra leucosticta* collected by us in the Mediterranean Sea in the South of Spain. As could be expected an *SOS1* gene existed in *P. leucosticta* and its sequence contained that of the EST from the *P. yezoensis* cDNA library (results to be published elsewhere).

In contrast with *Porphyra*, in the green alga *Chlamydomonas reinhardtii* (http://genome.jgi-psf.org/chlre2/chlre2.home.html), we found only one EST that could encode a IIC ATPase (EST accession number BI726894) and the corresponding gene sequence (C_200014 [chlre2:1594789]), and many that could encode H$^+$-ATPases. In *Chlamydomonas* an H$^+$-ATPase has been described (Campbell *et al.*, 2001) and another was identified in our search (accession number CAC19368). For both types of H$^+$-ATPases, ESTs were abundant. The sequence of the IIC-type ATPase of *C. reinhardtii* showed maximal similarity to the *U. petiolata* sequence and lower similarity to the sequences of the Stramenopile organisms *P. aphanidermatum*, *H. akashiwo*, and *Phytophthora sojae* (see below).

In oomycetes, only two ESTs that could correspond to IIC-type ATPases were found in *Phytophthora infectans* (EST accession numbers, CF106736 and CV925264), but none in *Phytophthora sojae*. Interestingly, the genome of *Phytophthora sojae* contains a gene encoding a protein similar to PaKPA1 (NaK2, estExt_fgenes1_pg.C_230124
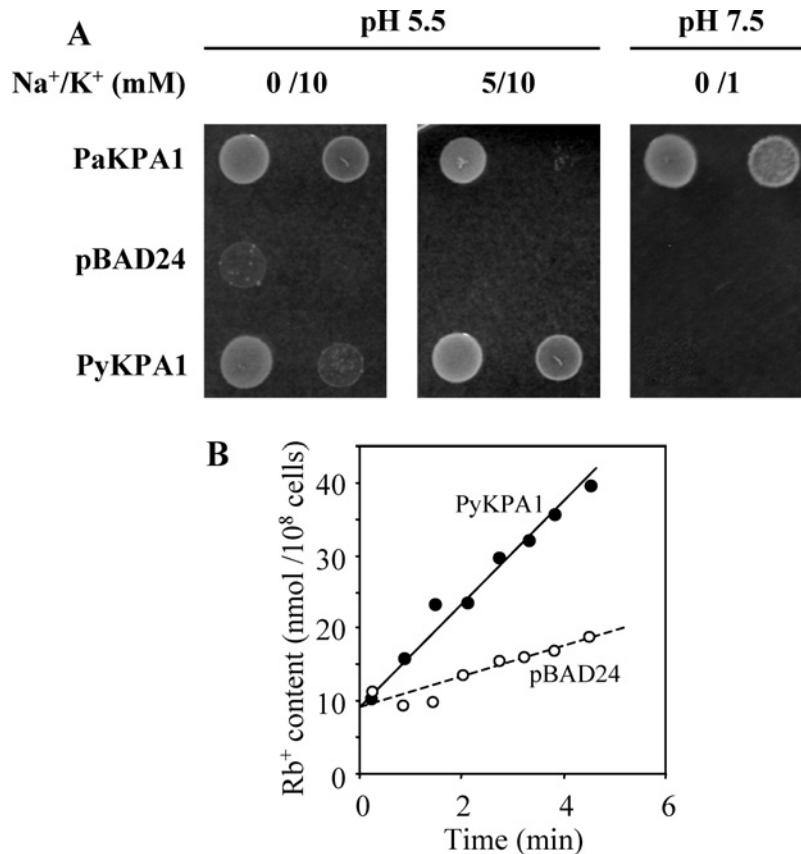
Fig. 5. (A) PaKPA1 and PyKPA1 mediated bacterial growth at low K$^+$. The *E. coli* strain TKW4205, defective for K$^+$ transport, was transformed with the empty plasmid pBAD24 and with the plasmid containing the *PaKPA1* gene or the *PyKPA1* cDNA under the control of an arabinose-responsive promoter. Two drops with serial dilutions of the bacteria were inoculated on minimal medium containing 0/10 or 5/10 mM Na$^+$/K$^+$ adjusted to pH 5.5, or 0/1 mM Na$^+$/K$^+$ adjusted to pH 7.5. In all cases 13 mM arabinose was present. (B) Time course of Rb$^+$ uptake by *E. coli* strain TKW4205 expressing *PyKPA1*. Experiments with K$^+$-starved cells in K$^+$-free minimal medium pH 5.5 carried out after addition of 10 mM RbCl as described in Materials and Methods. Open symbols correspond to the Rb$^+$ uptake of a control experiment carried out with the TKW4205 strain transformed with the empty plasmid pBAD24.

[sojae1:132981]), and another encoding a protein similar to the Na,K-ATPase of *H. akashiwo* (NaK1, estExt_fgenesh1_pg.C_50216 [sojae1:128719]), http://genome.jgi-psf.org/sojae1/sojae1.info.html). Moreover, several ESTs that could encode H$^+$-ATPases were found in oomycetes. In *Phytophthora* we found three different types, one type (estExt_fgenes1_pg. C-1370010 [sojae1:14336]) showed maximum similarity to *Trypanosoma* H$^+$-ATPase (accession number, AAP30857) and the other two (estExt_Genewise1.C_10281 [sojae1:10043] and estExt_fgenesh1_pg.C_103338 [sojae1:127358]) showed maximum similarity to plant and green alga *Dunaliella* H$^+$-ATPases type, respectively.

Although our work was focused primarily on oomycetes and algae, *D. discoideum* was an attractive organism because of the existence of many ESTs in databases. In this organism the search revealed a high number of ESTs that could encode IIC (e.g., accession numbers, AU062008 or AU060727) and H$^+$-ATPases (e.g., accession numbers AU062013 and BJ418991).

Phylogenetic Analysis of IIC ATPases

The six IIC or related ATPases from *Pythium* (oomycete), *Phytophthora* (oomycete), *Porphyra* (red marine alga), *Udotea* (green marine alga), and

Table I. Numbers of EST of Putative Na$^+$, K$^+$-ATPases and Putative H$^+$-ATPases in Selected Eukaryotic Organisms

Species	Total ESTs	ESTs of putative Na$^+$, K$^+$-ATPases	ESTs of putative H$^+$-ATPases
Dictyostelium discoideum	155,032	59	166
Chlamydomonas reinhardtii	167,641	1	62
Porphyra yezoensis	20,979	111	0
Phytophthora sojae	28,092	0	26
Phytophthora infectans	80,005	2	24

Note. BLAST searches were carried out against the translated EST sequences in http://www.ncbi.nlm.nih.gov/. Putative Na$^+$,K$^+$-ATPases were identified using the Iona sequence of *Dictyostelium discoideum* (accession number AAO51268) and the *Heterosigma akashiwo* Na$^+$. K$^+$-ATPase (accession number BAA82752) as query sequences. Putative H$^+$-ATPases were identified using the H$^+$-ATPase from *Dictyostelium discoideum* (accession number P54679), *Chlamydomonas reinhardtii* (accession number AJ300672), and *Saccharomyces cerevisiae* (accession number 1203382A) as query sequences.

Chlamydomonas (terrestrial green alga) identified in this study prompted us to study their phylogenetic relationships with animal and previously identified ATPases from the chitridiomycete *Blastocladiella*, the slime mold *Dictyostelium*, and the Stramenopile marine alga *Heterosigma*. The phylogenetic analysis (Fig. 6) revealed that the sequences from *Porphyra, Udotea, Chlamydomonas*, and one ATPase from *P. sojae* could be included in cluster IIC (Axelsen and Palmgren, 1998) together with the *Dictyostelium* and *Heterosigma* ATPases. The sequences of *Pythium*, Na,K2 from *P. sojae* and the *Blastocladiella* ATPases, BePAT1 and BePAT2, were less similar to the animal ATPases and their possible inclusion in group IIC needs further study. In any case, the ATPases from *P. aphanidermatum* and *P. sojae*, and the two from *Blastocladiella* ATPases do not form a common cluster. The most likely possibility is that they are members of two different groups that diverged similarly from each other and from animal group IIC ATPases.

DISCUSSION

In this report we identify and achieve a preliminary functional characterization of IIC P-type ATPases in three nonanimal eukaryotic organisms, belonging to oomycete, red alga, and green alga groups. The greatest difficulty for an extensive study aimed at determining the mechanisms involved in ion transport and energy conservation

in the plasma membrane of different types of eukaryotic cells is the absence of a simple procedure for the functional characterization of the cloned ATPases. This can be achieved either by gene disruption or by heterologous expression of the cloned gene or cDNA in a well characterized organism, bacterial or yeast mutants, *Xenopus* oocytes, insect cells, etc. The former procedure is practically unattainable in organisms that have not been extensively studied and in which homologous recombination is probably of low frequency, and the latter has presented technical difficulties to date. The procedure reported here, using the TKW4205 mutant of *E. coli* and an expression vector in which the tested transporter is expressed under the control of the arabinose-responsive promoter P$_{BAD}$, was described for expressing plant K$^+$ transporters (Senn *et al.*, 2001). In these systems, both putative ATPases, PaKPA1 and PyKPA1, partially suppressed the defect of the mutant and PyKPA1 mediated Rb$^+$ uptake. These results reasonably support that both putative ATPases pump K$^+$ into the cell. Furthermore, considering the enhancing effects of Na$^+$ on the bacterial clone expressing PyKPA1, it can be suggested that PyKPA1 is a Na$^+$-K$^+$-ATPase. This proposal is also supported by the yeast expression experiments (Fig. 4) and by the conserved amino acid and sequence similarities (also that of UpKPA1) with Na$^+$,K$^+$-ATPases (Figs. 3 and 6). In the case of PaKPA1, the conserved amino acids suggested that the function might be similar to that of H$^+$,K$^+$-ATPases (Fig. 3).

Another conclusion of our study is that plasma membrane energization in eukaryotes is complex and cannot be accounted for exclusively by the two models (see Introduction section) that perfectly explain the bioenergetics of the plasma membrane of animal, plant, and fungal cells. In algae, the existence of H$^+$-ATPases is well established (Campbell *et al.*, 2001; Ohta *et al.*, 1997; Wada *et al.*, 1996; Weiss and Pick, 1996; Wolf *et al.*, 1995) and this suggests that algae and plants follow the same model. However, our findings suggest that *P. yezoensis* contradicts this conclusion because it expresses a Na$^+$,K$^+$-ATPase instead of expressing an H$^+$-ATPase. The assumption that marine algae follow the animal model because they adapted to the animal cell environment may be also an oversimplification of the situation because the *P. yezoensis* model does not apply to some marine algal species of *Dunaliella*, in which expression of H$^+$-ATPases has been described (Wada *et al.*, 1996; Wolf *et al.*, 1995) while a Na$^+$-pump expression has never been found (Katz and Pick, 2001). In contrast, the green alga *C. reinhardtii* expresses a Na$^+$,K$^+$-ATPase (Fig. 6, Table I), although the expression is weak (according to the EST isolation frequency), and two putative H$^+$-ATPases
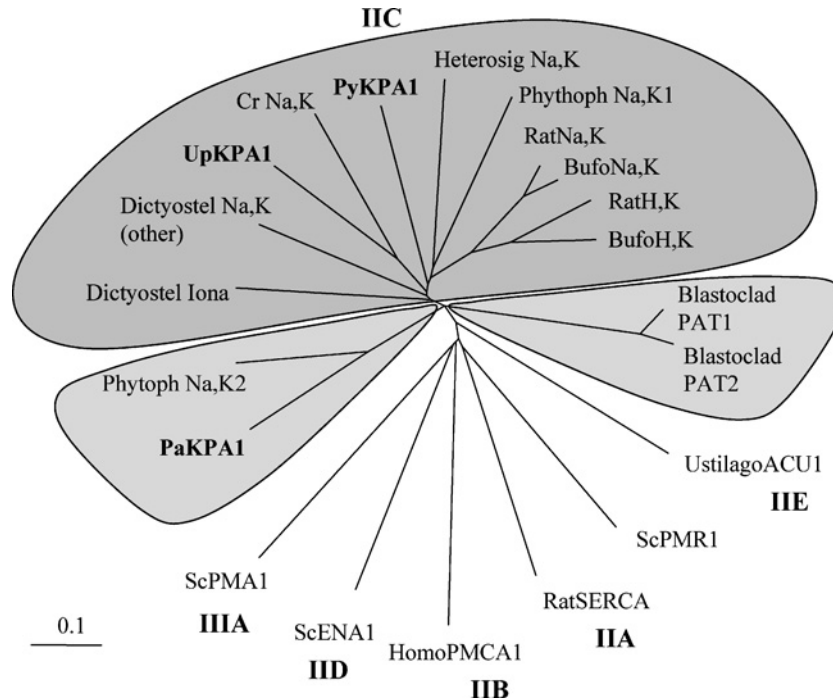
Fig. 6. Phylogenetic relationships of KPA1 ATPases with P-type ATPases of groups II and IIIA. Accession numbers (ordered clockwise from the representative of group IIE): *Ustilago maydis* ACU1, AJ622829; *Saccharomyces cerevisiae* PMR1, P13586; RatSERCA, 1910193A; Homo PMCA1, M95541.1; *S. cerevisiae* ENA1, P13587; *S. cerevisiae* PMA1, 1203382A; *Pythium aphanidermatum* KPA1, AJ972678; *Phytophthora sojae* Na,K2, (estExt_fgenesh1_pg. C_230124 [sojae1:132981]) (in http://genome.jgi-psf.org/sojae1/sojae1.info.html); *Dictyostelium discoideum* Iona, AAO51268; *D. discoideum* Na,K, JC1a27f12.r1 (in http://genome.imb-jena.de); *Udotea petiolata* KPA1, AJ972675; *Chlamydomonas reinhardtii* Na,K, C_200014 [chlre2:159478] (in http://genome.jgi-psf.org/chlre2/chlre2.home.html); *Porphyra yezoensis* KPA1, AJ972674; *Heterosigma akashiwo* Na,K, BAA82752; *Phytophthora sojae* Na,K1, (estExt_fgenesh1_pg. C_50216 [sojae1:128719]) (in http://genome.jgi-psf.org/sojae1/sojae1.info.html); Rat Na,K, M14511.1; *Bufo marinus* Na,K, S24650; Rat H,K, HKV94912.1; *Bufo marinus* H,K, 2003318A; *Blastocladiella emersonii* PAT1, T43025; *B. emersonii* PAT2, AF205944.1.

(Table I). This suggests that some terrestrial algae may express a Na^+,K^+-ATPase for some unknown functions but that the energetics of the plasma membrane is dominated by H^+-ATPases. Interestingly, the Stramenopile marine species *Heterosigma*, which is phylogenetically related to oomycetes, uses a Na^+,K^+-ATPase (Shono *et al.*, 2001) related to one of *P. infectans* (Fig. 6) and also a H^+-ATPase (Wada *et al.*, 1994).

In heterotrophic eukaryotic terrestrial organisms, the diversity is also clear. Our results indicate that oomycetes express ATPases in group IIC (the *Phytophthora* sequence in Fig. 6) and others in a related group (PaKPA1 and the *Phytophthora* sequence in Fig. 6) but that the expression of a H^+-ATPase is stronger (Table I). In the chytridiomycete *Blastocladiella* the model may be that the membrane potential is established by the K^+ equilib-

rium potential (Brunt *et al.*, 1982) and the K^+ asymmetry may be established by a K^+-ATPase. This function might be carried out by BePAT1 and BePAT2 ATPases whose sequences do not cluster with typical Na^+,K^+-ATPases, in group IIC (Fig. 6). In *Dictyostelium* the model is ambiguous because it has a IIC ATPase and two H^+-ATPases, and at least, one of them is important at acidic pH values but not at neutral pH (Coukell *et al.*, 1997).

To sum up, the two models for plasma membrane energization described so far, the animal cell model, in which Na^+,K^+-ATPases play the dominant role and the plant cell model, in which the H^+-ATPases are dominant, seem to apply to algal species. The former apply to the red alga *P. yezoensis* and the latter to the green alga *D. salina*. In addition, other eukaryotes, *Dictyostelium, Heterosigma, Chlamydomonas*, and *Phytophthora*,

express both Na^+,K^+-ATPases and H^+-ATPases to a greater or lesser extent and may define intermediate models that have not yet been described.

Finally, the existence of an *SOS1* gene in *P. yezoensis* of Japanese origin (EST accession number AU192232), and by an almost identical gene in a *Porphyra leucosticta* collected on the South coast of Spain (unpublished results), links *Porphyra* to higher plants. The plant SOS1 Na^+/H^+ exchanger has sequence similarity with NHX exchangers that may locate to the tonoplast (Yokoi *et al.*, 2002) but all SOS1 exchangers are very similar in sequence and the *Porphyra* sequence clearly belongs to the SOS1 group. This poses a question about the function of a weakly expressed Na^+/H^+ exchanger working parallel with a highly expressed Na^+,K^+-ATPase.

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