Presence of an isoform of H^+-pyrophosphatase located in the alveolar sacs of a scuticociliate parasite of turbot: physiological consequences

NATALIA MALLO[1], JESÚS LAMAS[2], ANA-PAULA DEFELIPE[1], MARIA-EUGENIA DECASTRO[3], ROSA-ANA SUEIRO[1,2] *and* JOSÉ-MANUEL LEIRO[1]*

[1] *Departamento de Microbiología y Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, 15782 Santiago De Compostela, Spain*
[2] *Departamento de Biología Celular y Ecología, Facultad de Biología, Instituto de Acuicultura, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[3] *Departamento de Biología Celular y Molecular, Facultad de Ciencias, Universidad de A Coruña, 15701 A Coruña, Spain*

(*Received 17 September 2015; revised 16 November 2015; accepted 18 December 2015*)

SUMMARY

H^+-pyrophosphatases (H^+-PPases) are integral membrane proteins that couple pyrophosphate energy to an electrochemical gradient across biological membranes and promote the acidification of cellular compartments. Eukaryotic organisms, essentially plants and protozoan parasites, contain various types of H^+-PPases associated with vacuoles, plasma membrane and acidic Ca^{+2} storage organelles called acidocalcisomes. We used Lysotracker Red DND-99 staining to identify two acidic cellular compartments in trophozoites of the marine scuticociliate parasite *Philasterides dicentrarchi*: the phagocytic vacuoles and the alveolar sacs. The membranes of these compartments also contain H^+-PPase, which may promote acidification of these cell structures. We also demonstrated for the first time that the *P. dicentrarchi* H^+-PPase has two isoforms: H^+-PPase 1 and 2. Isoform 2, which is probably generated by splicing, is located in the membranes of the alveolar sacs and has an amino acid motif recognized by the H^+-PPase-specific antibody PAB_{HK}. The amino acid sequences of different isolates of this ciliate are highly conserved. Gene and protein expression in this isoform are significantly regulated by variations in salinity, indicating a possible physiological role of this enzyme and the alveolar sacs in osmoregulation and salt tolerance in *P. dicentrarchi*.

Key words: H^+-PPase, *Philasterides dicentrarchi*, alveolar sacs, osmoregulation, turbot.

INTRODUCTION

Proton-translocating inorganic pyrophosphatases (H^+-PPases) are extremely hydrophobic integral membrane proteins that utilize the energy released by hydrolysis of pyrophosphate (PPi), which possesses a high-energy phosphoanhydride bond, to transport H^+ across the biological membranes against the electrochemical potential gradient (Maeshima, 2000; Belogurov and Lahti, 2002; Serrano *et al*. 2004; Gaxiola *et al*. 2007). The first H^+-PPase was discovered in membranes isolated from the photosynthetic bacterium *Rodospirillum rubrum* (Baltscheffsky *et al*. 1966). It was subsequently found in homogenates and in higher plant vacuoles (V-H^+-PPases) as a proton pump (Karlsson, 1975) and was later detected in acidocalcisomes of parasitic protozoa (Scott *et al*. 1998). Although this enzyme was long considered to occur only in plants and some photosynthetic bacteria (Drozdowicz *et al*. 2003), it has since been identified in a wide range of organisms including prokaryote extremophiles, fungi, some algae and protozoa (Maeshima, 2000; Drozdowicz and Rea, 2001). In plants, V-H^+-PPases are present in the vacuole membrane (tonoplast) and also in the plasma membrane (Rea and Poole, 1993; Long *et al*. 1995; Robinson *et al*. 1996). In protozoa, V-H^+-PPase is an integral membrane-associated protein that has been located in the acidocalcisomes and also in the Golgi apparatus, plasma membrane, digestive vacuoles and in a microneme maturation vacuolar compartment of trypanosomatids (Harper *et al*. 2006).

The first indication of the existence of the diversity and functional heterogeneity of V-H^+-PPases was found in the plant *Arabidopsis thaliana,* and two distinct enzyme categories were observed: AVP1 and AVP2 (Arabidopsis vacuolar H^+-pyrophosphatase). Phylogenetic analysis of these and V-H^+-PPases showed that AVP2, rather than being an isoform of AVP1, is representative of a novel category of AVP2-like (type II) V-PPases that coexist with AVP1-like (type I) V-H^+-PPases in plants and also in apicomplexan protists such as the malarial parasites (Drozdowicz *et al*. 2000). There is a clear evidence for the wide occurrence of V-H^+-PPase genes in hymenostomatid, peritrich and hypotrich ciliates (Pérez-Castiñeira *et al*. 2002); however,

* Corresponding author: Laboratorio de Parasitología, Instituto de Investigación y Análisis Alimentarios, c/ Constantino Candeira s/n, 15782 Santiago de Compostela (A Coruña), Spain. Tel: 34981563100. Fax: 34881816070. E-mail: josemanuel.leiro@usc.es

doi:10.1017/S0031182015001997

until recently the presence of V-H^+-PPase activity has only been demonstrated in the scuticociliate parasite of the turbot *Philasterides dicentrarchi* (Mallo *et al*. 2015).

More specifically, we report the results of a study showing, for the first time, the existence of two isoforms of H^+-PPase in *P. dicentrarchi*. One isoform of the enzyme was found in flat cortical sacs, designated 'alveolar sacs' in Ciliophora, and its gene expression was modulated by salt concentration.

MATERIALS AND METHODS

Parasites and experimental animals

Specimens of *P. dicentrarchi* (isolates B1, C1, D2, D3, I1, S1, P1; Iglesias *et al*. 2001; Budiño *et al*. 2011) were collected under aseptic conditions from peritoneal fluid obtained from experimentally infected turbot, *Scophthalmus maximus*, as previously described (Paramá *et al*. 2003). The ciliates were cultured at 21 °C in complete sterile L-15 medium, as previously described (Iglesias *et al*. 2003). In order to maintain the virulence of the ciliates, fish were experimentally infected every 6 months by intraperitoneal (ip) injection of 200 μL of sterile physiological saline containing 5×10^5 trophozoites, and the ciliates were recovered from ascitic fluid and maintained in culture as described above.

Turbot of approximately 50 g body weight were obtained from a local fish farm. The fish were held in 250-L tanks with aerated recirculating sea water maintained at 14 °C. They were subjected to a photoperiod of 12L:12D and fed daily with commercial pellets (Skretting, Burgos, Spain). The fish were acclimatized to laboratory conditions for 2 weeks before the experiments were started.

Eight to 10-week-old Institute for Cancer Research (ICR) (Swiss) CD-1 mice initially supplied by Charles River Laboratories (USA) were bred and maintained in the Central Animal Facility of the University of Santiago de Compostela (Spain) following the criteria of protection, control, care and welfare of animals and the legislative requirements relating to the use of animals for experimentation (EU Directive 86/609/EEC), the Declaration of Helsinki, and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The Institutional Animal Care and Use Committee of the University of Santiago de Compostela approved all experimental protocols.

Polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), real-time quantitative reverse transcriptase PCR (qRT-PCR)

P. dicentrarchi DNA was purified with DNAesy Blood and Tissue Kit (Qiagen) by following the manufacturer's instructions. DNA was analysed to estimate its quality, purity and concentration by A_{260} measurement in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA.).

The *P. dicentrarchi* trophozoites were incubated for 24 h in culture medium supplemented with different concentrations of NaCl: 4, 8 and 37‰. Total RNA was isolated from the trophozoites with a NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions. After purification of the RNA, the quality, purity and concentration were measured in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The reaction mixture used for cDNA synthesis (25 μL reaction mixture^{-1}) contained 1·25 μM random hexamer primers (Promega), 250 μM of each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol (DTT), 20 U of RNase inhibitor, 2·5 mM $MgCl_2$, 200 U of MMLV (Moloney murine leukemia virus reverse transcriptase (Promega) in 30 mM Tris and 20 mM KCl (pH 8·3) and 2 μg of sample RNA. PCR (for DNA and cDNA amplification) was performed with gene-specific primers for the H^+PPase gene: forward/reverse primer pair (FPiPh/RPiPh) 5′-CGGGACCAGAGGTATCTTTTA-3′/5′-ATTGATGTCAACGCCCCCTT-3′; and forward/reverse primer pair (F1qPiPh/R1qPiPh) 5′-GCCTACGAAATGGTCGAAGA-3′/5′-GCATCGGTGTATTGTCCAGA-3′ for real-time qRT-PCR. In parallel, real-time qRT-PCR was performed with β-tubulin as a reference gene, by including the primers for the β-tubulin gene (forward/reverse primer pair, 5′-ACCGGGGAATCTTAAACAGG-3′/5′-GCCACCTTATCCGTCCACTA-3′). The Primer 3Plus program was used, with default parameters, to design and optimize the primer sets. The PCR mixtures (25 μL) contained PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1·5 mM $MgCl_2$, pH 9·0), 0·2 mM of each deoxynucleoside triphosphate (dNTPs, Roche), 0·4 μM of each primer, 3 units of recombinant Taq polymerase (NZY Taq DNA polymerase, NZYTech, Portugal) and 50 ng of genomic DNA or 2μL of cDNA. The reactions were run in an automatic thermocycler (Biometra, Germany) as follows: initial denaturing at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 45 s and 72 °C for 1 min; and finally a 7 min extension phase at 72 °C. Quantitative PCR mixtures (10 μL) contained 5 μL Maxima SYBR green qPCR Master Mix (Thermo Scientific), 300 nM of the primer pair, 1 μL of cDNA and RNase-DNase-free water. Quantitative PCR was developed at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s, ending with a melting-curve analysis at 95 °C for 15 s, 55 °C for 15 s and 95 °C for 15 s. The specificity and size of PCR products were confirmed by 4% agarose gel electrophoresis. All

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

quantitative PCRs were performed in an Eco RT-PCR system (Illumina). Relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) applied with software conforming to minimum information for publication of qRT-PCR experiments (MIQE) guidelines (Bustin *et al*. 2009).

Production of recombinant H^+-PPase of *P. dicentrarchi* in yeast cells

The *P. dicentrarchi* RNA was purified using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and cDNA synthesis was performed as indicated in the previous section. The PCR was carried out with gene-specific primers designed from a partial sequence of the H^+-PPase of *P. dicentrarchi* (Mallo *et al*. 2015) (forward/reverse primer pair 5′-AAAGAAGAAGGGGTACCTTTGGATAA-AAGAattgatgtcaacgccccctt-3′/5′- TGGGACGCTC GACGGATCAGCGGCCGCTTAGTGGTGG-TGGTGGTGGTGgggaccagaggtatctttta-3′). These primers were designed and optimized using the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and by including a hybridization region with the yeast YEpFLAG-1 (Eastman Kodak Company) plasmid and a poly His region (lower case letters correspond with the gene annealing zone). The PCR reaction was initially developed at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1·5 min and 72 °C for 2 min and finally, a 7-min extension phase at 72 °C. The PCR products were purified using Gene Jet PCR Purification Kit (Fermentas, Life Sciences) according to the manufacturer's instructions.

Purified PCR products were cloned in YEpFLAG-1 (Eastman Kodak Company) yeast expression vector, a plasmid carrying a N-5′-phosphoribosylanthranilate isomerase (TRP1) gene that completes the auxotrophy for the tryptophan for the host yeast (López-López *et al*. 2010).

Linearized plasmid YEpFLAG-1 was digested with *EcoRI* and *SalI* (Takara) and used to transform *Saccharomyces cerevisiae* cells (strain BJ 3505) by the lithium acetate procedure (Ito *et al*. 1983). The procedure involves co-transformation of yeast cells with the linearized empty plasmid and the PCR-generated DNA fragment so that a recombination process occurs within the cell, yielding a plasmid bearing the desired insert. Positive colonies were selected using complete medium without tryptophan (CM-Trp) containing glucose (20 g L^{-1}), Yeast Nitrogen Base medium without amino acids (Sigma-Aldrich), supplemented with adenine (40 mg L^{-1}) and amino acids (histidine, leucine, tyrosine, 40 mg L^{-1} each; arginine, methionine, threonine 10 mg L^{-1} each; isoleucine and phenylalanine 60 mg L^{-1} each and lysine 40 mg L^{-1}).

Plasmid DNA was then extracted with Easy Yeast Plasmid Isolation Kit (Clonetech) according to the manufacturer's instructions. The purified and cloned DNA fragment was subjected to sequencing analysis (Sistemas Genómicos, Spain).

Recombinant protein of H^+-PPase of *P. dicentrarchi* was purified from transformed *S. cerevisiae* cultures, which had been incubated for 72 h in modified Yeast Peptone High Stability Expression Medium containing 1% glucose, 3% glycerol, 1% yeast extract and 8% peptone, at 30 °C, in Erlenmeyer flasks filled with 20% volume of culture medium (López-López *et al*. 2010). As the inoculum, a suitable volume of a pre-culture was added to obtain an initial OD_{600} of 0·1. The cell suspension was centrifuged at 7500 ***g*** for 15 min and the cleared supernatant was purified by immobilized metal affinity chromatography on a pre-charged Ni-Sepharose Histrap column (ÄKTAprime plus, GE Healthcare Life Sciences). The column was initially equilibrated with 25 mL of binding buffer (20 mM sodium phosphate, 0·5 M NaCl, 20 mM imidazole, pH 7·4). One hundred mL of culture medium was then passed through the column, and the protein bound to the column was finally eluted in 10 mL of elution buffer (20 mM sodium phosphate, 0·5 M NaCl, 250 mM imidazole, pH 7·4) (Mallo *et al*. 2015). The fractions thus obtained were analysed by 12·5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and dialyzed overnight in 2 L of bidistilled water. The dialyzed sample was concentrated in an Amicon Ultra centrifugal filter device (Millipore, USA) with a 10-kDa cut-off membrane. The final protein concentration was calculated by the Bio-Rad Protein Assay, which is based on the Bradford assay (Bradford, 1976).

Peptide synthesis

A peptide of 17 amino acid long corresponding to domain HKAAVIGDTIGDPLKDT (peptide HK) of the *P. dicentrarchi* H^+-PPase was synthesized and conjugated to keyhole-limpet haemocyanin (KLH), a carrier protein, to ensure maximum immunogenicity (ProteoGenix, France). A cysteine amino acid was added to two sequences to enable conjugation to KLH. The peptide was synthesized and conjugated to KLH by coupling with sulpho-SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate) reagent to yield 10–20 mg of product >85% purity, which was lyophilized and stored at −20 °C until use.

Immunization and serum extraction

A group of five ICR (Swiss) CD-1 mice were immunized by i.p. injection with 200 μL per mouse of a 1:1 (v/v) mixture of Freund complete adjuvant (Sigma-Aldrich) and a solution containing 500 μg of purified

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

recombinant H^+-PPase (rH^+-PPase) or with 400 μg of KLH conjugated to the synthetic peptide HK in phosphate buffered saline (PBS). The same dose of purified protein and peptide was prepared in Freund's incomplete adjuvant and injected i.p in mice 15 and 30 days after the first immunization. The mice were bled via the retrobulbar venous plexus 7 days after the second immunization (Piazzon *et al*. 2011). In order to obtain the polyclonal anti-rH^+-PPase (PAB_{rPPase}) or anti-HK (PAB_{HK}) antisera, the blood was left to coagulate overnight at 4° C before the serum was separated by centrifugation (2000 × ***g*** for 10 min), mixed 1:1 with glycerol and stored at −20 °C until use. In some experiments, a commercial rabbit polyclonal serum against KLH-conjugated synthetic peptide derived from the AVP1 isoform (UniProt P311414) of *Arabidopsis thaliana* V-PPase (PAB_{AVP1}, Agrisera, Sweden) was also used.

Western-blot analysis

Ciliate membrane-associated proteins (MAPs) were extracted by phase separation in Triton X-114 solution (Bordier, 1981), by a previously described method (Mallo *et al*. 2013). Specifically, 10^7 cells were resuspended in 1 mL of ice-cold 10 mM Tris–HCl buffer, pH 7·5, to which 1 mL of ice-cold extraction buffer (300 mM NaCl, 20 mM Tris–HCl, pH 7·5, 2% Triton X-114) was subsequently added. The cytoskeletal elements were eliminated by centrifugation at 16 000 × ***g*** for 10 min at 4 °C. The supernatant was then transferred to 1·5 mL Eppendorf tubes, which were heated for 5 min at 37 °C. At the end of this period, the solution became cloudy due to condensation of detergent micelles. The sample was then placed in 0·5 mL Eppendorf tubes (200 μL $tube^{-1}$) containing 300 μL of sucrose cushion (6% sucrose, 150 mM NaCl, 10 mM Tris–HCl, pH 7·5, 0·06% Triton X-114). The detergent and aqueous phases were separated by centrifugation at 300 ***g*** for 4 min at room temperature (RT). The resulting supernatants lying on the sucrose cushion of each tube were carefully removed and mixed in new 1·5 mL Eppendorf tubes. The extraction process was repeated by adding sufficient Triton X-114 to the aqueous mixture to yield a final concentration of 0·5%. The mixture was re-heated at 37 °C for 5 min. Once micellar condensation had taken place, the mixture was distributed among the original Eppendorf tubes containing the sucrose cushion and the detergent phase that was separated in the first extraction. The tubes were then recentrifuged at 300 ***g*** for 4 min at RT. The resulting supernatant was discarded and the proteins contained in the detergent phase were precipitated (by adding 9 volumes of cold acetone), resuspended (by vortexing) and finally incubated for 30 min on ice. The precipitated membrane proteins were then collected by centrifugation, at 16 000 ***g*** for 15 min at 4 °C, and dried in a speed vacuum concentrator (MiVac, GeneVac, UK). Finally, the extracts obtained were resuspended in 10 mM Tris–HCl, pH 7·5, and stored at −80 °C until use. The protein concentration of preparation was determined by the Bradford assay.

Samples from MAPs were separated under non-reducing conditions on linear SDS-PAGE 12·5% gels (Piazzón *et al*. 2008). On completion of the electrophoresis, the gels were stained with Thermo Scientific GelCode Blue Safe Protein Stain (Thermo Fisher, USA) for qualitative determination of the protein bands. At the same time, a gel was immunoblotted at 15 V for 35 min to Immobilon-P transfer membranes (0·45 μm; Millipore, USA) in a trans-blot S.D. transfer cell (Bio-Rad, USA) with transference buffer (48 mM Tris, 29 mM glycine, 0·037% SDS and 20% methanol, pH 9·2). The membrane was washed with Tris buffer saline (TBS; 50 mM Tris, 0·15 M NaCl, pH 7·4) and immediately stained with Ponceau S to verify transfer. After membrane destaining with bidistilled water, a blocking solution consisting of TBS containing 0·2% Tween 20 and 3% bovine serum albumin (BSA) was added, and the membrane was incubated for 1·5 h at RT. The membrane was then washed in TBS and incubated overnight with PAB_{HK} and PAB_{rPPase}, diluted 1/100, at 4 °C. The membrane was washed again with TBS and incubated with rabbit anti-mouse IgG (Dakopatts; dilution 1:6000) for 1 h at RT. After the membrane was washed 5 times for 5 min with TBS, it was incubated for 1 min with enhanced luminol-based chemiluminiscent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, USA), before being visualized and photographed with a FlourChem® FC2 imaging system (Alpha Innotech, USA).

Immunofluorescence, Immunoelectron microscopy, fluorescent enzyme linked immunoassay (FELISA), and fluorescent staining with pH-sensitive dye

For immunolocalization of H^+-PPase isoforms, an immunofluorescence assay was performed following the previously described protocol (Mallo *et al*. 2015). Briefly, 5×10^6 ciliates were centrifuged at 750 × ***g*** for 5 min, washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich) and fixed for 5 min in a solution of 4% formaldehyde in DPBS. The ciliates were then washed twice with DPBS, resuspended in a solution containing 0·1% Triton X-100 (PBT) for 3 min and then washed twice with DPBS. They were then incubated with 1% BSA for 30 min. After this blocking step, the ciliates were incubated at 4 °C overnight with a solution containing a 1:100 dilution of anti- PAB_{rPPase}, PAB_{HK} or PAB_{AVP1}. The ciliates were then washed 3 times with DPBS, before being incubated for 1 h at RT, with a 1:100 dilution of fluorescein

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

isotiocyanate (FITC) conjugated rabbit/goat anti-mouse/rabbit IgG-FITC antibody (Sigma). After three washes in DPBS, the samples were double-stained with 0·8 mg mL^{-1} 4′, 6-diamidine-2-phenylindole (DAPI; Sigma-Aldrich) in DPBS for 15 min at RT (Paramá *et al*. 2007). After another three washes in DPBS, the samples were mounted in PBS-glycerol (1:1) and visualized by fluorescence microscopy (Zeiss Axioplan, Germany) and/or confocal microscopy (Leica TCS-SP2, LEICA Microsystems Heidelberg GmbH, Mannheim, Germany).

For immunoelectron microscopy, an aliquot of 5×10^6 ciliates obtained from cultures in exponential growth phase was centrifuged at $750 \times \boldsymbol{g}$ for 5 min and washed in two changes of Sörensen buffer (SB; 0·1 M sodium/potassium phosphate buffer, pH 7·3) at RT. The resulting pellet was fixed for 60 min in 4% paraformaldehyde and 0·1% glutaraldehyde in SB at 4 °C. The fixed samples were washed in two changes of SB (10 min each) and incubated with 0·02 M glycine in SB for 10 min at RT. The ciliates were dehydrated in series of pre-cooled ethanol solutions (30, 50, 70, 80, 96 and 100% of 10 min each). The dehydrated pellet was placed in a 2:1 mixture of ethanol and resin (LR White uncatalyzed, Santa Cruz Biotechnology, USA; Philimonenko *et al*. 2002) for 20 min and then in pure resin for 2 h. Samples were infiltrated overnight with fresh resin at 4 °C. The resin was replaced the next day and the mixture was allowed to polymerize at 65 °C in vacuum for 48 h. Thin sections (80 nm thick) were cut, with a diamond knife, on a Reichert Ultracut E (Leica Microsystems AG, Germany). Thin sections were collected on 300 mesh nickel grids (Sigma-Aldrich) and were blocked by preincubation with 10% normal goat serum (NGS) in PBS-10% albumin and 0·1% Tween-20 (PBTB) for 30 min at RT. The sections were incubated for 1 h with PAB_{HK} in PBTB at 1:100 dilution, washed in PBS-albumin, and incubated with 10 nm gold-labelled goat anti-rabbit IgG (Sigma) at 1:50 dilution for 60 min. Finally, the sections were washed in distilled water, stained with uranyl acetate (1% in distilled water) and lead citrate (4·5 mM in 4·5 mM NaOH), and observed under a JEOL-JEM-2010 transmission electron microscope operated at 120 kV (JEOL, Japan). Negative controls were included to establish the specificity of the polyclonal antibodies used in these immunoassays. Some sections were processed with an unrelated antibody or were incubated in the presence of the secondary antibody only. No positive staining was observed in these controls.

For quantification of the expression of the alveolar H^+-PPase, we used a FELISA. One μg of MAPs (isolated from trophozoites incubated for 24 h in culture media with different saline concentrations: 4, 8 and 37‰) was added to 100 μL of carbonate-bicarbonate buffer (pH 9·6) in 1 × 8 well ELISA-strips (high binding, Greiner Bio-One, Germany) and incubated overnight at 4 °C. The wells were then washed three times with TBS, blocked 1 h with TBS containing 0·2% Tween 20 (TBS-T_1), 5% non-fat dry milk, incubated for 30 min at RT in a microplate shaker for ELISA (Stuart, UK) at 750 rpm with 1:100 dilution (in TBS-T_1 containing 1% non-fat dry milk) of PAB_{HK}, and washed five times with TBS containing 0·05% Tween 20. Bound anti-mouse antibodies were detected with FITC-conjugated rabbit anti-mouse Ig (Sigma) at 1:1000 dilution in TBS-T_1, and incubated for 30 min with shaking. After five washes in TBS, 100 μL of TBS was added to each well, and fluorescence was measured in a microplate fluorescence reader (Bio-Tek Instruments, USA) with an excitation wavelength of 490 nm, emission wavelength of 525 nm and a sensitivity of 70%. The results were expressed in arbitrary fluorescence units.

For identification of the acidic compartments on trophozoites of *P. dicentrarchi* we used a fluorescent stain assay with the pH-sensitive dye Lysotracker Red DND-99. An aliquot of 5×10^5 ciliates was centrifuged at $700 \times \boldsymbol{g}$ for 5 min, washed twice with PBS and stained for 10 min with 75 nM Lysotracker Red DND-99 (Lifetechnologies) solution. The ciliates were then observed in a fluorescence microscopy with an excitation filter (BP 546 nm), dichroic mirror (FT 580 nm) and emission filter (LP 590 nm).

Bioinformatic and statistical analysis

The amino acid sequences obtained for the H^+-PPase gene were aligned using the Clustal Omega multiple sequence alignment program (Sievers *et al*. 2011). Genetic distances were calculated in order to quantify sequence divergence between isolates by use of Kimura's (1980) two-parameter model, as implemented in MEGA version 6.0 (Tamura *et al*. 2013). Phylogenetic trees were constructed with the MEGA programme, by applying the neighbour-joining (NJ) method to the Kimura two-parameter correction model (Kimura, 1980) by bootstraping with 1000 replicates (Felsenstein, 1985).

The results are expressed as means ± standard error of the mean (S.E.M.). The data were examined by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons, and differences were considered significant at $\alpha = 0{\cdot}05$.

RESULTS

Cellular localization of H^+-PPase of *P. dicentrarchi* in acidic compartments

A cDNA fragment encoding 169 aa located between positions 305 and 474 of the amino acid sequence of the H^+-PPase (GenBank accession AHH28243) containing domain HKAVIGDTIGDPLKDTS was cloned in a yeast expression vector YepFlag-1.

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

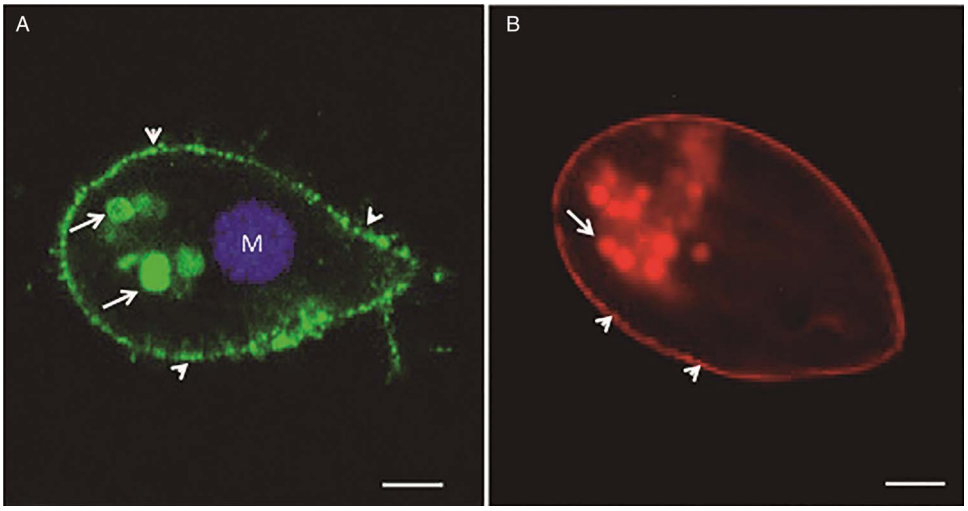

Fig. 1. (A, B) Detection by immunofluorescence and confocal microscopy of H^+-PPase in a ciliate trophozoite by using a mouse antibody anti-recombinant H^+-PPase expressed in the yeast *Saccharomyces cerevisiae* (PAB_{rPPase}) and a secondary polyclonal antibody anti-mouse Ig conjugated with FITC. After the immunofluorescence assay, trophozoites were counterstained with DAPI to identify the macronucleus (M). (A) Arrows indicate immunolocalization of H^+-PPase in the vacuoles and arrowheads indicate the presence of specific immunostaining appearing as a dotted line coinciding with the alveolar sacs. (B) Lysotracker Red DND-99 staining of acidic compartments of *P. dicentrarchi* showing fluorescent labelling of the posterior vacuoles (arrows) and the alveolar sacs (arrowheads). Scale bars = 10 μm. Abbreviations: DAPI, 4′, 6-diamidine-2-phenylindole; FITC, fluorescein isotiocyanate.

The mouse anti-rH^+-PPase polyclonal antibodies (PAB_{rPPase}), generated after immunization with the recombinant protein fragment, produced intense fluorescent staining of vacuoles located on the basal part of trophozoites and in alveolar sacs located under the plasma membrane (Fig. 1A).

Incubation of *P. dicentrarchi* trophozoites with the pH sensitive dye Lysotracker Red DND-99 produced intense staining in the vacuoles and in the alveolar sacs (Fig. 1B).

Immunohistochemical pattern of the H^+-PPase revealed by polyclonal antibodies (PAB_{HK})

Indirect immunofluorescence studies with a mouse polyclonal antibody generated against a KLH-conjugated synthetic peptide of the conserved amino acid domain HK (PAB_{HK}; Fig. 2A) and the polyclonal antibody anti-AVP1 (PAB_{AVP1}), a KLH-conjugated synthetic peptide derived from *Arabidopsis thaliana* V-PPase (Fig. 2B), revealed a unique labelling pattern on the surface of the parasite. Immunoelectron microscopic analysis with PAB_{HK} revealed specific labelling in the membranes of the alveolar sacs (Fig. 2C and D).

Sequence characteristics of H^+-PPase isoforms

To investigate the possible existence of H^+-PPase isoforms in the posterior vacuoles and in the alveolar sacs of *P. dicentrarchi*, we amplified a fragment of this gene by PCR and also amplified several cDNA from RNA using the FPiPh/RPiPh primer pair. Figure 3A shows the results of the nucleotide sequence amplified by PCR corresponding to a partial open reading frame (ORF) of H^+-PPase gene and its amino acid translation. Analysis of the DNA fragment amplified with primers FPiPh/RPiPh on 4% agarose gel revealed a single band of 558 nucleotides; however, when the cDNA generated from total RNA was amplified with the same primers, two bands were obtained, one of identical size to that obtained after DNA amplification (558 nucleotides) and a second band of 495 nucleotides (Fig. 3A). Sequencing of the two bands obtained by RT-PCR showed that the nucleotide sequence of the larger fragment corresponded exactly to the sequence obtained from genomic DNA by PCR. Sequencing of the minor band showed the disappearance of a 63 nucleotide fragment that was present in the largest band. After translation of both fragments to aa, the lower band produced a protein containing the complete domain HKAAVIGDTIGDPLKDTS, while the largest band generated a protein with this fragmented domain plus an internal sequence of 21 amino acids (Fig. 3A).

Polyclonal antibodies generated in mice after immunization with the synthetic peptide corresponding to the HK domain (PAB_{HK}) and with the recombinant fragment of H^+-PPase (PAB_{rPPase}) recognized a single protein band of approximately 62 kD on MAPs in Western blots (Fig. 3B).

Phylogenetic analysis of H^+-PPases in several strains of *P. dicentrarchi*

To determine the degree of protein conservation between isolates of *P. dicentrarchi*, we used PCR,

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

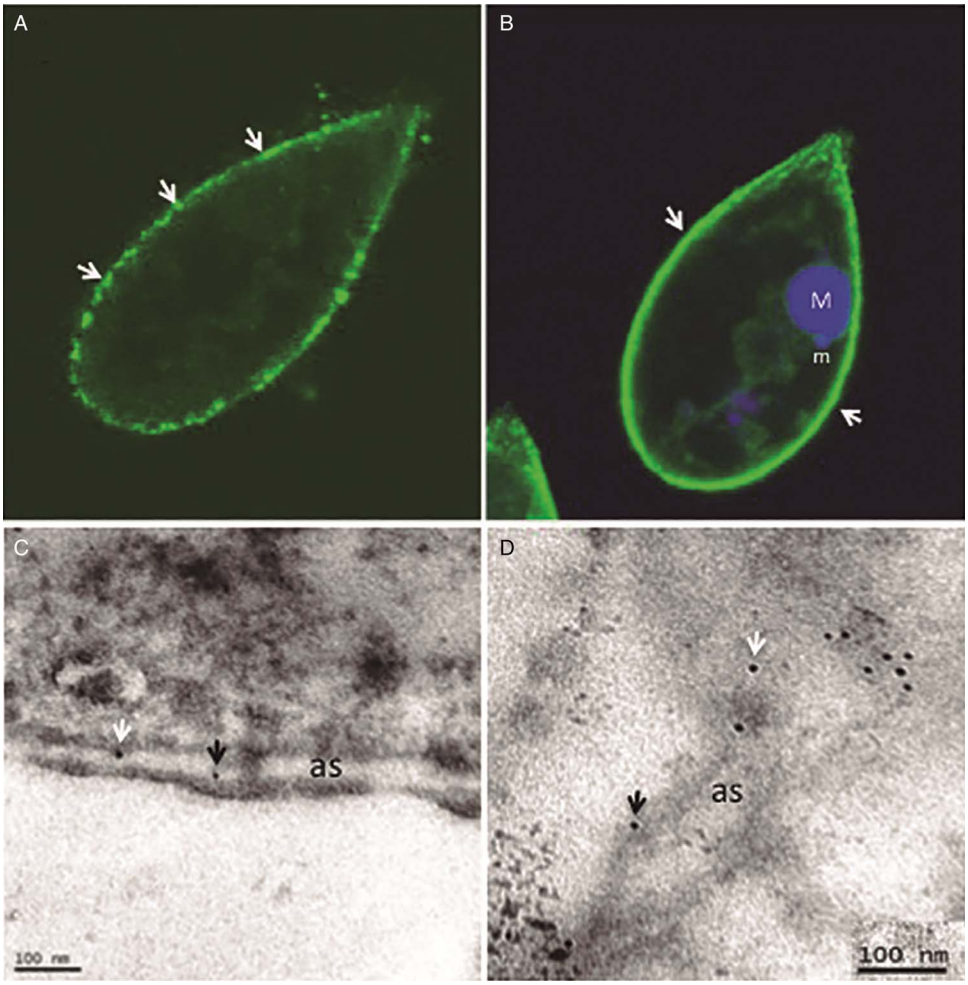

Fig. 2. Detection by immunofluorescence and confocal microscopy of a H^+-PPase isoform with a mouse polyclonal antibody generated to conserved amino acid domain HK (PAB_{HK}) (A) and with the commercial anti-AVP1 polyclonal antisera corresponding to AVP1 isoform of *A. thaliana* (PAB_{AVP1}) (B). A pattern of discontinuous fluorescence can be seen on the surface of trophozoites (arrows). (C, D) Immunoelectron microscopic localization of the H^+-PPase isoform using PAB_{HK} and which shows specific staining (arrows) on the membrane of the alveolar sacs (as).

with the FPiPh/RPiPh primer pair, to amplify the DNA of seven isolates. After obtaining the nucleotide sequence of each isolate and its translation into aa, we used the Clustal Omega program for multiple alignment of the amino acid sequences. The results showed a very high degree of conservation between the aa sequences of the isolates analysed (Fig. 4A), with a very small genetic distance between isolates. Construction of the phylogenetic tree by the NJ method revealed five isolates with 100% homology (I1, B1, D3, P1 and S1 isolates) and that D2 and C1 constituted two phylogenetically different groups (Fig. 4B).

Effect of salt concentration on expression of H^+-PPase

The results of assays of the expression levels of RNA corresponding to the H^+-PPase of *P. dicentrarchi* trophozoites cultivated in saline medium containing different concentrations of NaCl (between 4, 8 and 37‰, are shown in Fig. 5A. Relative mRNA levels (quantified by qPCR) of H^+-PPase remain unchanged at NaCl concentrations between 8 and 37‰; however, when the medium contained a low concentration of NaCl (e.g. 4‰), expression of H^+-PPase increased significantly relative to levels obtained in ciliates incubated at concentrations of NaCl between 8 and 37‰ (Fig. 5A).

The effect of salinity on the expression of the H^+-PPase containing the HK domain was analysed by a FELISA immunoassay under the same conditions of salinity as in the previous experiment with the PAB_{HK} antiserum. The levels of the enzyme containing the HK domain were highest in the ciliates incubated with the lowest concentration of salt (4‰) (Fig. 5B).

DISCUSSION

H^+-PPases are enzymes that translocate H^+ across a membrane by using potential energy liberated on hydrolysis of the phosphoanhydride bond of

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

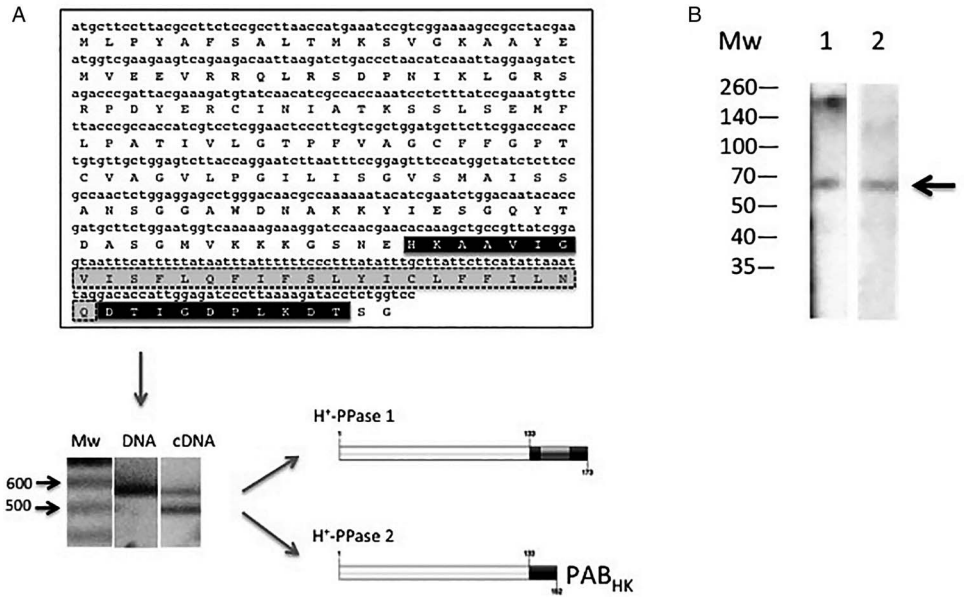

Fig. 3. (A) Nucleotide sequence belonging to a gene region of the H^+-PPase of *P. dicentrarchi* together with its corresponding translation into amino acids, containing a sequence motif recognized by the PAB_{HK} (black box). Agarose gel (4%) containing the PCR and RT-PCR products obtained using DNA and RNA as templates and the primers FPiPh/RPiPh is shown in the lower part of the figure. Two bands were observed when cDNA was used as a template, corresponding respectively to isoform 1 (the largest), and isoform 2 (the smallest). Isoform 2 contains the complete motif recognized by PAB_{HK}. (B) Western blot with PAB_{rPPase} (lane 1) and PAB_{HK} (line 2) on ciliate MAPs of trophozoites subjected to SDS-PAGE under non-reducing conditions and which recognizes a single band of approximately 62 kD (arrow). Mw: molecular weight markers. Abbreviations: MAPs, membrane-associated proteins; PAB_{rPPase}, polyclonal anti-rH^+-PPase; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR.

inorganic phosphate (Rea and Poole, 1993). They are widely distributed among land plants and have been found in several protozoan parasites including the scuticociliate parasite of turbot, *P. dicentrarchi* (Mallo *et al*. 2015). In eukaryotes, H^+-PPases are associated with certain acidic compartments of the endomembrane system, namely the vacuole and lysosomes of plant cells and the acidocalcisomes of trypanosomatides and apicomplexan protozoans (Scott and Docampo, 2000; Pérez-Castiñeira *et al*. 2002; Docampo *et al*. 2005). The functions of acidocalcisomes include cation storage, Ca^{2+} homeostasis, maintenance of intracellular pH homeostasis, and osmoregulation (Moreno and Docampo, 2009). In parasitic protozoans, acidocalcisomes also interact with other organelles such as the contractile vacuole and other vacuoles associated with the endosomal/lysosomal pathway (Moreno and Docampo, 2009; Docampo *et al*. 2010). In ciliates, such as *Paramecium caudatum,* acidification of phagocytic vacuoles occurs through fusion of nonlysosomal vesicles, known as acidosomes, which accumulate neutral red as well as acridine orange, indicating the acidic content of the vacuoles (Allen and Fok, 1983). Sequencing of the whole genome of several species of ciliates enables identification of genes encoding the V-ATPase, a proton pump that drives H^+ across membranes and that is crucial for acidifying food vacuoles (Plattner, 2010); however, although there is some evidence for the existence of H^+-PPases in ciliates, as several sequences are deposited in nucleotide databases (e.g. *Tetrahymena thermophila,* GenBank accession XM_001011583; *Tetrahymena pyriformis,* GenBank accession AJ251772), little is known about the occurrence of membrane-bound H^+-PPases and their physiological role in these protozoa (Pérez-Castiñeira *et al*. 2001). Acidocalcisomes and some of their transport activities have not yet been characterized in ciliates (Plattner *et al*. 2012). The present study clearly showed that the H^+-PPase in *P. dicentrarchi* is located in vacuoles and alveolar sacs and that both of these structures are acidic cellular components (as indicated by staining with the pH sensitive dye Lysotracker Red DND 99).

Subcellular location of members of the H^+-PPase family is mainly in endocellular membranes (vacuolar tonoplast) and acidocalcisomal membranes of eukaryotes (algae, plants and protozoa) (Maeshima, 2000; Drozdowicz and Rea, 2001; Docampo *et al*. 2005) and plasma membrane invaginations of both bacteria and archaea (Baltscheffsky *et al*. 1999; Serrano *et al*. 2004). However, evidence for a differential subcellular localization of the AVP1 (vacuole) and AVP2 (Golgi complex and lysosomes) isoforms has only been reported in plant cells (Rea *et al*. 1992; Mitsuda *et al*. 2001). Indirect immunofluorescence microscopy with polyclonal antibodies, to investigate the subcellular localization of V-H^+-PPase in *Plasmodium falciparum*, indicated that VP1 is

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

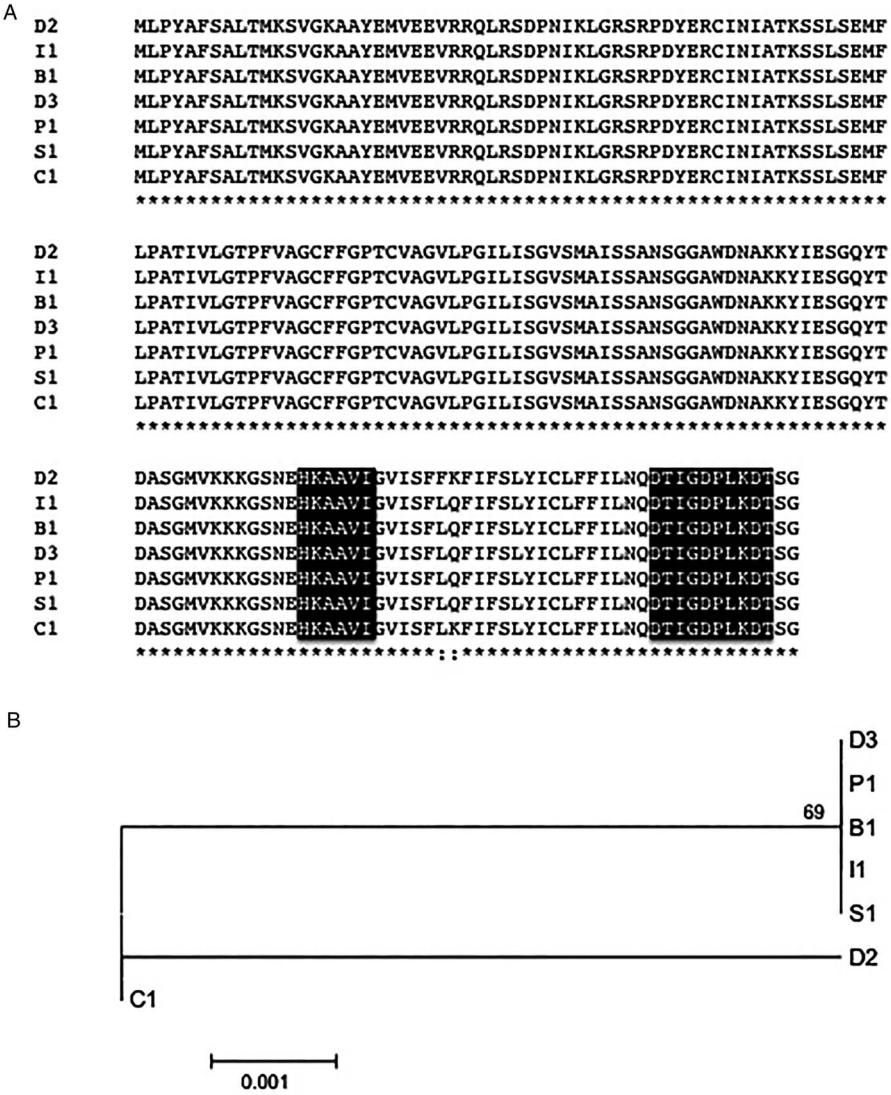

Fig. 4. (A) Multiple sequence alignment (Clustal Omega) of aa sequences obtained from a partial ORF of the gene of H^+-PPase from several isolates (B_1, D_2, D_3, C_1, I_1, S_1 and P_1) of *P. dicentrarchi*. The boxes highlighted in bold indicate the motif recognized by the PAB_{HK} antibody. (B) Phylogenetic comparison of *P. dicentrarchi* H^+-PPase isolates. Aligned aa sequences were subjected to phylogenic analysis by the NJ method. The numbers at the nodes represent bootstrap values out of 1000 resampled values in the NJ analysis with the Kimura two-parameter correction model. Abbreviations: NJ, neighbour joining; ORF open reading frame.

present within the vacuolar membrane and, possibly, in food vacuoles (Luo *et al*. 1999; MacIntosh *et al*. 2001). The proton pumps V-H^+-PPase and V-H^+-ATPase also appear to be colocalized in acidic organelles such as acidocalciomes and food vacuoles in malarian parasites (Marchesini *et al*. 2000; Moriyama *et al*. 2003; Saliba *et al*. 2003). In the present study, we used immunofluorescence and immunoelectron microscopy to demonstrate that the PAB_{HK} sera, which recognizes the highly conserved domain HKAAVIDTIGDPKDT, generates specific labelling only in the alveolar sacs of ciliates, which suggests that this domain is not found in the H^+-PPase vacuoles. Thus, immunostaining with PAB_{HK} may indicate the possible existence of two isoforms of H^+-PPase in *P. dicentrarchi*.

The existence of multiple isoforms of H^+-PPases has been demonstrated in plants (Venter *et al*. 2006). Thus, at least two genes encoding H^+-PPases have been detected in the *rice (Oryza sativa L.)* genome (Sakakibara *et al*. 1996), three isoforms have been found in tobacco (Lerchl *et al*. 1995), two in red beet (*Beta vulgaris* L.) (Kim *et al*. 1994), two in barley (*Hordeum vulgare* L.) (Fukuda *et al*. 2004), two in grapevine (*Vitis vinifera* L.) (Venter *et al*. 2006) and two in cacao (*Theobroma cacao* L.) (Motamayor *et al*. 2013). All of these show high homology within the coding region but differ strongly in the untranslated regions, and their expression is probably regulated in different ways (Maeshima, 2000). Plants have two phylogenetically distinct V-H^+-PPases that can

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

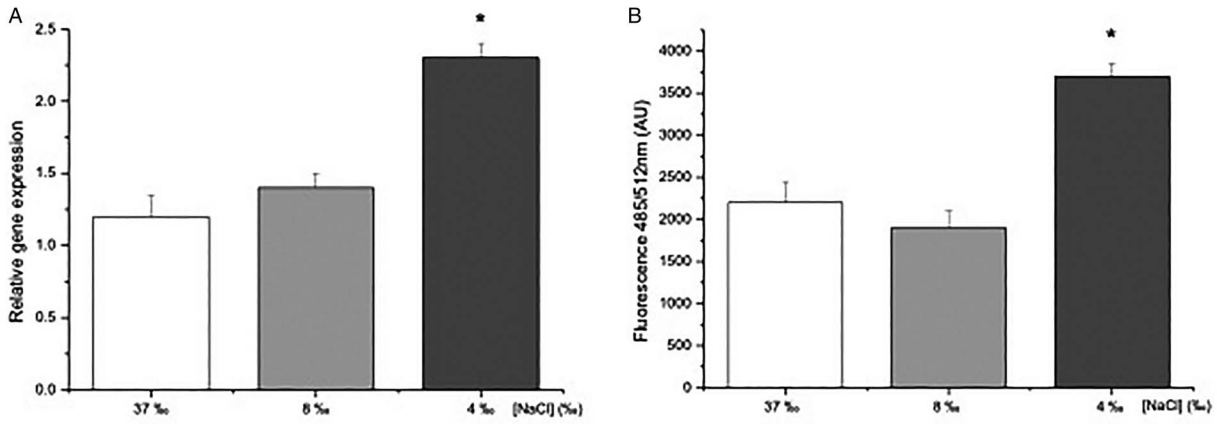

Fig. 5. (A) Relative gene expression levels of *P. dicentrarchi* H^+-PPase, determined by real-time qRT-PCR in trophozoites incubated for 24 h in medium containing different concentrations of NaCl (4, 8 and 37‰). Gene expression was normalized to the reference gene (*P. dicentrarchi* β-tubulin) and normalized data are expressed in AU. (B) Expression levels of the H^+-PPase containing the HK domain quantified by FELISA immunoassay with the PAB_{HK} antisera. Expression was measured on ciliate MAPs of trophozoites incubated for 24 h in medium containing NaCl at 4, 8 and 37‰ and is shown as fluorescence units. Values shown are means ± standard error (S.E.) from five assays. $*P < 0{\cdot}01$ relative to ciliates incubated in the medium with 37‰ NaCl. Abbreviations: AU, arbitrary units; FELISA, fluorescent enzyme linked immunoassay; MAPs, membrane-associated proteins; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

be classified into two subclasses: AVP1, which depends on cytosolic K^+ for its activity and is moderately sensitive to inhibition by Ca^{2+}; and AVP2, which is K^+-independent but extremely Ca^{+2}-sensitive (Sarafian *et al*. 1992; Drozdowicz *et al*. 2000; Gaxiola *et al*. 2007). Two genes encoding VP1 and VP2 have been identified in the malarian parasite *P. falciparum* (MacIntosh *et al*. 2001), and the sequence of isoform 2 corresponding to the himenostomatid ciliate *Tetrahymena pyriformis* is also available (GenBank accession AJ251471).

There is an almost-complete conservation between AVP1/AVP2 of the amino acid sequences recognized by polyclonal antibody PAB_{HK} (HKAAVIGDTIGPLK motif), further justifying the suggestion that these antibodies are universal reagents for detecting V-PPase polypeptides (Drozdowicz and Rea, 2001). We have previously shown that the *P. dicentrarchi* H^+-PPase has a motif in common with the polyclonal antibody PAB_{HK} specific to AVP1 (Mallo *et al*. 2015). In the present study, we found that this motif is encoded by a gene containing an intercalated nucleotide sequence that can be transcribed into two mRNA molecules, generating two isoforms: one isoform produces a protein with the fragmented motif and the other produces a protein containing the complete motif. The total size of the proteins produced by gene isoforms varies between 62 and 64 kD (the 2 kD difference is the estimated size of the peptide intercalated between the motif HK); however, these would probably be overlooked by SDS-PAGE or Western blot analysis because of the limited ability of these techniques to separate proteins of very similar molecular size (Dauly *et al*. 2006). The hypothesis proposed here to explain the presence of both H^+-PPase isoforms is based on the differential recognition of the H^+-PPase in the alveolar sacs by PAB_{HK} and also on the presence of two amino acid sequences in the cDNAs generated by RT-PCR with the FPiPh/RPiPh primers. Presence of the isoform containing the complete HK motif in the H^+-PPase of the alveolar sacs may be explained by a splicing mechanism. Isoform 1 of H^+-PPase present in the vacuoles does not undergo this process, generating a protein with a fragmented motif that would not be recognized by PAB_{HK}. There are some examples of genes that generate isoforms transcribed from alternate promoter sites within the gene which may mediate cell signalling and induce translocation to various cellular localizations (Saitoh *et al*. 2002). Furthermore, it is well-known that splicing regulation can be modulated by several sequence elements in both exons and introns that either activate (exonic splicing enhancer, ESE; introning splicing enhancer, ISE) or repress (exonic splicing silencer, ESS; intronic splicing silencer, ISS) (Poulos *et al*. 2011). However, introns are removed and exons are added during the splicing process, and therefore introns should not appear in the final mature mRNA. The results of the present study suggest that the mRNA fragment eliminated by splicing during processing of H^+-PPase mRNA is also a coding region. The existence of introns with features of both introns and protein-coding exons and referred to as exitrons (exonic introns) has recently been described in *Arabidopsis* and humans (Marquez *et al*. 2015). This unusual alternative splicing event may be a novel source of alternative transcripts and protein isoforms for genes

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

annotated as intronless and may explain the generation of the two H^+-PPase isoforms in *P. dicentrarchi*.

Description of nucleotide sequences of H^+-PPase genes from plants, bacteria and archaea highlighted an unusually high degree of sequence conservation (Serrano *et al*. 2007). In the present study, we also found a high level of sequence conservation of the H^+-PPase gene among several isolates of *P. dicentrarchi*, which could also be used in conjunction with other highly conserved genes, such as *β*-tubulin, to detect intraspecific genetic variation within populations of scuticociliates that infect cultured turbot (Budiño *et al*. 2011).

In plants, it is known that efficient exclusion of excess Na^+ from the cytoplasm and accumulation of vacuolar Na^+ are the most important steps involved in maintaining ion homeostasis within cells, and both tonoplast and plasma membrane Na^+/H^+ antiporters exclude Na^+ from the cytosol driven by the H^+-motive force generated by the plasma membrane H^+-ATPase and H^+-PPase (Silva and Gerós, 2009). Algal and plant H^+-PPases are induced under anoxia, chilling and salt-induced stress (Carystinos *et al*. 1995; Fukuda *et al*. 2004), and overexpression of the vacuolar H^+-PPase isoform AVP1 in the model plant *Arabidopsis* has been claimed to confer increased saline and drought tolerance (Gaxiola *et al*. 2001). As euryhaline organisms, ciliates are well adapted to variations in salinity and tolerate salinity levels as low as 4‰ or as high as 62‰ (i.e. 27‰ higher than seawater) (Hu, 2014). We found that *P. dicentrarchi* is able to respond to saline-induced stress by modifying expression of the H^+-PPase isoforms located in the alveolar sacs, indicating a potential role of these structures in salt tolerance in marine scuticociliates.

In conclusion, the H^+-PPase of *P. dicentrarchi* is located in the membranes of the phagocytic vacuole and alveolar sacs, thus promoting acidification of these cellular compartments. Specifically, an isoform of ionic pump H^+-PPase containing a highly conserved amino acid motif generated by splicing is located in the alveolar sacs and is recognized by a polyclonal antibody, PAB_{HK}. Gene and protein expression of this isoform are regulated by changes in salinity, which suggests that the isoform must play an important physiological role in the adaptive responses of these marine ciliates in maintaining both intracellular pH homeostasis and osmoregulation.

FINANCIAL SUPPORT

This study was financially supported by the Ministerio de Economía y Competitividad (Spain) under grant agreement AGL2014-57125-R, the European Union's Horizon 2020 research and innovation programme under grant agreement No. 634429 (PARAFISHCONTROL), and by the Xunta de Galicia (Spain) under grant agreement GPC2014/069. All authors benefitted from these grants.

REFERENCES

Allen, R. D. and Fok, A. K. (1983). Nonlysosomal vesicles (acidosomes) are involved in phagosome acidification in *Paramecium*. *The Journal of Cell Biology* **97**, 566–570.

Baltscheffsky, H., von Stedingk, L.-V., Heldt, H. W. and Klingenberg, M. (1966). Inorganic pyrophosphate formation in bacterial photophosphorylation. *Science* **153**, 1120–1122.

Baltscheffsky, M., Schultz, A. and Baltscheffsky, H. (1999). H^+-PPases a tightly membrane-bound family. *FEBS Letters* **457**, 527–533.

Belogurov, G. A. and Lahti, R. (2002). A lysine substitute for K^+. *Journal of Biological Chemistry* **277**, 49651–49654.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *Journal of Biological Chemistry* **256**, 1604–1607.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 284–254.

Budiño, B., Lamas, J., Pata, M. P., Arranz, J. A., Sanmartín, M. L. and Leiro, J. (2011). Intraspecific variability in several isolates of *Philasterides dicentrarchi* (syn. *Miamiensis avidus*), a scuticociliate parasite of farmed turbot. *Veterinary Parasitology* **175**, 260–272.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611–622.

Carystinos, G. D., McDonald, H. R., Monroy, A. F., Dhindsa, R. S. and Poole, R. J. (1995). Vacuolar H^+-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. *Plant Physiology* **108**, 641–649.

Dauly, C., Perlman, D. H., Costello, C. E. and McComb, M. E. (2006). Protein separation and characterization by np-RP-HPLC followed by intact MALDI-TOF mass spectrometry and peptide mass mapping analyses. *Journal of Proteome Research* **5**, 1688–1700.

Docampo, R., de Souza, W., Miranda, K., Rohloff, P. and Moreno, S. N. (2005). Acidocalcisomes – conserved from bacteria to man. *Nature Reviews Microbiology* **3**, 251–261.

Docampo, R., Ulrich, P., Moreno, S. N. J. (2010). Evolution of acidocalcisomes and their role in polyphosphate storage and osmoregulation in eukaryotic microbes. Philosophical Transactions of Royal Society *B* **365**, 775–784.

Drozdowicz, Y. M., Kissinger, J. C. and Rea, P. A. (2000). AVP2, a sequence-divergent, K^+ insensitive H^+-translocating inorganic pyrophosphatase from *Arabidopsis*. *Plant Physiology* **123**, 353–362.

Drozdowicz, Y. M. and Rea, P. A. (2001). Vacuolar H(+) pyrophosphatases: from the evolutionary backwaters into the mainstream. *Trends in Plant Science* **6**, 206–211.

Drozdowicz, Y. M., Shaw, M., Nishi, M., Striepen, B., Liwinski, H. A., Roos, D. S. and Rea, P. A. (2003). Isolation and characterization of TgVP1, a type I vacuolar H+-translocating pyrophosphatase from *Toxoplasma gondii*. The dynamics of its subcellular localization and the cellular effects of a diphosphonate inhibitor. *The Journal of Biological Chemistry* **278**, 1075–1085.

Felsenstein, J. (1985). Confidence limits on phylogenies: and approach using the bootstrap. *Evolution* **39**, 783–791.

Fukuda, A., Chiba, K., Maeda, M., Nakamura, A., Maeshima, M., Tanaka, Y. (2004). Effect of salt and osmotic stresses on the expression of gene for the vacuolar H^+-pyrophosphatase, H^+-ATPase subunit A, and Na^+/H^+ antiporter from barley. *Journal of Experimental Botany* **55**, 585–594.

Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G., Alper, S. L. and Fink, G. R. (2001). Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proceedings of the National Academy of Sciences of the United States of America **98**, 11444–11449.

Gaxiola, R. A., Palmgren, M. G. and Schumacher, K. (2007). Plant proton pumps. *FEBS Letters* **581**, 2204–2214.

Harper, J. M., Huynh, M. H., Coppens, I., Parussini, F., Moreno, S. and Carruthers, V. B. (2006). A cleavable propeptide influences *Toxoplasma* infection by facilitating the trafficking and secretion of the TgMIC2-M2AP invasion complex. *Molecular Biology of the Cell* **17**, 4551–4563.

Hu, X. (2014). Ciliates in extreme environments. *Journal of Eukaryotic Microbiology* **61**, 410–418.

Iglesias, R., Paramá, A., Álvarez, M. F., Leiro, J., Fernández, J. and Sanmartín, M. L. (2001). *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot *Scophthalmus maximus* in Galicia (NW Spain). *Diseases of Aquatic Organisms* **46**, 47–55.

Iglesias, R., Paramá, A., Álvarez, M. F., Leiro, J., Aja, C. and Sanmartín, M. L. (2003). *In vitro* growth requirements for the fish

https://doi.org/10.1017/S0031182015001997 Published online by Cambridge University Press

pathogen *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida). *Veterinary Parasitology* **111**, 19–30.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology* **153**, 163–168.

Karlsson, J. (1975). Membrane-bound potassium and magnesium ion-stimulated inorganic pyrophosphatase from roots and cotyledons of sugar beet (*Beta vulgaris* L.). *Biochimica et Biophysica Acta* **399**, 356–363.

Kim, Y., Kim, E. J. and Rea, P. A. (1994). Isolation and characterization of cDNAs encoding the vacuolar H^+-pyrophosphatase of *Beta vulgaris*. *Plant Physiology* **106**, 375–382.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Lerchl, J., Kónig, S., Zrenner, R. and Sonnewald, U. (1995). Molecular cloning, characterization and expression of isoforms encoding tonoplast-bound proton-translocating inorganic pyrophosphatase in tobacco. *Plant Molecular Biology* **28**, 833–840.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_T$ Method. *Methods* **25**, 402–408.

Long, A. R., Williams, L. E., Nelson, S. J. and Hall, J. L. (1995). Localization of membrane pyrophosphatase activity in *Ricinus communis* seedlings. *Journal of Plant Physiology* **146**, 629–638.

López-López, O., Fuciños, P., Pastrana, L., Rúa, M. L., Cerdán, M. E. and González-Siso, M. I. (2010). Heterologous expression of an esterase from *Thermus thermophilus* HB27 in *Saccharomyces cerevisiae*. *Journal of Biotechnology* **145**, 226–232.

Luo, S., Marchesini, N., Moreno, S. N. and Docampo, R. (1999). A plant-like vacuolar H(+)-pyrophosphatase in *Plasmodium falciparum*. *FEBS Letters* **460**, 217–220.

Maeshima, M. (2000). Vacuolar H^+-pyrophosphatase. *Biochimica et Biophysica Acta* **1465**, 37–51.

Mallo, N., Lamas, J. and Leiro, J. M. (2013). Evidence of an alternative oxidase pathway for mitochondrial respiration in the scuticociliate *Philasterides dicentrarchi*. *Protist* **164**, 824–836.

Mallo, N., Lamas, J., Piazzon, C. and Leiro, J. M. (2015). Presence of a plant-like proton-translocating pyrophosphatase in a scuticociliate parasite and its role as a possible drug target. *Parasitology* **142**, 449–462.

Marchesini, N., Luo, S., Rodrigues, C. O., Moreno, S. N. J. and Docampo, R. (2000). Acidocalcisomes and vacuolar H^+-pyrophosphatase in malaria parasites. *Biochemical Journal* **347**, 243–253.

MacIntosh, M. T., Drozdowicz, Y. M., Laroiya, K., Rea, P. A. and Vaidya, A. B. (2001). Two classes of plant-like vacuolar-type H(+)-pyrophosphatases in malaria parasites. *Molecular and Biochemical Parasitology* **114**, 183–195.

Marquez, Y., Höpfler, M., Ayatollahi, Z. and Barta, A. (2015). Unmasking alternative splicing inside protein-coding exons defines exitrons and their role in proteome plasticity. *Genome Research* **25**, 995–1007.

Mitsuda, N., Enami, K., Nakata, M., Takeyasu, K. and Sato, M. H. (2001). Novel type *Arabidopsis thaliana* H^+-PPase is localized to the Golgi apparatus. *FEBS Letters* **488**, 29–33.

Moreno, S. N. J. and Docampo, R. (2009). The role of acidocalcisomes in parasitic protists. *Journal of Eukaryotic Microbiology* **56**, 208–213.

Moriyama, Y., Hayashi, M., Yatsushiro, S. and Yamamoto, A. (2003). Vacuolar proton pumps in malaria parasite cells. *Journal of Bioenergetics and Biomembranes* **35**, 367–375.

Motamayor, J. C., Mockaitis, K., Schmutz, J., Haiminen, N., Livingstone, D., Cornejo, O., Findley, S. D., Zheng, P., Utro, F., Royaert, S., Saski, C., Jenkins, J., Podicheti, R., Zhao, M., Scheffler, B. E., Stack, J. C., Feltus, F. A., Mustiga, G. M., Amores, F., Phillips, W., Marelli, J. P., May, G. D., Shapiro, H., Ma, J., Bustamante, C. D., Schnell, R. J., Main, D., Gilbert, D., Parida, L. and Kuhn, D. N. (2013). The genome sequence of the most widely cultivated cacao type and its use to identify candidate genes regulating pod color. *Genome Biology* **14**, r53.

Paramá, A., Iglesias, R., Álvarez, M. F., Leiro, J., Aja, C. and Sanmartín, M. L. (2003). *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida): experimental infection and possible routes of entry in farmed turbot (*Scophthalmus maximus*). *Aquaculture* **217**, 73–80.

Paramá, A., Castro, R., Lamas, J., Sanmartín, M. L., Santamarina, M. T. and Leiro, J. M. (2007). Scuticociliate proteinases may modulate turbot immune responses by inducing apoptosis in pronephric leucocytes. *International Journal For Parasitology* **37**, 87–95.

Pérez-Castiñeira, J. R., Gómez-García, R., López-Marqués, R. L., Losada, M. and Serrano, A. (2001). Enzymatic systems of inorganic pyrophosphatase bioenergetics in photosynthetic and heterotrophic protists: remnants or metabolic cornestones? *International Microbiology* **4**, 135–142.

Pérez-Castiñeira, J. R., Alvar, J., Ruiz-Pérez, L. M. and Serrano, A. (2002). Evidence for a wide occurrence of proton-translocating pyrophosphatase genes in parasitic and free-living protozoa. *Biochemical and Biophysical Research Communications* **294**, 567–573.

Philimonenko, V. V., Janácek, J. and Hozák, P. (2002). LR White is preferable to Unicryl for immunogold detection of fixation sensitive nuclear antigens. *European Journal of Histochemistry* **46**, 359–364.

Piazzón, C., Lamas, J., Castro, R., Budiño, B., Cabaleiro, S., Sanmartín, M. L. and Leiro, J. (2008). Antigenic and cross-protection studies on two turbot scuticociliate isolates. *Fish and Shellfish Immunology* **25**, 417–424.

Piazzon, C., Lamas, J. and Leiro, J. M. (2011). Role of scuticociliate proteinases in infection success in turbot, *Psetta maxima* (L.). *Parasite Immunology* **33**, 535–544.

Plattner, H. (2010). Membrane trafficking in protozoa SNARE proteins, H^+-ATPase, actin, and other key players in ciliates. *International Review of Cell and Molecular Biology* **280**, 79–184.

Plattner, H., Sehring, I. M., Mohamed, I. K., Miranda, K., De Souza, W., Billington, R., Genazzani, A. and Ladenburger, E. M. (2012). Calcium signaling in closely related protozoan groups (Alveolata): non-parasitic ciliates (*Paramecium, Tetrahymena*) *vs* parasitic Apicomplexa (*Plasmodium, Toxoplasma*). *Cell Calcium* **51**, 351–382.

Poulos, M. G., Batra, R., Charizanis, K. and Swanson, M. S. (2011). Developments in RNA splicing and disease. *Cold Spring Harbor Perspectives in Biology* **3**, a000778.

Rea, P. A. and Poole, R. J. (1993). Vacuolar H^+-translocanting pyrophosphatase. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 157–180.

Rea, P. A., Kim, Y., Sarafian, V., Poole, R. J., Davies, J. M. and Sanders, D. (1992). Vacuolar H^+-translocating pyrophosphatases: a new category of ion translocase. *Trends in Biochemical Sciences* **17**, 348–353.

Robinson, D. G., Haschke, H. P., Hinz, G., Hoh, B., Maeshima, M. and Marty, F. (1996). Immunological detection of tonoplast polypeptides in the plasma membrane of pea cotyledons. *Planta* **198**, 95–103.

Saitoh, O., Murata, Y., Odagiri, M., Itoh, M., Itoh, H., Misaka, T. and Kubo, Y. (2002). Alternative splicing of RGS8 gene determines inhibitory function of receptor type-specific Gq signaling. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10138–10143.

Saliba, K. J., Allen, R. J., Zissis, S., Bray, P. G., Ward, S. A. and Kirk, K. (2003). Acidification of the malaria parasite's digestive vacuole by a H^+-ATPase and a H^+-pyrophosphatase. *The Journal of Biological Chemistry* **278**, 5605–5612.

Sarafian, V., Kim, Y., Poole, R. J. and Rea, P. A. (1992). Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 1775–1779.

Scott, D. A. and Docampo, R. (2000). Characterization of isolated acidocalcisomes of *Trypanosoma cruzi*. *The Journal of Biological Chemistry* **275**, 24215–24221.

Serrano, A., Pérez-Castiñeira, J. R., Baltscheffsky, M. and Baltscheffsky, H. (2007). H^+-PPases: yesterday, today and tomorrow. *IUBMB Life* **59**, 76–83.

Serrano, A., Pérez-Castiñeira, R., and Baltscheffsky, H. (2004). Proton-pumping inorganic pyrophosphatases in some archea and other extremophilic prokaryotes. *Journal of Bioenergetics and Biomembranes* **36**, 127–133.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., López, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D. and Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* **7**, 539.

Silva, P. and Gerós, H. (2009). Regulation by salt of vacuolar H^+-ATPase and H^+-pyrophosphatase activities and Na^+/H^+ Exchange. *Plant Signaling and Behavior* **4**, 718–726.

Scott, D. A., de Souza, W., Benchimol, M., Zhong, I., Lu, H. G., Moreno, S. N. and Docampo, R. (1998). Presence of a plant-like proton-pumping pyrophosphatase in acidocalcisomes of *Trypanosoma* cruzi. *The Journal of Biological Chemistry* **273**, 22151–22158.

Sakakibara, Y., Kobayashi, H. and Kasamo, K. (1996). Isolation and characterization of cDNAs encoding vacuolar H^+-pyrophosphatase isoforms from rice (*Oryza sativa* L.). *Plant Molecular Biology* **31**, 1029–1038.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Venter, M., Gronewald, J.-H. and Botha, F. C. (2006). Sequence analysis and transcriptional profilling of two vacuolar H^+-pyrophosphatase isoforms in *Vitis vinifera*. *Journal of Plant Research* **119**, 469–478.