

EVOLUTION OF VACUOLAR PYROPHOSPHATASES AND VACUOLAR H⁺-ATPASES IN DIATOMS

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ABSTRACT

To cope with changing environments and maintain optimal metabolic conditions, the control of the intracellular proton gradients has to be tightly regulated. Among the important proton pumps, vacuolar H⁺-ATPases (V-ATPases) and H⁺-translocating pyrophosphatases (H⁺-PPases) were found to be involved in a number of physiological processes, and shown to be regulated at the expression level and to exhibit specific sub-cellular localizations. Studies of the role of these transporters are relatively scarce in algae and nearly absent in diatoms. Phylogenetic analyses disclose that diatoms, with both K⁺-dependent and K⁺-independent membrane integral pyrophosphatases, including proteins with high homology with a novel class of Na⁺,H⁺-PPases. Analyses of *Phaeodactylum tricorutum* EST libraries show that the gene putatively encoding a Na⁺,H⁺-PPase is over-expressed in urea adapted condition. Genome-wide investigations of the reservoir of V-ATPases encoding subunits demonstrate that diatoms display an expanded number of genes encoding for the proteolipid subunits c of the V₀ subcomplex. Preliminary localization studies show that one of V₀-c subunits is associated to the endoplasmic reticulum membrane in *P. tricorutum*. Altogether our data highlight that the combination of comparative and functional genomic approaches reach promises to provide new information to the roles of membrane proton pumps in diatoms.

I. INTRODUCTION

Eukaryotic cells have acquired specialized functions by creating a number of specialized membrane-bound organelles

(vacuoles, endosomes, Golgi apparatus, mitochondria, plastid, etc.). The creation of specific membrane-bound compartments, which is part of the consequences of endosymbiotic events, might also have evolved from the need of creating distinct subcellular environments to maintain appropriate conditions for specific metabolic pathways.

In eukaryotes several proton pumps that generate H⁺ electrochemical gradients in organelles create electrochemical gradients to power ATP production and to transport substances across membranes. For example, vacuolar H⁺-ATPases (V-ATPases) use ATP as energy source to translocate protons inside the organellar lumen [18, 20]. V-ATPase comprises two domains: a cytosolic V₁ domain and a membrane V₀ domain. The large cytosolic V₁ domain, which has eight different subunits (A through H), is involved in ATP hydrolysis that is coupled to the pumping of protons into a compartment via the membrane-bound V₀ complex (common V₀ subunits are: a, c, c', d and e) (Fig. 1). Intracellular V-ATPases play important roles in normal physiological processes such as receptor-mediated endocytosis, intracellular membrane trafficking, pro-hormone processing, protein degradation, and to couple uptake and release of ions and metabolites [63]. V-ATPases also pump protons across the plasma membrane into the extracellular space in a variety of specialized cells, including some kidney cells, specific epididymis cells or osteoclasts [24]. The identification of V-ATPases in cells presenting an invasive phenotype, is thought to be important for the development of novel therapeutic approaches to treat cancer metastasis [47]. A related family to these proton transporters has been found on the membrane of bacteria [66]. Even if prokaryotic V-ATPases are made up of a simpler subunit composition than the eukaryotic enzymes, each subunit shows significant sequence similarity to its eukaryotic counterpart [66].

In addition to the V-ATPase, a second type of endomembrane proton pumps, the H⁺-pyrophosphatase (H⁺-PPase), uses the energy of hydrolysis PPi to translocate H⁺ across membranes. H⁺-PPases are divided into two subfamilies, the type I corresponds to the K⁺-dependent and the type II to the K⁺-independent. The K⁺-dependent proton pumps were also shown to transport sodium [38]. More recently, a subclade of prokaryotic type I, were shown to simultaneously transport

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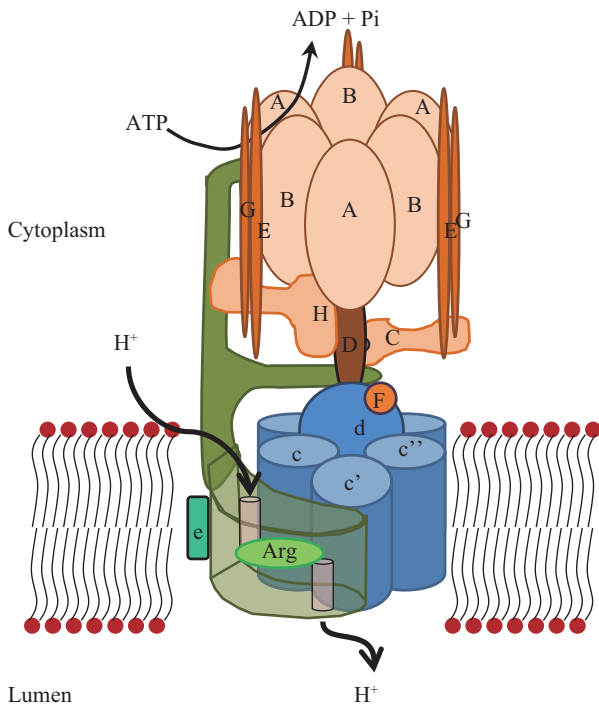


Fig. 1. Structure of the V-ATPase complex. In most organisms, the V-ATPase complex is composed by two subcomplexes: V_1 which is composed by 8 subunits from A to H localized in the cytoplasm, and V_0 which is integrated in the plasmic membrane. The V_0 complex contains a hexameric ring of the proteolitic subunits (V_0 -c, V_0 -c' and V_0 -c'') completed by the proteins V_0 -a, V_0 -d and V_0 -e.

both sodium and proton (H^+ , Na^+ -PPases) [37]. In *Arabidopsis thaliana*, a K^+ -dependent isoforms were shown to be localized at the vacuolar membrane and therefore be involved in lumen acidification, and K^+ -independent isoforms to the Golgi apparatus and the trans-Golgi network [58]. Vacuolar H^+ -PPase also appears to be involved in auxin-dependent organ development [19, 34], and in resistance against stresses [20, 68]. It was also suggested that H^+ -PPase activity contributed to the regulation of the internal pH of acidocalcisomes that are electron dense organelles conserved in eukaryotes, and involved in the storage of cations and phosphorus [14]. Membrane embedded PPases might also contribute to the regulation of the cellular pH of both Bacteria and Archaea [59].

Despite the multiple physiological roles played by V-ATPases and H^+ -PPase detailed studies in algae have been limited. In the green alga *Chlamydomonas reinhardtii* both pyrophosphatases and V-ATPases have been shown to be localized at the plasma membrane [53] and in polyphosphate vacuoles fractions of the vacuole [54]. V-ATPase activity was found to be associated with the function of the contractile vacuoles in the green alga *Scherffelia dubia* [7]. The role of vacuolar proton pumps was investigated in the halotolerant green alga *Dunaliella viridis* [43]. In the red alga *Cyanidioschyzon merolae* V-ATPases and H^+ -PPase were identified among the 32 proteins from a fraction largely enriched in vacuoles [65]. However, the role of these transporters has

been rarely investigated in brown microalgae or macroalgae. It was only recently that a first role for a V-ATPase in copper detoxification was proposed in the macroalgae *Fucus vesiculosus* [45].

Diatoms are unicellular photoautotrophic eukaryotes that can dominate marine and freshwater microalgal communities, and believed to be responsible for 20% of global carbon fixation on Earth, and thus play important roles as primary producers [16, 17, 52, 60]. Diatoms that are members of the supergroup of Chromalveolates (including Heterokonts, Cryptophytes, Haptophytes and Alveolates), are secondary symbionts, thought to derive from the engulfment by a heterotrophic eukaryote host cell of a red alga [10, 27]. The sequencing of a number of diatom genomes, including the centrics (cell with radial symmetry) *Thalassiosira pseudonana* [6, 31] and *T. oceanica* [36], and the pennates (cell with bi-lateral symmetry) *Phaeodactylum tricorutum* [9], *Pseudonitzschia multiseriis* and *Fragilariopsis cylindrus*, is revealing the chimeric nature of their genomes, with a number of genes found from green or red algal origin but also from prokaryotic origin. In complement to the genome information, gene transformation techniques have been developed for several species [44, 46, 48, 51, 67] and gene silencing using RNA interference is becoming an important techniques for functional analyses [2, 12, 23, 32], opening up the possibilities to perform functional studies in diatoms.

To better understand the role of membrane proton transporters, we report a genome-wide survey of the V-ATPase and H^+ -PPase gene families in diatoms, and compare the gene reservoir to other algae and Stramenopiles. We also analyzed EST databases in *P. tricorutum* and found that membrane-integral pyrophosphatases exhibit differential expression pattern depending on growth conditions. In this diatom species we also report the first localization of a member of the V_0 -c subunit to the ER-membrane.

II. MATERIAL AND METHODS

1. Cell Culture

Culture of *Phaeodactylum tricorutum* strain used corresponds mainly to the oval morphotype, and is available at the Algae Culture Collection of Göttingen University (strain 1090-1a). Cells were grown at 19°C in an enriched artificial sea water media [64], 75 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity, and under a light:dark regime of 12h:12h.

2. Plasmid Construction and Diatoms Transformation

To clone the gene encoding for a V_0 -c subunit, total RNAs were extracted using Trizol (Invitrogen), treated with DNase I (Biolabs), and then reverse amplified using Superscript III (Invitrogen), according to the protocols from the manufacturer. The cDNA was amplified using high fidelity pfx DNA polymerase (Invitrogen). The specific primers used for the V_0 -c gene are: (5'-CCGGAATTCAACATGAGTGTCGAAATGGAACTTGCCCGG-3') and (5'-GCTCTAGAGCCGTTGTT

CCCCTCGCACACGAACGAGTTC-3'). The amplified DNA fragment was first purified and then cloned in frame with EGFP gene into a pPTEGfp plasmid [67]. In the resulting construct the transgenic gene is under the control of a *fcpA* promoter, and the resistant gene *sh ble* used for selection is under *fcpB*. After transformation by tungsten microparticle bombardment at 1550 psi (Bio-Rad Model PDS-1000/He Biolistic Particle Delivery System) the desired transgenic diatoms were repleted with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ of phleomycin, following described protocols [3]. Since the integration of plasmid was stable over time, the cultures of transgenic cell lines were maintained without selection. The transgenic strain expressing the BiP:EGFP chimeric protein was kindly provided by Dr. P. Kroth [4], and was maintained in appropriate condition to favor the transition from fusiform to oval morphology.

3. Image Acquisition

Images were obtained with a Leica DM-IRB microscope as previously described [13]. The set of filters used for GFP were 485 \pm 25 nm excitation (Ex) and 535 \pm 30 nm emission (Em), and for the chlorophyll fluorescence signal we used 485 \pm 25 nm (Ex) and 675 \pm 50 nm (Em). Image analyses were performed using a combination of software including Metamorph software 7.5 (Molecular Devices), Fiji, and Amira 5.1. All the images correspond to Z-D projections.

4. Collection of Sequences and *in silico* Analyses

Cyberscreening for pyrophosphatase and V-ATPases was performed using National Center of Biotechnology Information and European Bioinformatics Institute databases with basic local alignment search tools (tblastn, blastp, blastn). For the 24 algae and Stramenopiles species studied in more details, the gene/protein searches were performed at the specific genome browsers from DOE Joint Genome Institute (<http://genome.jgi-psf.org/>) or the Phytozome 7.0 (www.phytozome.net/). The proteins identification number (ID) were retrieved from *Aureococcus anophagefferens* (V1.0), *Phytophthora capsici* (V11), *Phytophthora ramorum* (V1.1), *Phytophthora sojae* (V1.1), *Aplanochytrium kerguelense* PBS07 (v1.0), *Aurantiochytrium limacinum* ATCC MYA-1381 (v1.0), *Schizochytrium aggregatum* ATCC 28209 (v1.0), *Guillardia theta* CCMP2712 (V1.0), *Volvox carteri* (V1), *Ostreococcus lucimarinus* (V2.0), *Ostreococcus* RCC809 (V2.0), *Ostreococcus tauri* (V2.0), *Bigeloviella natans* CCMP2755 (V1.0), *Micromonas pusilla* CCMP1545 (V2.0), *Chlorella* sp. NC64C (V2.0), *Coccomyxa* sp. C-169 (V2.0), *Micromonas* sp. RCC299 (V3.0), *Emiliania huxleyi* CCMP1516 (V1.0), and the diatoms *Phaeodactylum tricorutum* (V2.0), *Thalassiosira pseudonana* (V3.0), *Pseudo-nitzschia multiseriis* (v1.0), and the haploid models of *Fragilariopsis cylindrus* (V1.0). The search for genes in the unicellular red algae *Cyanidioschyzon merolae* was performed at <http://merolae.biol.s.u-tokyo.ac.jp/>, and for the diatom *Thalassiosira oceanica* at <http://bose.geomar.de/cgi-bin/gbrowse/Toceanica/>. For proteins, for which the

gene models were considered to be incomplete, manual editing of the models were performed. When no model was present, the search was repeated as tblastn search against the entire genomes, using a low stringent e-value $>1e^{-3}$; if no gene model could be predicted we refer to a missing gene in Table 1. For phylogenetic analyses the maximum likelihood (ML) topology was computed using Mega 5.2 [62] based on the JTT matrix-based model [25]. Non-parametric bootstrap support was estimated from 1,000 iterations. In addition, the tree topology was compared to the ones obtained from Neighbor-Joining method [55].

Detailed data for over 130,000 ESTs from *P. tricorutum* and the description of the conditions used to derive the 16 libraries are available at <http://www.diatomics.biologie.ens.fr/EST3/est3.php> [39].

III. RESULTS AND DISCUSSION

1. Evolution of Membrane-Integral Pyrophosphatases in Diatoms

Membrane embedded H^+ -PPase uses PPi as its energy source in the generation of proton gradients, and corresponds to a single highly hydrophobic polypeptide believed to function as homodimers [20, 29, 35, 42]. Based on phylogenetic analyses and biochemical evidences, H^+ -transporting pyrophosphatase were divided into K^+ -dependent and K^+ -independent subfamilies [8, 20, 38, 40, 50], but both types require Mg_2^+ as a cofactor. Site direct mutagenesis experiments revealed that modification of single residue, a change of an Ala residue in position 460 of the bacterium *Carboxydotherrhus hydrogeniformans* into Lys, abolish the potassium dependence [8]. Some K^+ -dependent PPases were shown to function as Na^+ -pumps [38, 40], and the specificity to work as a H^+ - or as a Na^+ -pump involved specific Glu residues in the transmembrane domains 5 and 6 [38]. More recently it was shown that a subfamily of PPases, named Na^+ - H^+ -PPases, are capable of transporting both Na^+ and H^+ ions in a non-competitive manner [37].

To gain more information on the evolution of the membrane pyrophosphatases family in diatoms, we conducted a genome-wide survey from five species for which the complete genome is available: *Phaeodactylum tricorutum* [9], *Thalassiosira pseudonana* [6], *Fragilariopsis cylindrus*, *Pseudo-nitzschia multiseriis*, and *T. oceanica* [36]. The results are compared with data obtained from green or red unicellular algae and Chromalveolate species for which the full-genome sequence is available. The full dataset comprised 9 Chlorophytes algae (3 fresh water species: *Chlorella* NC64A, *Coccomyxa* sp. C-169, and *Volvox carteri*, 5 marine species: *Micromonas pusilla*, *Micromonas* sp. RCC299, *Ostreococcus specises*, *Ostreococcus lucimarinus* and *Ostreococcus* RCC809, and the coastal lagoon *Ostreococcus tauri*), 10 Stramenopiles (the 5 above-mentioned Diatoms, the three Oomycetes *Phytophthora capsici*, *P. ramorum*, and *P. sojae*, the Labyrinthulomycetes *Aplanochytrium kerguelense*, *Aurantiochytrium limacinum*, *Schizochytrium aggregatum*, and the small marine

Table 1. Overview of Pyrophosphatases and V-ATPases gene families in green, red and brown algae and in other Stramenopiles.

Phylum	Species	Genome (Mb)	V-PPases		V ₀ -subunits					V-ATPases							
			K ⁺ -dep.	K ⁺ -ind.	a	c	c''	d	e	A	B	C	D	E	F	G	H
Stramenopiles/ Diatoms	<i>Phaeodactylum tricoratum</i>	26	1 + 1	1	1	8	2	1	1	1	1	1	1	1	1	1	2
	<i>Fragilariopsis cylindrus</i>	81	1 + 1	0	6	6	2	2	2	2	2	2	2	1	1	1	4
	<i>Pseudo-nitzschia multiseriata</i>	219	1 + 1	0	4	5	3	1	1	1	1	1	1	1	1	1	2
	<i>Thalassiosira pseudonana</i>	31	1 + 1	0	1	5	2	1	1	1	1	1	1	1	1	1	2
	<i>Thalassiosira oceanica</i>	82	1	0	1	2	1	1	NF	1	1	2	2	1	1	1	3
Stramenopiles/ Pelagophytes	<i>Aureococcus anophagefferens</i>	57	1 + 1	1	1	1	1	1	1*	1	1	1	1	1	1	1*	2
Stramenopiles/ Labyrinthulomycetes	<i>Aplanochytrium kerguelense</i>	35.8	3 + 0	3	1	3	1	1	1	1	1	1	1	1	1	1	1
	<i>Aurantiochytrium limacinum</i>	60.9	3 + 0	1	1	3	1	1	1	1	1	1	1	1	1	1	1
	<i>Schizochytrium aggregatum</i>	41	3 + 0	1	1	2	NF	1	1	1	1	1	1	1	1	1	1
Stramenopiles/ Oomycetes	<i>Phytophthora capsici</i>	56	1 + 0	1	1	2	1	1	1	1	1	1	1	1	1	1	1
	<i>Phytophthora ramorum</i>	65	1 + 0	1	1	2	1	2	1	1	1	1	1	1	1	1	1
	<i>Phytophthora sojae</i>	95	1 + 0	1	1	2*	1	1	1	2 [#]	1	1	1	1	1	1	1
Rhizaria/ Cercozoa	<i>Bigeloviella natans</i>	95	3 + 1	1	4	2	1	1	1*	1	1	1	1	1	1	NF	1
Haptophyceae/ Isochrysidales	<i>Emiliana huxleyi</i>	168	5 + 1	0	4	6	2	1	2*	1	1	1	2	1	1	2	1
Cryptophyta/ Pyrenomonadales	<i>Guillardia theta</i>	87.2	3	2	3	3	1	1	1	1	1	1	1	1	1	1	2
Viridiplantae/ Chlorophytes	<i>Chlorella variabilis</i> NC64A	46	1 + 0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorophytes	<i>Coccomyxa subellipsoidea</i>	49	1 + 0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	<i>Micromonas pusilla</i>	22	2 + 0	1	1	1	1	1	1	1	1	1	1	1	1	NF	1
	<i>Micromonas</i> sp. RCC299	21	2 + 0	1	1	2	1	1	1	1	1	1	1	1	1	1	1
	<i>Ostreococcus lucimarinus</i>	13	1 + 1	1	1	2	1	1	1	1	1	1	1	1	1	1	1
	<i>Ostreococcus</i> sp. RCC809	13	1 + 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	<i>Ostreococcus tauri</i>	12	1 + 1	1	1	2	1	2 [#]	1	1	1	1	1	1	1	1	1
	<i>Volvox carteri</i>	138	1 + 0	0	2	1	1	1	NF	1	1	1	1	1	1	1	1
Rhodophytes	<i>Cyanidioschyzon merolae</i>	17	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1

The # refers to incomplete gene sequence information, * to a gene(s) that was not annotated, and NF to a gene that could not be found using blastp or to blastn search engine and a set of proteins from different phylum.

non-motile Pelagophyte *Aureococcus anophagefferens*), one Rhodophyte (the hot-spring alga *Cyanidioschyzon merolae*), one Haptophyte (the Coccolith *Emiliana huxleyi*), one Cryptophyte (the small marine alga *Guillardia theta*), and one Rhizaria (the marine Chlorarachniophyte flagellate *Bigeloviella natans*). The number of gene encoding for membrane-integral pyrophosphatases was found to be small all species, with a maximum of 6 genes in the haploid genome of *E. huxleyi*, and a single gene in *C. merolae* (Table 1).

Interestingly, all the analyzed organisms contain at least one gene encoding for a putative enzyme of each types of pyrophosphatases, with the exception of the unicellular red alga *C. merolae*, that has a single gene that encodes a K⁺-independent enzyme. To understand whether all red algae possess only one type of pyrophosphatase or whether this is a consequence of the highly reduced genome of *C. merolae*, we search for putative K⁺-dependent candidates using available ESTs information. Sequences that with the determinants of K⁺-independent enzymes could be found in the unicellular red alga *Galdieria sulphuraria* (contig_849_Oct13_2005:g33.t1; <http://dbdata.rutgers.edu/dragon/>) or *Porphyridium cruentum* (GenBank: HS789509.1). However, ESTs corresponding to K⁺-dependent enzymes could be found in the unicellular alga *Porphyridium purpureum* (HS853118.1), *Cyanidium caldarium*

(CAH58649.1), and from the foliose red alga *Porphyra yezoensis* (CAD24772.1). Our data reveal that the two subfamilies of H⁺-PPases are likely to be present in all algae lineages.

To further understand the evolution of PPases in diatoms, we constructed a Bayesian phylogeny of the protein superfamily using a subset of 65 proteins that includes most of the genes found in the diatoms lineage (Fig. 2). The phylogeny reveals the existence of several clades within the K⁺-dependent subfamily, and demonstrates that putative proteins from several diatoms and other algae fall into a separate clade, that correspond to the Na⁺,H⁺-PPase subfamily (Fig. 2, and data not shown). Na⁺,H⁺-PPase subfamily has been proposed to have evolved from bacterial Na⁺-PPases [37]. In such scenario, the presence of prokaryotic-like Na⁺,H⁺-PPases in the genome of several microalgae from different lineages suggests independent acquisition of genes from bacterial origin. Alternatively, it can be proposed that this subfamily of K⁺-dependent PPases might have an ancient green algae origin and have been transferred into the genome of Chromalveolates. This latest hypothesis also implies that Na⁺,H⁺-PPase encoding genes have been independently lost in several green algae and Chromalveolates (for discussion of green algae genes in the Chromalveolates see [15, 28, 49] and references therein). In future, it will be important to perform biochemical studies to

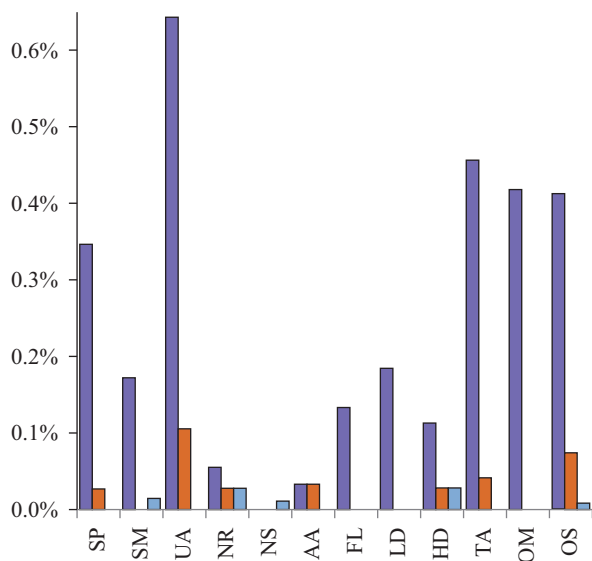


Fig. 3. Expression levels of the genes encoding for membrane pyrophosphatases in *P. tricornutum*. Histograms showing the expression levels of the K-dependent (ID 26190) in blue, K-dependent Na⁺/H⁺ (ID 15815) in purple and K-independent (ID 21183) in orange. The expression levels of each gene in the different libraries were normalized by the libraries size. Abbreviations for library conditions are: Silica plus (SP), Silica minus (SM), Urea adapted (UA), Nitrate replenished (NR), Nitrate starved (NS), Ammonium adapted (AA), Iron limitation (FL), Low decadienal (LD), High decadienal (HD), Tropical accession (TA), Oval morphology (OM) and Old library (OS).

3. Evolution of V-ATPases

The V-ATPase is a large multi-subunit complex, composed by two functional domains, V₁ and V₀. V₁ is composed of eight different subunits in a stoichiometry of A₃B₃CDE₃FG₃H and is located on the cytoplasmic side of the membrane [30] (Fig. 1). The V₁ domain corresponds to the rotary domain that houses the catalytic ATP binding sites, whereas proton translocation occurs through the membrane integral V₀ domain (Fig. 1). The V₀ domain is composed of six different subunits, with a stoichiometry of ac₄c'c''de in the yeast [21]. Detailed expression analyses have shown that subunit isoforms might result from alternatively spliced. Completion of several genome projects also revealed that they can be encoded by multiple genes. Genome-wide survey of V-ATPase genes identified 15 genes in *Saccharomyces cerevisiae* [26], 33 genes in *Drosophila melanogaster* [1], and 26 genes in *Arabidopsis thaliana* [61]. More recently, the conservation of genes encoding V-ATPase subunits from 17 different higher plants and from the green algae *Chlamydomonas reinhardtii* revealed that the total number of genes identified ranges from 54 in the soybean *Glycine max* (its genome size is estimated to be 1.1 Gb [56]) to 14 in *C. reinhardtii* [57].

With a few exception of the V₁ subunit G (V₁-G) subunit in *Micromonas pusilla* and *Bigelowiella natans*, the V₀ subunit e (V₀-e) in *Volvox carteri* and *T. oenica*, and V₀-c'' in

Schizochytrium aggregatum, the 13 subunits were identified in the 24 genomes analyzed (Table 1). The analyses of the chromosomal localization of the V-ATPase genes in different algae and Oomycetes reveal that several orthologous genes might have arisen by tandem or segmental duplication (not shown). In yeast the V-ATPase proteolipid subunits c, c' and c'' have been shown to be homologous to each other and to the F-ATPase subunit c, from which they appear to have been derived by gene duplication [22, 41]. Segmental duplication of regions of the chromosome encoding for V₀ subunits have been, for example, reported in *A. thaliana* [61] or in *D. melanogaster* [24]. Evidence for duplication of genes in the same chromosome segment were found for one V₀-c gene in *P. sojae*, one gene in *E. huxleyi*, one in *T. pseudonana*, two in *P. tricornutum* and in *F. cylindrus*, and for one V₀-a gene in *F. cylindrus*. For several of the V₀-c genes from diatoms, we found evidences for recent tandem duplications; two genes were considered to be the result of a tandem duplication when the distance between the paralogous gene pairs was below 1 kb. For example, the calculated protein identity between duplicated V₀-c was high and vary between 54% (106/195 aminoacids; comparing *P. tricornutum* proteins ID 15145/ID 22609), 98% (167/170; for *F. cylindrus* proteins ID 205176/ID 267224) and 100% (170/170; ID 21882/ID 29011, and 171/171; *T. pseudonana* proteins ID 39417/ID 2233). During evolution duplicate genes are retained because the redundancy conferred by the duplicate genes might facilitate species adaptation, genetic robustness against null mutations, or to obtain subfunctionalization or new functions. We found that the V₀-c genes family was particularly enriched in diatoms, compared to the other genomes analyzed here (Table 1). Bayesian phylogeny of the proteolipid subunit reveals that diatoms possess a single copy of the subunit V₀-c'' and several copies of the V₀-c type (Fig. 4). Using the present dataset we also found that diatoms proteolipid subunit can be phylogenetically classified into three major clades (Fig. 4) (such result was confirmed using a larger set of 485 sequences; not shown). Surprisingly, we also found that some V₀-c and V₀-c* subunits were substitution of the conserved glutamic acid residue (*Saccharomyces cerevisiae*, VMA3 Glu137 or VMA11 Glu148) whose carboxyl side chain was shown to be important for proton transport activity [22] (Fig. 4); suggesting that some of the diatoms genes might be pseudogenes, or that new amino acid residues, yet unknown, contribute to protons transport. In future, comparative protein structure modeling might help to gain new information on the structure of the "divergent" V₀-c* subunits found in diatoms.

4. Subcellular Localization of a V₀-c Proteolipid Subunit in *P. tricornutum*

Over the last decades, studies in a number of model organisms, from yeast to flies or higher plants, have shown that V-ATPases are important regulators in energizing secondary active transport and in membrane trafficking. It was also demonstrated that different V₀ et V₁ subunits can display

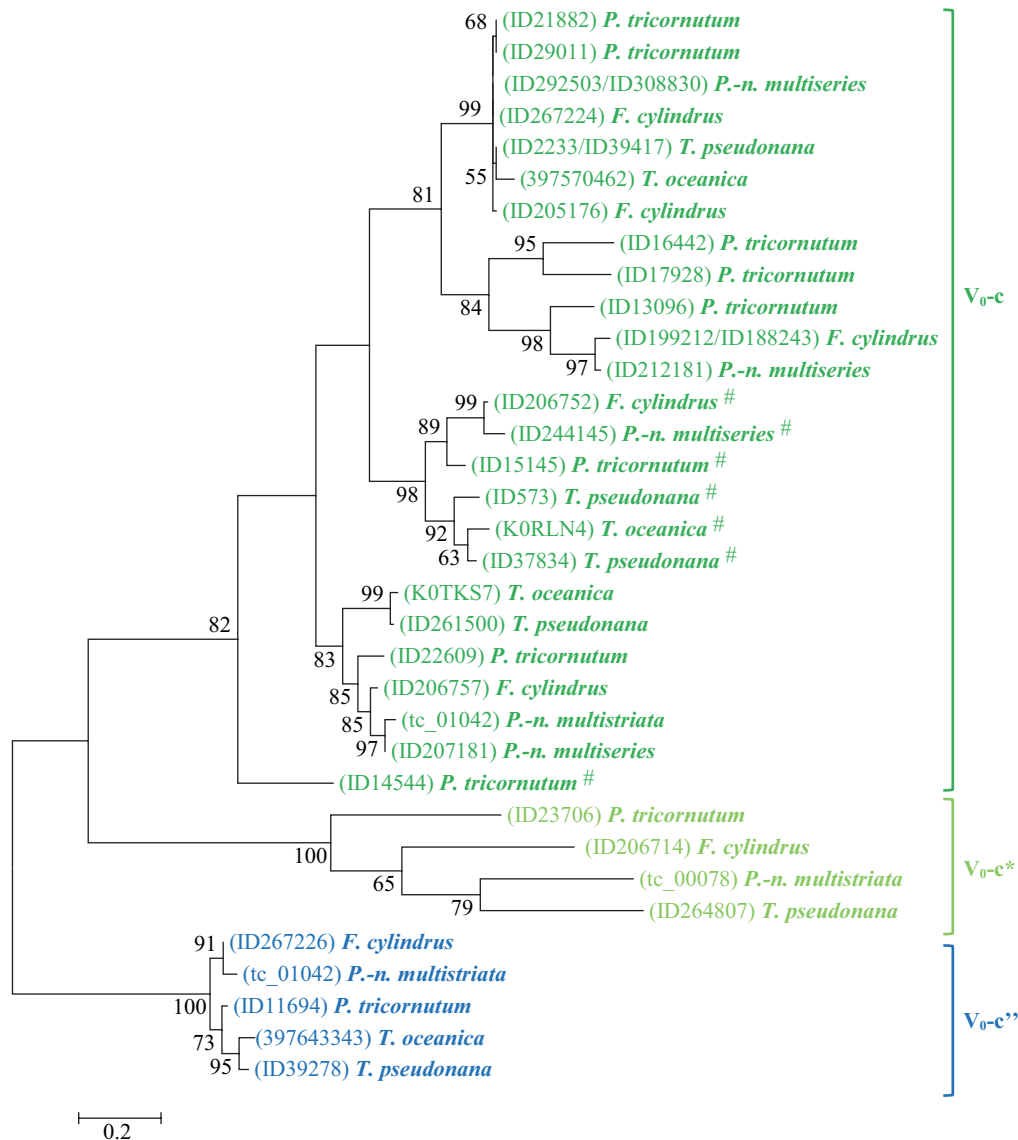


Fig. 4. Molecular phylogenetic analysis of V₀-c and V₀-c'' subunits families in diatoms. The Maximum Likelihood, based on the JTT matrix-based model, analysis involved 34 sequences, with a total of 133 amino acid positions in the final dataset. Bootstrap values above 50% (1000 replicates) are indicated at the corresponding nodes. The # indicates protein sequences that present in the fourth trans-membrane domain a substitution of the glutamic residue shown to be important for H⁺ transport. The scale bar represents the branch length corresponding to 0.2 substitution/site.

specific subcellular localization, show tissue-specific expression, respond to environmental stresses or be expressed at different developmental stages (for reviews see: [24, 33, 57, 63]).

In a first attempt to characterize V-ATPases in *P. tricornutum*, one gene encoding for a V₀-c subunit (ID 29011) was fused to EGFP and the corresponding transgenic line was analyzed for subcellular localization. Localization studies show that V₀-c:EGFP accumulates in a network of membranes traversing the entire length of the cell (Fig. 5, upper panel); that was likely to correspond to the endoplasmic reticulum. Indeed, the localization of V₀-c was found to be very similar to the one of the ER-localized chaperone BiP (compare Fig. 5 upper and lower panels). We are convinced that further lo-

calization studies coupled to functional analyses are becoming very important to decipher the role of the different V-ATPase paralogous genes in diatom.

IV. CONCLUDING REMARKS AND FUTURE DIRECTIONS

V-ATPase and H⁺-PPases which are ubiquitous proton pumps were shown to be essential to acidify intracellular compartments, to transport protons across the plasma membrane, to be implicated in response to salt stress, to be required for full activation of the PKA pathway in response to glucose, and to be implicated in vesicular trafficking and membrane

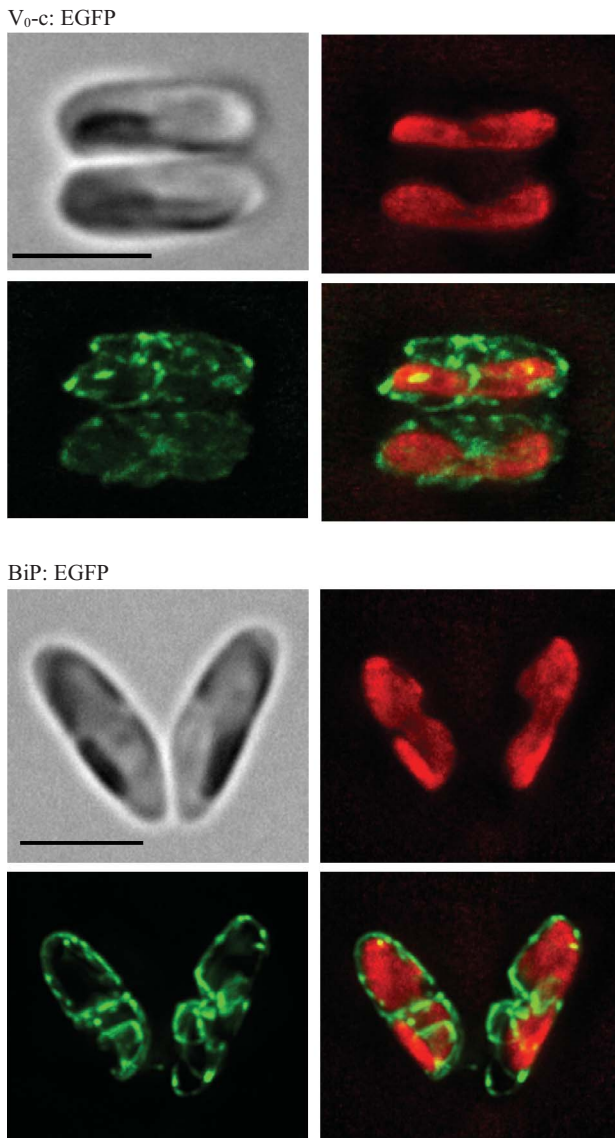


Fig. 5. Localization of the V_0 -c and BiP in the endoplasmic reticulum of *P. tricornutum*. Accumulation of a chimeric protein V_0 -c: EGFP (upper panel) or BiP: EGFP (lower panel) in the endoplasmic reticulum (ER). The images correspond to the Nomarski image, the chlorophyll auto-fluorescence (red), the GFP fluorescence (green), and the merged channel. The scale bars represent 5 μ m.

fusion. We believe that these important roles found in various organisms correspond to a vast source of inspiration to design informative experiments in algae.

Based on the results presented here, we propose that future researches should focus on three topics: (i) the expression of the different V-ATPase subunits and H^+ -PPases in response to stress, to nutrient availability or in adaptation to ocean acidification; (ii) for the membrane integral pyrophosphatases, biochemical characterization of their catalytic activity is required to understand whether these genes confer an advantage in acclimation and adaptation to the marine environments; (iii) more investigation should also be made in the elucidation of

the localization of the different V-ATPases subunits in diatoms. Subcellular localization of V_0 -c isoforms may demonstrate specific localization linked to the particular endomembrane system found in diatoms and in other Heterokonts. V-ATPases have been shown to be active at the Coccolith vesicle membrane of *Pleurochrysis*, and proposed to play a role in calcification [5, 11]. It will therefore be very interesting to further localize V-ATPases subunits in diatoms, and to study their expression during the course of frustule formation.

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