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# EVOLUTION OF VACUOLAR PYROPHOSPHATASES AND VACUOLAR $H^+$-ATPASES IN DIATOMS

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Key words: V-ATPases, $H^+$-PPases, endoplasmic reticulum, vacuole, algae.

## 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 tricornutum* 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 expended number of genes encoding for the proteolipid subunits c of the $V_0$ subcomplex. Preliminary localization studies show that one of $V_0$-c subunits is associated to the endoplasmic reticulum membrane in *P. tricornutum*. 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 $V_1$ domain and a membrane $V_0$ domain. The large cytosolic $V_1$ 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 $V_0$ complex (common $V_0$ 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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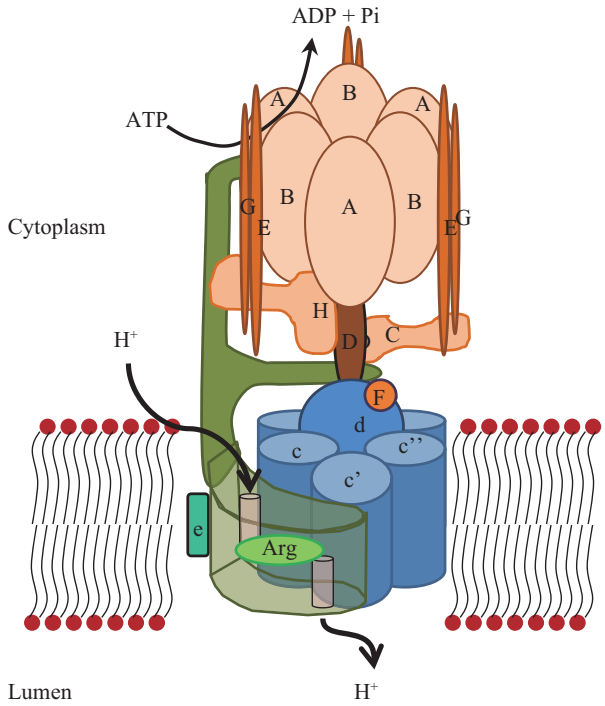


**Fig. 1. Structure of the V-ATPase complex. In most organisms, the V-ATPase complex is composed by two subcomplexes: $V_1$ which is composed by 8 subunits from A to H localized in the cytoplasm, and $V_0$ which is integrated in the plasmic membrane. The $V_0$ complex contains a hexameric ring of the 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 *Arabdopsis 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 tricornutum* [9], *Pseudonitschia multiseries* 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. tricornutum* 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 tricornutum* 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 $\mu E.m^{-2}.s^{-1}$ 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'-CCGGAATTCAACATGAGTGTCGAAATGG AAACTTGCCCGG-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 $\mu g.ml^{-1}$ 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 morphotype.

### 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 ± 25 nm excitation (Ex) and 535 ± 30 nm emission (Em), and for the chlorophyll fluorescence signal we used 485 ± 25 nm (Ex) and 675 ± 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 Bioinfortmatics 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), *Bigelowiella 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 tricornutum* (V2.0), *Thalassiosira pseudonana* (V3.0), *Pseudo-nitzschia multiseries* (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. tricornutum* 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 *Carboxydothermus hydrogenoformans* 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 tricornutum* [9], *Thalassiosaria pseudonana* [6], *Fragilariopsis cylindrus*, *Pseudo-nitzschia multiseries*, 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-ATPases | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $V_0$-subunits | | | | | $V_1$-subunits | | | | | | |
| | | | $K^+$-dep. | $K^+$-ind. | a | c | c" | d | e | A | B | C | D | E | F | G | H |
| Stramenopiles/ Diatoms | *Phaeodactylum tricornutum* | 26 | 1 + 1 | 1 | 1 | 8 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| | *Fragilariopsis cylindrus* | 81 | 1 + 1 | 0 | 6 | 6 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 4 |
| | *Pseudo-nitzschia multiseries* | 219 | 1 + 1 | 0 | 4 | 5 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| | *Thalassisira pseudonana* | 31 | 1 + 1 | 0 | 1 | 5 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| | *Thalassisira oceanica* | 82 | 1 | 0 | 1 | 2 | 1 | 1 | NF | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 3 |
| Stramenopiles/ Pelagophytes | *Aureococcus anophagefferens* | 57 | 1 + 1 | 1 | 1 | 1 | 1 | 1 | 1* | 1 | 1 | 1 | 1 | 1 | 1 | 1* | 2 |
| Stramenopiles/ Labyrinthulomycetes | *Aplanochytrium kerguelense* | 35.8 | 3 + 0 | 3 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Aurantiochytrium limacinum* | 60.9 | 3 + 0 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Schizochytrium aggregatum* | 41 | 3 + 0 | 1 | 1 | 2 | NF | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Stramenopiles/ Oomycetes | *Phytophthora capsici* | 56 | 1 + 0 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Phytophthora ramorum* | 65 | 1 + 0 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Phytophthora sojae* | 95 | 1 + 0 | 1 | 1 | 2* | 1 | 1 | 1 | 2# | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Rhizaria/ Cercozoa | *Bigelowiella natans* | 95 | 3 + 1 | 1 | 4 | 2 | 1 | 1 | 1* | 1 | 1 | 1 | 1 | 1 | 1 | NF | 1 |
| Haptophyceae/ Isochrysidales | *Emiliania huxleyi* | 168 | 5 + 1 | 0 | 4 | 6 | 2 | 1 | 2* | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 1 |
| Cryptophyta/ Pyrenomonadales | *Guillardia theta* | 87.2 | 3 | 2 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Viridiplantae/ | *Chlorella variabilis* NC64A | 46 | 1 + 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Chlorophytes | *Coccomyxa subellipsoidea* | 49 | 1 + 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Micromonas pusilla* | 22 | 2 + 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | NF | 1 |
| | *Micromonas* sp. RCC299 | 21 | 2 + 0 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Ostreococcus lucimarinus* | 13 | 1 + 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Ostreococcus* sp. RCC809 | 13 | 1 + 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Ostreococcus tauri* | 12 | 1 + 1 | 1 | 1 | 2 | 1 | 2# | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | *Volvox carteri* | 138 | 1 + 0 | 0 | 2 | 1 | 1 | 1 | NF | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Rhodophytes | *Cyanidioschyzon merolae* | 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 *Emiliania huxleyi*), one Cryptophye (the small marine algae *Guillardia theta*), and one Rhizaria (the marine Chlorarachniophyte flagellate *Bigelowiella 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. huxlei*, 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

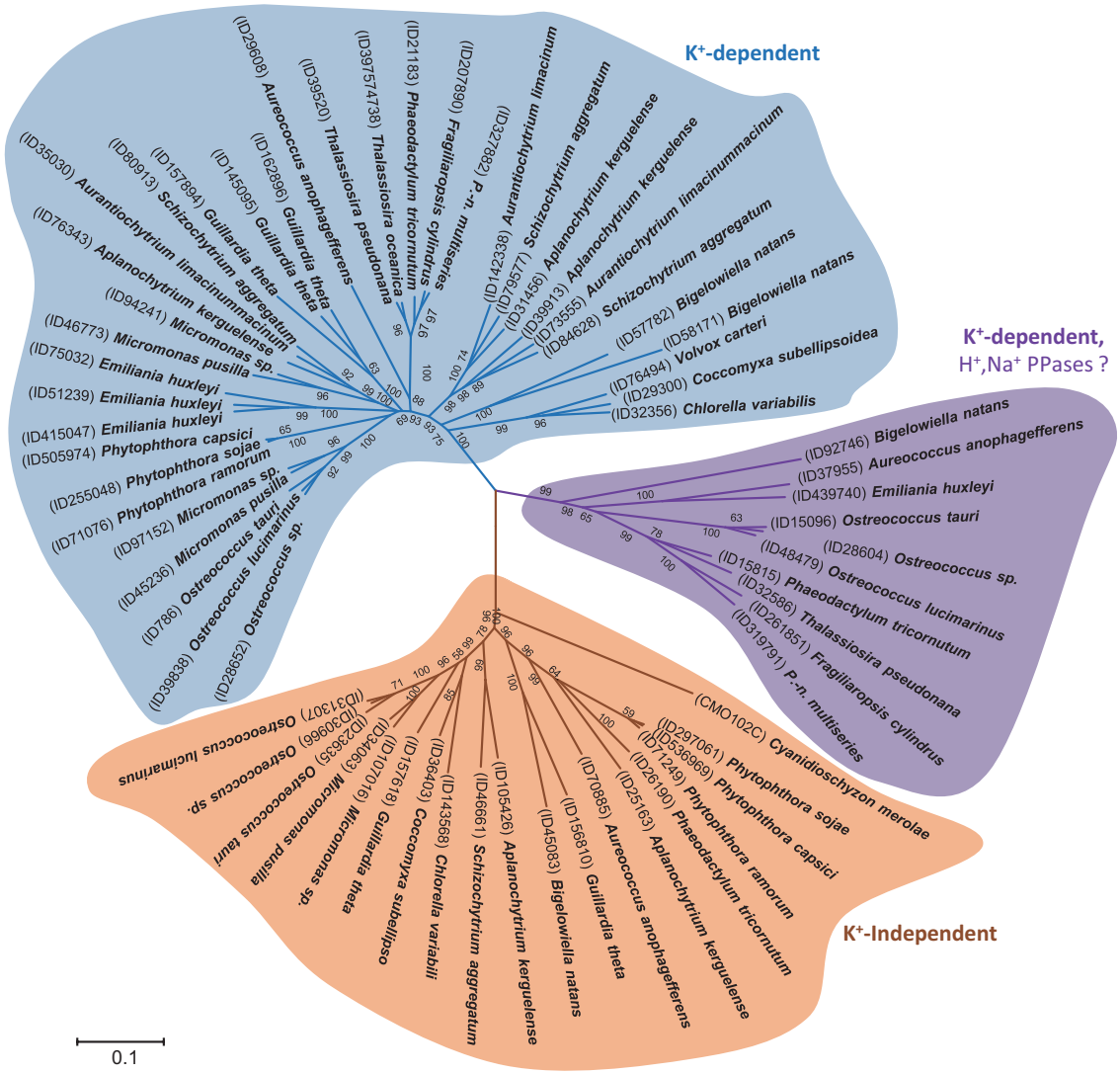


**Fig. 2. Maximum Likelihood best tree of $K^+$-dependent and $K^+$-independent pyrophosphatases. The data correspond to a subset of 65 proteins sequences (24 taxa), and the phylogeny corresponds to 474 amino acid aligned residues. For clarity reason only the boostrap values above 50% (1000 replicates) are showed. The protein reference (JGI protein IDs or GenBank accession number) follows the name of the organisms. The scale bar shows the correspondence between branch length and rate of amino acid substitution per site.**

test the intriguing possibility that diatoms, and several microalgae species, have membrane-integral pyrophosphatases capable of transporting sodium and proton ions.

## 2. Expression Pattern of Membrane-Integral Pyrophosphatases under Different Physiological Conditions in *P. tricornutum*

The expression of $H^+$-PPases genes in the pennate diatom *P. tricornutum*, was analyzed using available ESTs information that were generated to explore the responses to a range of growth conditions including several nutrient regimes of Si, N, Fe, and dissolved inorganic carbon (DIC), stresses (hyposalinity or low temperature), blue light, cells exposed to aldehyde decadienal, and from different morphotypes [39].

Analysis of the expression levels over the 16 libraries reveals that compared to the $K^+$-independent PPases (ID 26190) the two genes encoding $K^+$-dependent PPases, ID 21183 and ID 15815, were 6 times and 56 times more expressed, respectively. The highest expression of these two latest genes was found to be in the urea adapted condition, with 55 and 9 ESTs for the genes ID 21183 and ID 15815, respectively (Fig. 3). The existence of a functional ornithine-urea cycle (OUC) in Stramenopiles was first deduced from genomes analysis [6, 9]. Later it was demonstrated that the OUC serves as a distribution and repackaging hub for inorganic carbon and nitrogen in *P. tricornutum* [2]. Even if the ion selectivity and the physiological roles of membrane pyrophosphatases still have to be investigated in diatoms, it will be particularly interesting to test for their contribution in nutrient-use efficiency and in adaptation to abiotic stresses such as salinity or acidification.

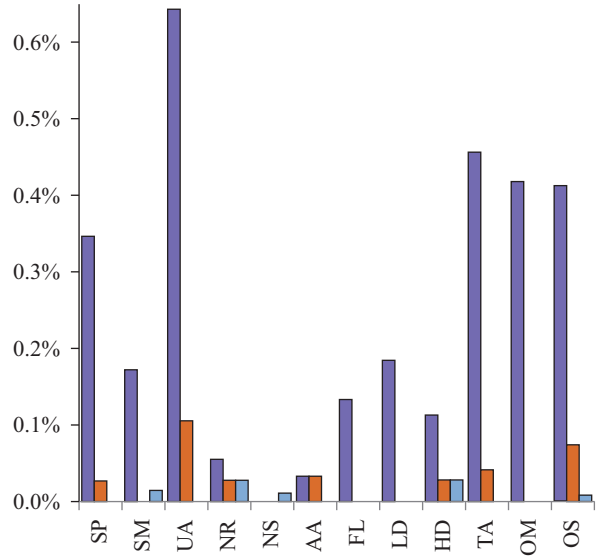


**Fig. 3. Expression levels of the genes encoding for membrane pyrophosphatases in *P. tricornutum*. Histograms showing the expression levels of the K-dependent (ID 26190) in blue, K-dependent $Na^+/H^+$ (ID 15815) in purple and K-independent (ID 21183) in orange. The expression levels of each gene in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

## 3. Evolution of V-ATPases

The V-ATPase is a large multi-subunit complex, composed by two functional domains, $V_1$ and $V_0$. $V_1$ is composed of eight different subunits in a stoichiometry of $A_3B_3CDE_3FG_3H$ and is located on the cytoplasmic side of the membrane [30] (Fig. 1). The $V_1$ domain corresponds to the rotary domain that houses the catalytic ATP binding sites, whereas proton translocation occurs through the membrane integral $V_0$ domain (Fig. 1). The $V_0$ domain is composed of six different subunits, with a stochiometry of $ac_4c'c''de$ 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 $V_1$ subunit G ($V_1$-G) subunit in *Micromonas pusilla* and *Bigelowiella natans*, the $V_0$ subunit e ($V_0$-e) in *Volvox carteri* and *T. ocenica*, and $V_0$-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 $V_0$ 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 $V_0$-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 $V_0$-a gene in *F. cylindrus*. For several of the $V_0$-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 $V_0$-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 $V_0$-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 $V_0$-c" and several copies of the $V_0$-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 $V_0$-c and $V_0$-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" $V_0$-c* subunits found in diatoms.

## 4. Subcellular Localization of a $V_0$-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 $V_0$ et $V_1$ subunits can display

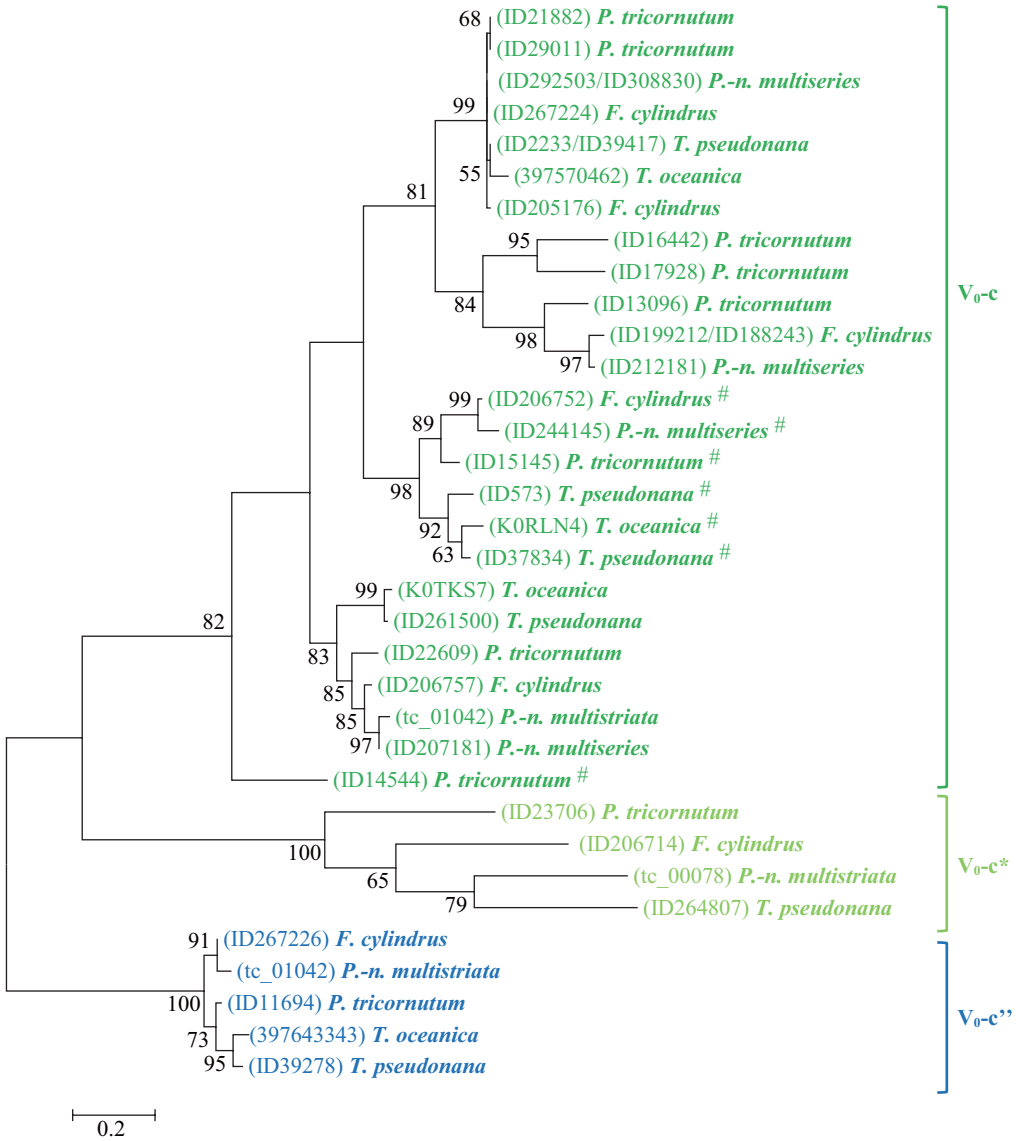


**Fig. 4. Molecular phylogenetic analysis of $V_0$-c and $V_0$-c" subunits families in diatoms. The Maximum Likelihood, based on the JTT matrix-based model, analysis involved 34 sequences, with a total of 133 amino acid positions in the final dataset. Bootstrap values above 50% (1000 replicates) are 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 $V_0$-c subunit (ID 29011) was fused to EGFP and the corresponding transgenic line was analyzed for subcellular localization. Localization studies show that $V_0$-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 $V_0$-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 localization 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

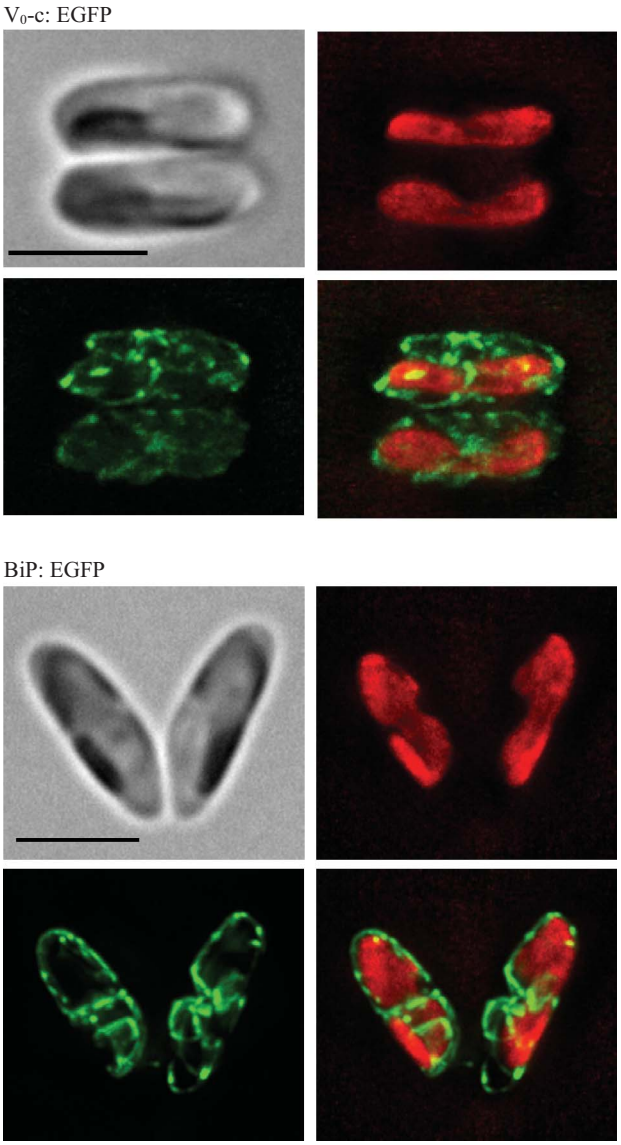


**Fig. 5. Localization of the $V_0$-c and BiP in the endoplasmic reticulum of *P. tricornutum*. Accumulation of a chimeric protein $V_0$-c: EGFP (upper panel) or BiP: EGFP (lower panel) in the endoplasmic reticulum (ER). The images correspond to the Nomarsky image, the chlorophyll auto-fluorescence (red),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

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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## REFERENCES

1. Allan, A. K., Du, J., Davies, S. A., and Dow, J. A., "Genome-wide survey of V-ATPase genes in *Drosophila* reveals a conserved renal phenotype for lethal alleles," *Physiological Genomics*, Vol. 22, pp. 128-138 (2005).
2. Allen, A. E., Dupont, C. L., Obornik, M., Horak, A., Nunes-Nesi, A., McCrow, J. P., Zheng, H., Johnson, D. A., Hu, H., Fernie, A. R., and Bowler, C., "Evolution and metabolic significance of the urea cycle in photosynthetic diatoms," *Nature*, Vol. 473, pp. 203-207 (2011).
3. Apt, K. E., Kroth-Pancic, P. G., and Grossman, A. R., "Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*," *Molecular and General Genetics*, Vol. 252, pp. 572-579 (1996).
4. Apt, K. E., Zaslavkaia, L., Lippmeier, J. C., Lang, M., Kilian, O., Wetherbee, R., Grossman, A. R., and Kroth, P. G., "*In vivo* characterization of diatom multipartite plastid targeting signals," *Journal of Cell Science*, Vol. 115, pp. 4061-4069 (2002).
5. Araki, Y. and Gonzalez, E. L., "V-and P-type $Ca^{2+}$-stimulated ATPases in a calcifying strain of *Pleurochrysis* sp. (Haptophyceae)," *Journal of Phycology*, Vol. 34, pp. 79-88 (1998).
6. Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S., Allen, A. E., Apt, K. E., Bechner, M., Brzezinski, M. A., Chaal, B. K., Chiovitti, A., Davis, A. K., Demarest, M. S., Detter, J. C., Glavina, T., Goodstein, D., Hadi, M. Z., Hellsten, U., Hildebrand, M., Jenkins, B. D., Jurka, J., Kapitonov, V. V., Kroger, N., Lau, W. W., Lane, T. W., Larimer, F. W., Lippmeier, J. C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M. S., Palenik, B., Pazour, G. J., Richardson, P. M., Rynearson, T. A., Saito, M. A., Schwartz, D. C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F. P., and Rokhsar, D. S., "The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism," *Science*, Vol. 306, pp. 79-86 (2004).
7. Becker, B. and Hickisch, A., "Inhibition of contractile vacuole function by brefeldin A" *Plant and Cell Physiology*, Vol. 46, pp. 201-212 (2005).
8. Belogurov, G. A. and Lahti, R., "A lysine substitute for $K^+$. A460K mutation eliminates $K^+$ dependence in $H^+$-pyrophosphatase of *Carboxydothermus hydrogenoformans*," *The Journal of Biological Chemistry*, Vol. 277, pp. 49651-49654 (2002).
9. Bowler, C., Allen, A. E., Badger, J. H., Grimwood, J., Jabbari, K., Kuo, A., Maheswari, U., Martens, C., Maumus, F., Otillar, R. P., Rayko, E., Salamov, A., Vandepoele, K., Beszteri, B., Gruber, A., Heijde, M., Katinka, M., Mock, T., Valentin, K., Verret, F., Berges, J. A., Brownlee, C., Cadoret, J. P., Chiovitti, A., Choi, C. J., Coesel, S., De Martino, A., Detter, J. C., Durkin, C., Falciatore, A., Fournet, J., Haruta, M., Huysman, M. J.,

Jenkins, B. D., Jiroutova, K., Jorgensen, R. E., Joubert, Y., Kaplan, A., Kroger, N., Kroth, P. G., La Roche, J., Lindquist, E., Lommer, M., Martin-Jezequel, V., Lopez, P. J., Lucas, S., Mangogna, M., McGinnis, K., Medlin, L. K., Montsant, A., Oudot-Le Secq, M. P., Napoli, C., Obornik, M., Parker, M. S., Petit, J. L., Porcel, B. M., Poulsen, N., Robison, M., Rychlewski, L., Rynearson, T. A., Schmutz, J., Shapiro, H., Siaut, M., Stanley, M., Sussman, M. R., Taylor, A. R., Vardi, A., von Dassow, P., Vyverman, W., Willis, A., Wyrwicz, L. S., Rokhsar, D. S., Weissenbach, J., Armbrust, E. V., Green, B. R., Van de Peer, Y., and Grigoriev, I. V., "The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes," *Nature*, Vol. 456, pp. 239-244 (2008).

10. Cavalier-Smith, T., "Chloroplast evolution: secondary symbiogenesis and multiple losses," *Current Biology*, Vol. 12, pp. R62-R64 (2002).
11. Corstjens, P. L. A. M., Araki, Y., and Gonzalez, E. L., "A coccolithophorid calcifying vesicle with a vacuolar-type ATPase proton pump: cloning and immunolocalization of the $V_0$ subunit c," *Journal of Phycology*, Vol. 37, pp. 71-78 (2001).
12. De Riso, V., Raniello, R., Maumus, F., Rogato, A., Bowler, C., and Falciatore, A., "Gene silencing in the marine diatom *Phaeodactylum tricornutum*," *Nucleic Acids Research*, Vol. 37, p. e96 (2009).
13. Descles, J., Vartanian, M., El Harrak, A., Quinet, M., Bremond, N., Sapriel, G., Bibette, J., and Lopez, P. J., "New tools for labeling silica in living diatoms," *New Phytologist*, Vol. 177, pp. 822-829 (2008).
14. Docampo, R. and Moreno, S. N., "Acidocalcisomes," *Cell Calcium*, Vol. 50, pp. 113-119 (2011).
15. Dorrell, R. G. and Smith, A. G., "Do Red and Green Make Brown?: Perspectives on Plastid Acquisitions within Chromalveolates," *Eukaryotic Cell*, Vol. 10, pp. 856-868 (2011).
16. Falkowski, P. G., Katz, M. E., Knoll, A. H., Quigg, A., Raven, J. A., Schofield, O., and Taylor, F. J., "The evolution of modern eukaryotic phytoplankton," *Science*, Vol. 305, pp. 354-360 (2004).
17. Field, C. B., Behrenfeld, M. J., Randerson, J. T., and Falkowski, P., "Primary production of the biosphere: integrating terrestrial and oceanic components," *Science*, Vol. 281, pp. 237-240 (1998).
18. Forgac, M., "Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology," *Nature Reviews Molecular Cell Biology*, Vol. 8, pp. 917-929 (2007).
19. Fukuda, A. and Tanaka, Y., "Effects of ABA, auxin, and gibberellin on the expression of genes for vacuolar $H^+$-inorganic pyrophosphatase, $H^+$-ATPase subunit A, and $Na^+/H^+$ antiporter in barley," *Plant Physiology and Biochemistry*, Vol. 44, pp. 351-358 (2006).
20. Gaxiola, R. A., Palmgren, M. G., and Schumacher, K., "Plant proton pumps," *FEBS Letters*, Vol. 581, pp. 2204-2214 (2007).
21. Graham, L. A., Powell, B., and Stevens, T. H., "Composition and assembly of the yeast vacuolar H(+)-ATPase complex," *The Journal of Experimental Biology*, Vol. 203, pp. 61-70 (2000).
22. Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y., "VMA11 and VMA16 encode second and third proteolipid subunits of the *Saccharomyces cerevisiae* vacuolar membrane $H^+$-ATPase," *The Journal of Biological Chemistry*, Vol. 272, pp. 4795-4803 (1997).
23. Huysman, M. J., Fortunato, A. E., Matthijs, M., Costa, B. S., Vanderhaeghen, R., Van den Daele, H., Sachse, M., Inze, D., Bowler, C., Kroth, P. G., Wilhelm, C., Falciatore, A., Vyverman, W., and De Veylder, L., "AUREOCHROME1a-mediated induction of the diatom-specific cyclin dsCYC2 controls the onset of cell division in diatoms (*Phaeodactylum tricornutum*)," *Plant Cell*, Vol. 25, pp. 215-228 (2013).
24. Jefferies, K. C., Cipriano, D. J., and Forgac, M., "Function, structure and regulation of the vacuolar ($H^+$)-ATPases," *Archives of Biochemistry and Biophysics*, Vol. 476, pp. 33-42 (2008).
25. Jones, D. T., Taylor, W. R., and Thornton, J. M., "The rapid generation of mutation data matrices from protein sequences," *Computer Applications in the Biosciences*, Vol. 8, pp. 275-282 (1992).
26. Kane, P. M., "The where, when, and how of organelle acidification by the yeast vacuolar $H^+$-ATPase," *Microbiology and Molecular Biology Reviews*, Vol. 70, pp. 177-191 (2006).
27. Keeling, P. J., "The endosymbiotic origin, diversification and fate of plastids," *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*, Vol. 365, pp. 729-748 (2010).
28. Keeling, P. J. and Palmer, J. D., "Horizontal gene transfer in eukaryotic evolution" *Nature Reviews Genetics*, Vol. 9, pp. 605-618 (2008).
29. Kellosalo, J., Kajander, T., Kogan, K., Pokharel, K. and Goldman, A., "The structure and catalytic cycle of a sodium-pumping pyrophosphatase," *Science*, Vol. 337, pp. 473-476 (2012).
30. Kitagawa, N., Mazon, H., Heck, A. J., and Wilkens, S., "Stoichiometry of the peripheral stalk subunits E and G of yeast V1-ATPase determined by mass spectrometry," *The Journal of Biological Chemistry*, Vol. 283, pp. 3329-3337 (2008).
31. Koester, J. A., Swanson, W. J., and Armbrust, E. V., "Positive selection within a diatom species acts on putative protein interactions and transcriptional regulation," *Molecular Biology and Evolution*, Vol. 30, pp. 422-434 (2013).
32. Lavaud, J., Materna, A. C., Sturm, S., Vugrinec, S., and Kroth, P. G., "Silencing of the violaxanthin de-epoxidase gene in the diatom *Phaeodactylum tricornutum* reduces diatoxanthin synthesis and non-photochemical quenching," *PLoS ONE*, Vol. 7, p. e36806 (2012).
33. Lee, S. K., Li, W., Ryu, S. E., Rhim, T., and Ahnn, J., "Vacuolar H(+)-ATPases in *Caenorhabditis elegans*: what can we learn about giant $H^+$ pumps from tiny worms?" *Biochimica et Biophysica Acta*, Vol. 1797, pp. 1687-1695 (2010).
34. Li, J., Yang, H., Peer, W. A., Richter, G., Blakeslee, J., Bandyopadhyay, A., Titapiwantakun, B., Undurraga, S., Khodakovskaya, M., Richards, E. L., Krizek, B., Murphy, A. S., Gilroy, S., and Gaxiola, R., "*Arabidopsis* $H^+$-PPase AVP1 regulates auxin-mediated organ development," *Science*, Vol. 310, pp. 121-125 (2005).
35. Lin, S. M., Tsai, J. Y., Hsiao, C. D., Huang, Y. T., Chiu, C. L., Liu, M. H., Tung, J. Y., Liu, T. H., Pan, R. L., and Sun, Y. J., "Crystal structure of a membrane-embedded $H^+$-translocating pyrophosphatase," *Nature*, Vol. 484, pp. 399-403 (2012).
36. Lommer, M., Specht, M., Roy, A. S., Kraemer, L., Andreson, R., Gutowska, M. A., Wolf, J., Bergner, S. V., Schilhabel, M. B., Klostermeier, U. C., Beiko, R. G., Rosenstiel, P., Hippler, M., and Laroche, J., "Genome and low-iron response of an oceanic diatom adapted to chronic iron limitation," *Genome Biology*, Vol. 13, p. R66 (2012).
37. Luoto, H. H., Baykov, A. A., Lahti, R., and Malinen, A. M., "Membrane-integral pyrophosphatase subfamily capable of translocating both $Na^+$ and $H^+$," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 110, pp. 1255-1260 (2013).
38. Luoto, H. H., Belogurov, G. A., Baykov, A. A., Lahti, R., and Malinen, A. M., "$Na^+$-translocating membrane pyrophosphatases are widespread in the microbial world and evolutionarily precede $H^+$-translocating pyrophosphatases," *The Journal of Biological Chemistry*, Vol. 286, pp. 21633-21642 (2011).
39. Maheswari, U., Jabbari, K., Petit, J. L., Porcel, B. M., Allen, A. E., Cadoret, J. P., De Martino, A., Heijde, M., Kaas, R., La Roche, J., Lopez, P. J., Martin-Jezequel, V., Meichenin, A., Mock, T., Schnitzler Parker, M., Vardi, A., Armbrust, E. V., Weissenbach, J., Katinka, M., and Bowler, C., "Digital expression profiling of novel diatom transcripts provides insight into their biological functions," *Genome Biology*, Vol. 11, p. R85 (2010).
40. Malinen, A. M., Belogurov, G. A., Baykov, A. A., and Lahti, R., "$Na^+$-pyrophosphatase: a novel primary sodium pump," *Biochemistry*, Vol. 46, pp. 8872-8878 (2007).
41. Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y. C., Nelson, H., and Nelson, N., "cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of $H^+$-ATPases," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 85, pp. 5521-5524 (1988).
42. Martinoia, E., Maeshima, M., and Neuhaus, H. E., "Vacuolar transporters and their essential role in plant metabolism," J*ournal of Experimental Botany*, Vol. 58, pp. 83-102 (2007).
43. Meng, X., Xu, Z., and Song, R., "Molecular cloning and characterization of a vacuolar $H^+$-pyrophosphatase from *Dunaliella viridis*," *Molecular Biology Reports*, Vol. 38, pp. 3375-3382 (2011).

44. Miyahara, M., Aoi, M., Inoue-Kashino, N., Kashino, Y., and Ifuku, K., "Highly efficient transformation of the diatom *Phaeodactylum tricornutum* by multi-pulse electroporation," *Bioscience, Biotechnology, and Biochemistry*, Vol. 77, pp. 874-876 (2013).
45. Morris, C. A., Owen, J. R., Thomas, M. C., El-Hiti, G. A., Harwood, J. L., and Kille, P., "Intracellular localization and induction of a dynamic RNA-editing event of macro-algal V-ATPase subunit A (VHA-A) in response to copper," *Plant, Cell and Environment*, Vol. 37, pp. 189-203 (2013).
46. Muto, M., Fukuda, Y., Nemoto, M., Yoshino, T., Matsunaga, T., and Tanaka, T., "Establishment of a genetic transformation system for the marine pennate diatom *Fistulifera* sp. strain JPCC DA0580--a high triglyceride producer," *Marine Biotechnology* (NY), Vol. 15, pp. 48-55 (2013).
47. Nishisho, T., Hata, K., Nakanishi, M., Morita, Y., Sun-Wada, G. H., Wada, Y., Yasui, N., and Yoneda, T., "The a3 Isoform Vacuolar Type $H^+$-ATPase Promotes Distant Metastasis in the Mouse B16 Melanoma Cells," *Molecular Cancer Research*, Vol. 9, pp. 845-855 (2011).
48. Niu, Y. F., Yang, Z. K., Zhang, M. H., Zhu, C. C., Yang, W. D., Liu, J. S., and Li, H. Y., "Transformation of diatom *Phaeodactylum tricornutum* by electroporation and establishment of inducible selection marker," *Biotechniques*, Vol. 0, pp. 1-3 (2012).
49. Nosenko, T. and Bhattacharya, D., "Horizontal gene transfer in chromalveolates," *BMC Evolutionary Biology*, Vol. 7, p. 173 (2007).
50. Perez-Castineira, J. R., Lopez-Marques, R. L., Losada, M., and Serrano, A., "A thermostable K(+)-stimulated vacuolar-type pyrophosphatase from the hyperthermophilic bacterium *Thermotoga maritime*," *FEBS Letters*, Vol. 496, pp. 6-11 (2001).
51. Poulsen, N., Chesley, P. M., and Kroger, N., "Molecular Genetic manipulation of the diatom *Thalassiosira pseudonana* (Bacillariophyceae)," *Journal of Phycology*, Vol. 42, pp. 1059-1065 (2006).
52. Riebesell, U., "Photosynthesis. Carbon fix for a diatom," *Nature*, Vol. 407, pp. 959-960 (2000).
53. Robinson, D. G., Hoppenrath, M., Oberbeck, K., Luykx, P., and Ratajczak, R., "Localization of pyrophosphatase and V-ATPase in *Chlamydomonas reinhardtii*," *Botanica Acta*, Vol. 111, pp. 108-122 (1998).
54. Ruiz, F. A., Marchesini, N., Seufferheld, M., Govindjee, and Docampo, R., "The polyphosphate bodies of *Chlamydomonas reinhardtii* possess a proton-pumping pyrophosphatase and are similar to acidocalcisomes," *The Journal of Biological Chemistry*, Vol. 276, pp. 46196-46203 (2001).
55. Saitou, N. and Nei, M., "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Molecular Biology and Evolution*, Vol. 4, pp. 406-425 (1987).
56. Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X. C., Shinozaki, K., Nguyen, H. T., Wing, R. A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R. C., and Jackson, S. A., "Genome sequence of the palaeopolyploid soybean," *Nature*, Vol. 463, pp. 178-183 (2010).
57. Schumacher, K. and Krebs, M., "The V-ATPase: small cargo, large effects," *Current Opinion in Plant Biology*, Vol. 13, pp. 724-730 (2010).
58. Segami, S., Nakanishi, Y., Sato, M. H., and Maeshima, M., "Quantification, organ-specific accumulation and intracellular localization of type II H(+)-pyrophosphatase in *Arabidopsis thaliana*," *Plant and Cell Physiology*, Vol. 51, pp. 1350-1360 (2010).
59. Serrano, A., Pérez-Castiñeria, J. R., Baltscheffsky, H., and Baltscheffsky, M., "Proton-pumping inorganic pyrophosphatases in some archaea and other extremophilic prokaryotes," *Journal of Bioenergetics and Biomembranes*, Vol. 36, pp. 127-133 (2004).
60. Smetacek, V., "Diatoms and the ocean carbon cycle," *Protist*, Vol. 150, pp. 25-32 (1999).
61. Sze, H., Schumacher, K., Muller, M. L., Padmanaban, S., and Taiz, L., "A simple nomenclature for a complex proton pump: VHA genes encode the vacuolar H(+)-ATPase," *Trends in Plant Science*, Vol. 7, pp. 157-161 (2002).
62. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., "MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods," *Molecular Biology and Evolution*, Vol. 28, pp. 2731-2739 (2011).
63. Toei, M., Saum, R., and Forgac, M., "Regulation and isoform function of the V-ATPases," *Biochemistry*, Vol. 49, pp. 4715-4723 (2010).
64. Vartanian, M., Descles, J., Quinet, M., Douady, S., and Lopez, P. J., "Plasticity and robustness of pattern formation in the model diatom *Phaeodactylum tricornutum*," *New Phytologist*, Vol. 182, pp. 429-442 (2009).
65. Yagisawa, F., Nishida, K., Yoshida, M., Ohnuma, M., Shimada, T., Fujiwara, T., Yoshida, Y., Misumi, O., Kuroiwa, H., and Kuroiwa, T., "Identification of novel proteins in isolated polyphosphate vacuoles in the primitive red alga *Cyanidioschyzon merolae*," *The Plant Journal*, Vol. 60, pp. 882-893 (2009).
66. Yokoyama, K. and Imamura, H., "Rotation, structure, and classification of prokaryotic V-ATPase," *Journal of Bioenergetics and Biomembranes*, Vol. 37, pp. 405-410 (2005).
67. Zaslavskaia, L. A., Lippmeier, J. C., Kroth, P. G., Grossman, A. R., and Apt, K. E., "Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes," *Journal of Phycology*, Vol. 36, pp. 379-386 (2000).
68. Zhang, J., Li, J., Wang, X., and Chen, J., "OVP1, a vacuolar H+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance," *Plant Physiology and Bochemistry*, Vol. 49, pp. 33-38 (2011).