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Chapter 5

Evolutionary and functional analysis of a *Chara* plasma membrane H^+-ATPase

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Abstract

The plasma membrane (PM) H^+-ATPases are the main transporters in plants and fungi plasma membranes, comparable to the Na^+/K^+ ATPases in animal cells. At the molecular level, most studies on the PM H^+-ATPases were focused on land plants and fungi (yeast). The research of PM H^+-ATPases in green algae, as the ancestor of land plants, falls far behind due to the lack of genetic information. Here we isolated one potential PM H^+-ATPase gene (*CHA1*) from *Chara braunii*, a speice of green algae belonging to the division Charophyta, one of the closest ancestor of land plants. The gene deduces to a 107kDa protein, with all 6 P-type ATPase-specific motifs and a long, diverse C-terminal. A new amino acid sequence pattern R*****Q in transmembrane segment 5 was identified among the known PM H^+-ATPases from Charophyta and Chlorophyta algae, which is different from the typical PM H^+-ATPases in yeast or land plants. Plasmid-borne *CHA1* was transported and expressed in yeast systems. A complementation assay and a sub-cellular localization assay showed that CHA1 could successfully reach the cell membrane of yeast, though the activation as a proton pump could only be achieved when certain length (last 77-87 amino acid) of the C-terminal got truncated. These results suggest that an auto-inhibition domain is located in the C-terminal and that it has a different regulation mechanism than the models having been built up for yeast and land plants. Over-truncation of either N-terminal (e.g. more than 55 amino acids) or C-terminal (e.g. more than 98 amino acids) results in failure of this heterologous protein to reach the proper destination. Expression of CHA1 in Arabidopsis protoplast confirmed the plasma membrane localization in plant system.

Introduction

Plasma membrane (PM) H^+-ATPases, which belong to the least divergent subfamily of the P-type ATPase, P3A, have been identified in plants, fungi, and some protozoa, archaea. Similar to their counter-parts, the Na^+/K^+ ATPases in animal cells, H^+-ATPases act as primary transporters in both plants and fungi (Kuhlbrandt, 2004; Morth et al., 2011). Protons are used to create an electrochemical gradient, to balance and regulate the cytoplasmic pH and to facilitate secondary membrane transporters for e.g. uptake of nutrients. In higher plants, the PM H^+-ATPases, are under a tight regulation of biotic and abiotic stimuli, such as plant hormones and light, and are also well known as key regulators in processes such as cell expansion, stomatal opening and polarity development (Hohm et al., 2014).

With regard to the PM H^+-ATPase in plants, research into the protein sequence, the crystal structure and bio-chemical characters of this highly conserved subfamily of transport proteins shows that the functional proteins have five main domains aiding different functions (Buch-Pedersen et al., 2009; Ekberg et al., 2010). There is a transmembrane domain with 10 transmembrane helices and four cytosolic domains. The cytosolic domains are defined as the N (nucleotide binding) domain, the P (phosphorylation) domain, the A (actuator) domain, and the R (regulation) domain (Perdersen et al., 2007).

In a broader evolutionary perspective, the PM H^+-ATPases show patterns of diversity correlated to the evolutionary lines, especially in the R domain. It is reasonable to believe that these patterns may fit to the diversity of environments and are correlated to the evolution pattern. The R domain in vascular plants has been shown to function as an auto-inhibition domain, containing two conserved regions (Region I and II) and a penultimate threonine (named: pT H^+-ATPase) (Palmgren et al., 1991; Speth et al., 1997). This threonine can be phosphorylated by protein kinases, after which it becomes a binding site for 14-3-3 proteins (Fuglsang et al., 1999). The binding of 14-3-3 proteins abolishes the (auto) inhibition and activates the pumping (Baunsgaard et al., 1998). The binding of 14-3-3 proteins can be stabilized by the addition of the fungal toxin fusicoccin to create a long-lasting pump activation (Baunsgaard at al., 1998; Oecking and Hagemann, 1999). The penultimate threonine as a key regulation point is only present in land plant PM H^+-ATPases, and thus arose at a later time point in evolution. Experiments have shown that the moss *Physcomitrella patens* and

liverwort *Marchantia polymorpha*, as the basal lineages of extant land plants, contain both PM H^+-ATPases with and without the penultimate threonine, indicating that pT regulation mechanism might only have developed after the transition of plants from water to land (Okumura et al., 2012a). On the other hand, no evidence of pT was found among the known PM H^+-ATPases in lower lines such as algae (Okumura et al., 2012b). In the PM H^+-ATPases of Chlorophytes as well as the resembling protist, the whole R region regulation complex (Region I, II and pT) is missing, instead these PM H^+-ATPases can have a large variety of either short or long C-terminal cytoplasmic domains (Pedersen et al., 2012).

Regarding to the main function of PM H^+-ATPases, as a primary pump, there are several conserved essential residues creating a one-way-only passage for the transport of H^+. For example, based on the *Arabidopsis thaliana* H^+-ATPase2 (AHA2) model, residue Asp684 in transmembrane segment M6 functions as the H^+ acceptor/donor, and residue Asn106 in M2 is proposed and confirmed as a gatekeeper in cooperation with Asp684 to ensure the efficient transport of protons against the electrochemical gradient (Buch-Pedersen et al., 2000, 2003; Pedersen et al., 2007; Ekberg et al., 2013). So far, the Asp684 and Asn106 found in AtAHA2 are conserved among the known PM H^+-ATPases in land plants and algae. In addition, Arg655 in AtAHA2 has been proposed as a backflow preventer due to its position in the cavity opposite to the Asp684, and its positive charge, which may serve as positive plug preventing proton reflux (Buch-Pedersen and Palmgren, 2003; Pederson et al., 2012; Pedersen et al., 2007). Early evidence showed a conservation of R655 (AtAHA2) in all streptophyte pumps, but it is absent in typical protist and chlorophyte PM H^+-ATPases (Pederson et al., 2012). Coincidently, there is evidence of co-existence of both Na^+/K^+ and H^+ pumps in these protists and chlorophyte algae, despite the fact that the PM Na^+/K^+-ATPases or PM H^+-ATPases are strictly exclusively expressed in respectively animal cells or land plants/fungi (Pedersen, 2012). Based on this data, it was suggested that the missing R655 may fail the chlorophyte proton pump to build up a membrane potential, while the coexisting Na^+/K^+ -ATPases are the primary transporters to fulfill this function (Pedersen et al., 2012). Since not enough research has been done on the function of PM H^+-ATPases and Na^+/K^+-ATPases in algae, the above hypothesis is calling for further evidence.

In the evolutionary tree, there is a division of freshwater green algae, named Charophyta, which is believed to be the closest ancestor to the land plants

(Zhang and van Duijn, 2014). Not much is yet known about the molecular biological characters of the P3A H^+-ATPases in this essential group that bridges the unicellular algae (mainly the Chlorophyta algae) and the land plants, due to the lack of DNA sequence database. This leaves a gap in the P3A H^+-ATPases research in the evolutionary perspective.

Among the Charophyta, a group of branched, plant-like, multi-cellular green algae Charophyceae has already been used as a model system in plant physiology research for the past decades, credit to their huge internodal cells. For example, electrophysiological studies by measurement and control of potential difference (PD) across the plasma and tonoplast membranes of Characeae date back to the 1970s, offering the background knowledge of the plasma membrane transporters and channels including PM H^+-ATPases (Beilby and Casanova, 2014). The Characeae show the ability of acid/alkaline band formation along the internodal cells under the stimulation of light. PM H^+-ATPases are believed to be the key players behind this phenomenon by building up the acid band and facilitating the DIC (dissolved inorganic carbon species) uptake (Bulychev et al., 2001). In addition, there are significant differences to the land plants. The regulation mechanisms of PM H^+-ATPases seem to be different in *Chara corallina*, as earlier research showed no obvious PM H^+-ATPases stimulation by the plant hormone IAA or fusicoccin (Okumura et al., 2012; Zhang et al., 2016). This is consistent with the hypothesis that pT H^+-ATPases did not evolve earlier than in bryophytes (Okumura et al., 2012b). Altogether, this makes the *Chara* PM H^+-ATPase a good research target to fill in the gap between Chlorophyta and land plants, and to identify patterns in the evolution of PM H^+-ATPase functions and regulation mechanisms in plants.

In this study, one of the potential *Chara* PM H^+-ATPases gene (*CHA1*) of *Chara braunii* was isolated and the predicted protein sequence was analyzed. The alignment of CHA1 with other P3A H^+-ATPases from land plants, fungi and algae, showed both conservation and differences in the evolution pattern. We cloned this *CHA1* gene in the yeast expression system. Results showed that even though the wildtype gene can partially reach the plasma membrane of yeast, it could not replace the yeast PMA1 to support the growth. After deletion of the last 60 amino acids at the C-terminal, the pump could still reach the plasma membrane and is able to rescue the mutated yeast strain.

Materials and Methods

Plant material

Chara braunii was a kind gift from Prof. Ilse Foissner in Austria, and was cultured at room temperature in aquaria filled with sterilized forest soil covered with sand at the bottom and artificial pond water(APW) containing 0.1 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM NaCl (pH about 6.0) as described earlier (Berecki et al., 2001) under 16 hours photoperiod. Fresh internodal cells and branches were used for genomic DNA isolation.

Genomic DNA extraction and H^+-ATPase isolation

Chara genomic DNA was extracted from fresh *Chara* cells from the up-ground part (internodal cells and branches) using the CTAB DNA isolation protocol (de Pater et al. 2006).

Based on the sequences of three possible *Chara* PM H^+-ATPases contigs (transcript_4956, transcript_1405, transcript_181b) obtained from a sequencing experiment on *Chara braunii* RNAs (kindly offered by Holger Breuninger and Liam Dolan from Oxford, UK, unpublished data), the most likely open reading frames (ORF) were identified from the three hits with CLC workbench 7, naming transcript_4956 CDS (2775kb), transcript_1405 CDS (2952kb), transcript_181b CDS (1977kb). And based on these predicted CDSs (coding sequences), the forward, reverse primers were designed to amplify the fragments from genomics DNA (respectively, 4956_F, 4956_R, 1405_F, 1405_R, 181b_F and 181b_R (table 1)). When this failed, proton pump specific forward (PPs F1) and reverse (PPs R1) primers were designed based on the most conserved part from the three hits with approximately 1Kb in between. Other forward and reverse primers were designed to cover the whole sequence with specificity based on the hits sequences (table 1). Isolated *Chara* genome DNA was used as template and PCR reactions were performed using the Phusion polymerase (Thermo) with GC-buffer and recommended settings, temperatures were set based on the primers or the best tested results from the gradient-temperature PCR.

Tail PCR was carried out based on the description by Whittier et al. (1995), for the extension from the isolated and sequenced middle part to both the N-terminal and C-terminal. For N-terminal extension, forward primers NT_1, NT_2, NT_3 (table 1) were used successively for the three consecutive PCR

reactions, each with one of degenerative primers AD1, AD2 and AD3 (table 1), respectively. C-terminal extensions were carried out twice, stepwise, in the same way but with forward primers CT1_1, CT1_2, CT1_3 and CT2_1, CT2_2, CT2_3 (table 1).

All PCR products were purified by gel electrophoresis and recovered using a GeneJET Gel purification kit (Ehermo scientific). DNA fragments were cloned into the pJET Blunt cloning vector using the CloneJet PCR Cloning Kit (Thermo Scientific), and were subsequently sequenced (Macrogen Europe, Amsterdam, The Netherlands).

Sequence analysis and gene identification

PCR sequences were assembled and analyzed with CLC Main Workbench 7. The deduced protein sequence was analyzed by InterPro (including results from two independent tools of TMHMM server v.2.0 and Phobius) and the Protein Homology/analogy Recognition Engine Version 2 (PHYRE2).

Yeast strains and Culture Conditions

The yeast *S. cerevisiae* haploid null mutant strain YAK2 (*Matα, ade2-101, leu2Δ1, his3-Δ200, ura3-52, trp1Δ63, lys2-801pma1-Δ:: HIS3, pma2-Δ:: TRP1*) was kindly provided by Prof. Marc Boutry (University of Louvain, Belgium). This strain, lacking the two endogenous genomic copies of the H^+-ATPase gene *PMA1* and *PMA2*, and carrying the *PMA1* gene under the control of the GAL1-10 promoter on an URA3 centrometric plasmid for survival, was used for the complementation assay (de Kerchove d'Exaerde et al., 1995).

The yeast *S. cerevisiae* BY4743 (*MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, LYS2/lys2Δ0, met15Δ0/MET15, ura3Δ0/ura3Δ0*) was used for the CHA1 subcellular localization assay (Zhang, 2016).

Plasmids constructions

2up(PMA1)pma2 is a kind gift from Prof. Marc Boutry (University of Louvain, Belgium). *Nicotiana plumbaginifolia* proton pump *pma2* gene is under the control of the yeast *PMA1* promoter, with the *LEU2* gene for selection and the 2μ derived sequence for high copy replication in yeast (de Kerchove d'Exaerde et al., 1995; Lou et al.,1999).

2up(PMA1)CHA1: The plasmid contains the *Chara CHA1* gene (by replacing *pma2* gene from the plasmid 2up(PMA1)pma2) under the control of the yeast *PMA1* promoter, with the *LEU2* gene for selection and the 2μ derived sequence for high copy replication in yeast.

pUG34-GFP-CHA1: The *CHA1* gene was inserted as *Spe*I-*Sal*I fragment into single-copy yeast plasmid pUG34GFP (Sakalis, 2013) for the N-terminal fusion with GFP under the control of the MET25 promoter.

pART7(35S)YFP-CHA1: The *Chara att*B-flanked *CHA1* gene fragment was first inserted to pDONR207 through gateway BP recombination reaction. Then an LR recombination reaction was performed to transfer the *CHA1* from entry clone to destination vector pART7(35S)YFP-Gateway plasmid (Gleave, 1992; Yao, unpublished data), with N-terminal YFP fusion, under the control of 35S promoter.

CHA1 wild-type (wt) encoding sequence, C-terminal truncated (ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, ΔC887) and N-terminal truncated (ΔN55, ΔN64) DNA fragments were obtained by PCR with different primers (table 2) and pJET-CHA1 as the templet.

Complementation assay

The yeast H^+-ATPase gene *PMA1* is expressed under a galactose-dependent (*GAL1*) promoter, while the plasmid-borne *Chara* H^+-ATPase gene *CHA1* is expressed under the control of the yeast *PMA1* promoter with a LEU2 selection marker on multicopy 2-um plasmid 2up(PMA1)CHA1. An expression plasmid 2up(PMA1)pma2 equipped with yeast *PMA1* promoter and the tobacco *PMA2* gene, which is known to be able to rescue the yeast growth (de Kerchove d'Exaerde et al., 1995), was transformed as the positive control (C+). An empty expression plasmid (Yeplac) was transformed as the negative control (C-). Yeast transformation was carried out with the lithium acetate method. The transformed yeast was plated on solid selective medium (MY medium with addition of adenine and lysine) with either 2% galactose or 2% glucose for selective expression. Independent transformation was carried out at least three times.

Subcellular localization of CHA1 in yeast

The plasmids pUG34GFP and pUG34-GFP-CHA1 were transformed into yeast BY4743 cells by the lithium acetate method. Transformants were plated on solid MY medium containing methionine (MET) to suppress the expression of CHA1. After three days, clones were transferred to liquid MY medium containing methionine. The overnight liquid cultures were then centrifuged and the yeast cells were resuspended in fresh MY liquid medium without methionine to induce CHA1 expression for 1 hour. All yeast cultures were carried out at 30^0C. Cells were collected by centrifugation and resuspension in MilliQ water (to lower the background noise) for the GFP signal observation. In this study, a 63x magnification oil immersion objective on the Zeiss Imager microscope (LSM510) was used. Fluorescence at 488 nm excitation and 520 nm emission was analysed using ZEISS ZEN2009 software. The images were then processed with ImageJ (ImageJ National Institutes of Health, USA).

Arabidopsis protoplast transformation and microscopic analysis

Protoplasts were prepared from *Arabidopsis thaliana* Col-0 cell suspension cultures and transfected with the plasmid pART7-35S-YFP-CHA1 mediated by polyethylene glycol (PEG) as previously described (Schirawski et al., 2000). Transfected protoplasts were incubated at 25^0C for at least 16 hours in the dark prior to observing. The YFP signal was detected using an argon laser with 514 nm excitation and a band pass filter of 530-600 nm with a ZEISS confocal imaging microscope. Images were processed with ImageJ (ImageJ National Institutes of Health, USA).

Results and discussion

Potential *Chara* PM H^+-ATPase gene (*CHA1*) isolation

In order to identify possible PM H^+-ATPase genes in *Chara braunii*, we searched the transcriptome database generated by high throughput sequencing on RNAs isolated from *Chara braunii*, using the known plant PM H^+-ATPase sequences as a bait quarry (including *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*). This identified three contigs. Initial attempts to isolate the full ORF using primers based on the contig sequences failed for all possible PCR parameter modifications (temperature gradients or different concentrations of PCR solutions). Alignment of the three

contigs revealed a conserved sequence of approximately 1kb (Fig. 1A), proton pump specific forward primer (PPs F1) and reverse primer (PPs R1) (detailed in Fig. 1B) were used for PCR amplification. Sequencing result showed the highest resembling to transcript_181b CDS mentioned above (Fig. S1). Besides the points mutation, there is no sign of sequence insertion or deletion. Due to the lack of an official *Chara* sequence database, and the fact that there are more specie-level diversities among ecorticate species (Beilby and Casanova, 2014), we could not fully rely on the hits sequences information. Therefore, tail PCR specific primers (CT1_1, CT1_2, CT1_3) were designed to extend the C-terminal based on this 1kb known sequence information (Fig. 2A). Another C-terminal tail PCR was performed based on the result from the first tail PCR (with specific primers CT2_1, CT2_2, CT2_3) to reach the stop codon (Fig. 2A). Different combinations of forward and reverse primers (table 1) along the known full-length transcript_181b CDS were tried out. N-terminal tail PCR was carried out (with specific primers NT_1, NT_2, NT_3) to verify the beginning of the coding sequence. PCR results with different lengths from different primers together with the tail PCR results were then assembled (Fig. 2B). We managed to assemble one potential H^+-ATPase encoding sequence with an open reading frame length of 2958kb, which aligned well with parts of the transcript_1405 CDS and transcript_181b CDS (Fig. 3, S2). Comparing the isolated sequence from the genome DNA with the cDNA hits did not show signs of possible introns. Before further confirmation could be done, we took this 2958kb DNA sequence as a potential H^+-ATPase coding gene *CHA1* for further analysis. More information (eg. RNA seq) will be necessary to know how many H^+-ATPases homologous exist in *Chara*.

Protein Sequence analysis

From the final sequence of 2958kb a polypeptide with a calculated molecular mass of 107kDa was deduced. This polypeptide fits in the range of about 100kDa that can be found among other P3A H^+-ATPases and it contains all 6 P-type ATPase-specific amino acid motifs, and a conserved aspartic acid (corresponding to Asp684 in AtAHA2) as the H^+ acceptor/donor (Fig. S3) (Serrano,1989; Buch-Pedersen and Palmgren, 2003). The protein sequence was blasted in the NCBI database, with the confirmation of the highest homology to PM H^+-ATPases, showing 65% amino acid identity with the *Klebsormidium flaccidum* PM H^+-ATPase, and 57% or 35% amino acid identity with respectively

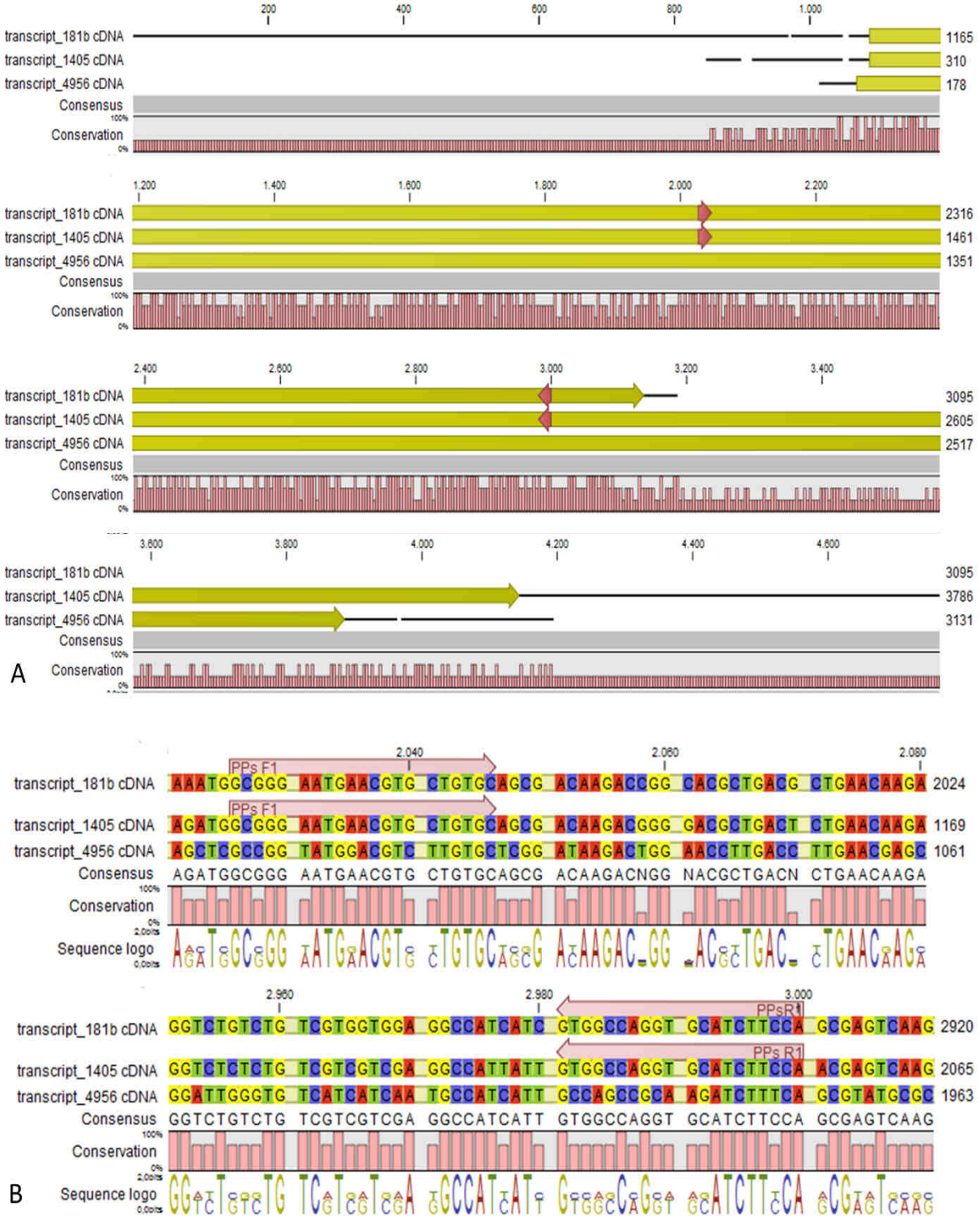

Figure 1. Alignment of the three contigs (transcript_181b, transcript_1405 and transcript_4956) with CLC workbench 7. (A) Alignment of the contigs with the predicted ORF in yellow and red arrows indicating the primers used for amplifying the conserved part. (B) Sequence information in detail regarding the forward and reverse primer (PPs F1 and PPs R1).

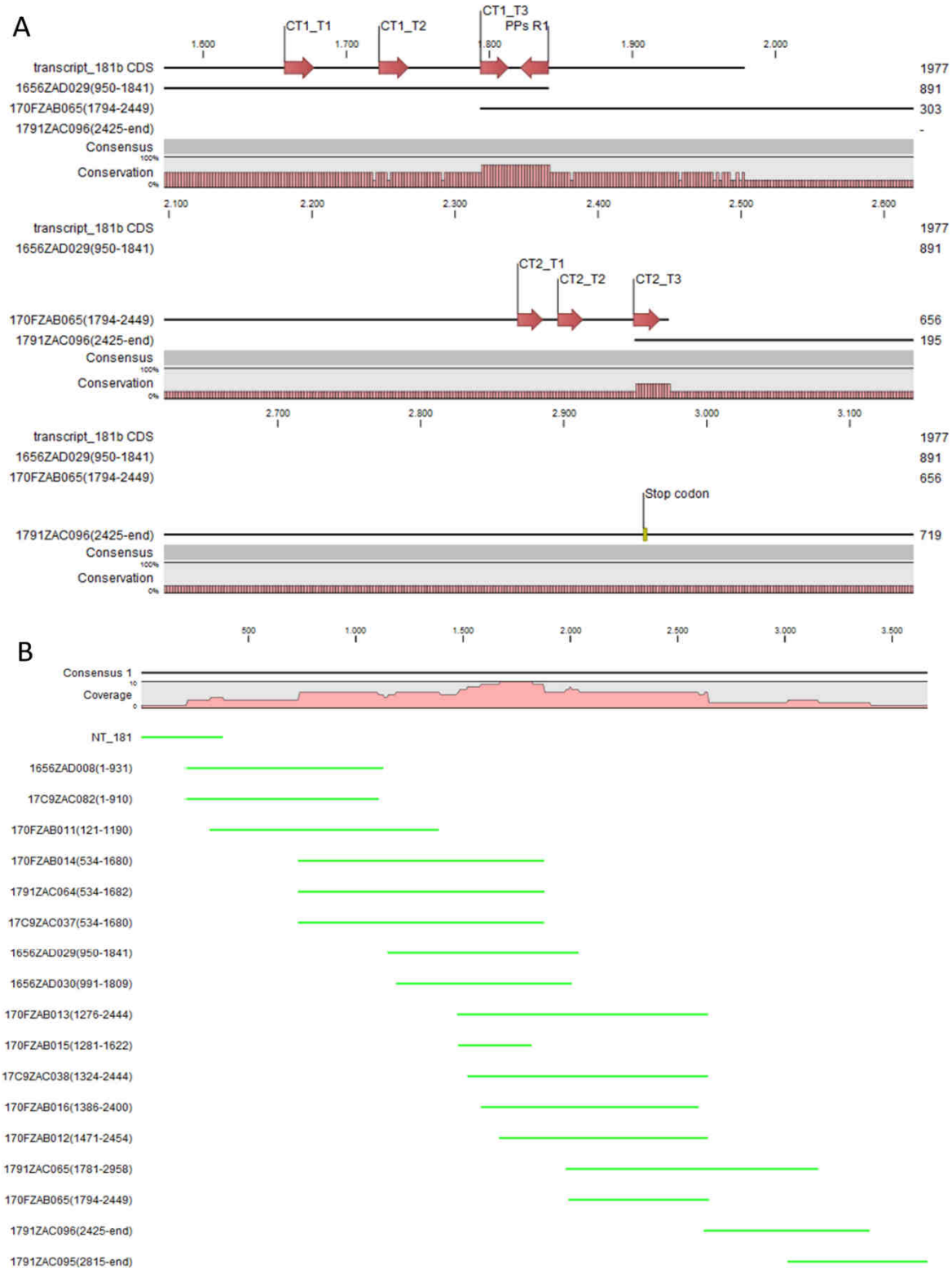

Figure 2. Alignment and assemble of PCR fragments for *CHA1* isolation. (A) Alignment of two step-wise tail PCR fragments for *CHA1* C-terminal extension with the primers indicating in red arrows. (B) Assemble of all PCR and tail PCR results for *CHA1*.

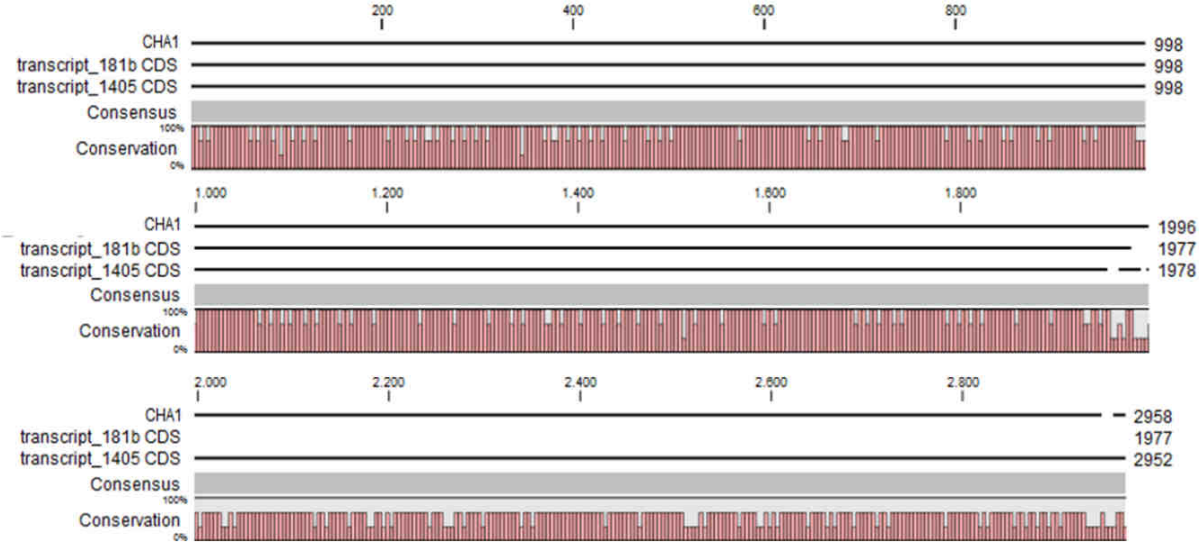

Figure 3. Sequence alignment on CLC workbench 7 of the assembled full *CHA1* (from start to stop codon) with predicted coding sequences of transcript_181b and transcript_1405.

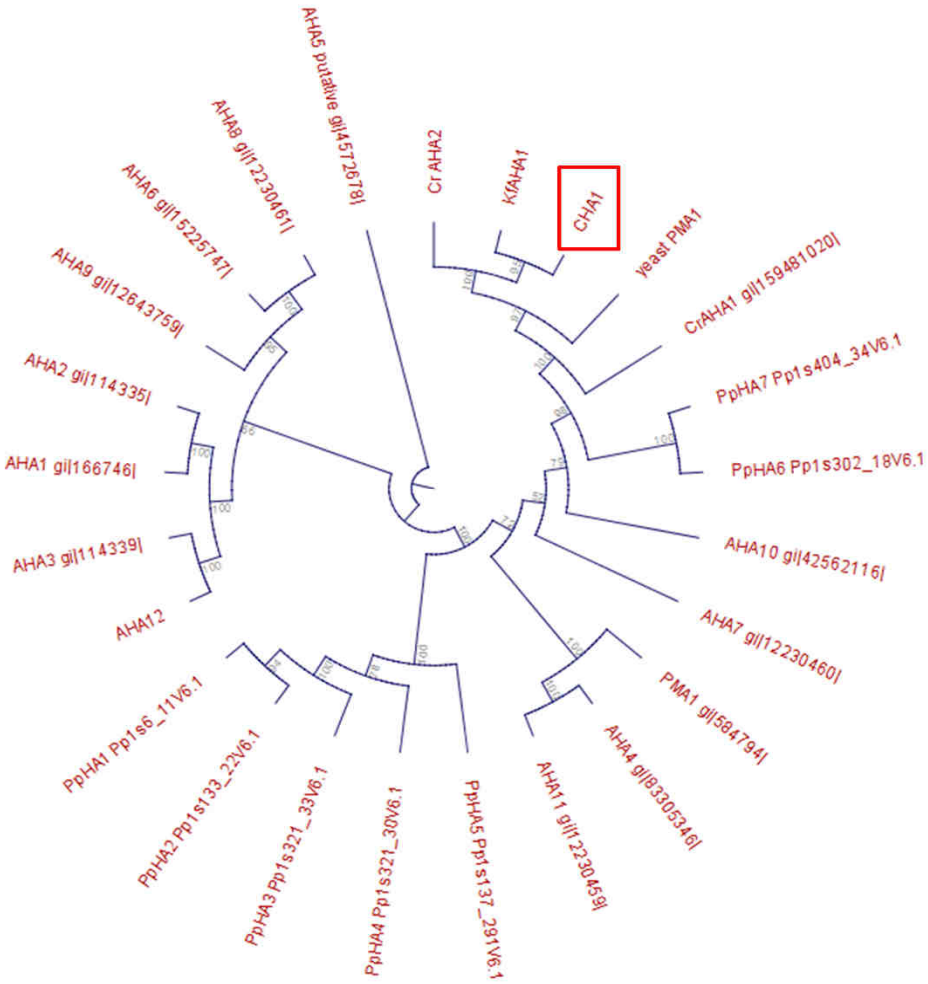

Figure 4. Phylogenetic tree of P3A ATPases, with *CHA1* highlighted in a red box.

the *Chlamydomonas reinhardtii* PM H^+-ATPase or the *Arabidopsis thaliana* PM H^+-ATPase10. The relationship between CHA1 and other P3A H^+-ATPases is indicated in the phylogenetic tree (Fig. 4).

Structure and function related sequence analysis

Transmembrane segments.

The structure of deduced protein CHA1 was analyzed with several online tools. Besides the relatively conserved N-, P- and A-domain, there are more varieties at the N- and C-terminus. Results showed the prediction of 9 (from TMHMM server v.2.0 and Phobius) transmembrane helixes in CHA1, instead of the conserved 10 transmembrane helixes in other P3A H^+-ATPases among the plants and fungi (Fig. 5A). Instead of two transmembrane helixes (M1, M2) before the small loop of other PM proton pumps, e.g. AtAHA2 (Fig. 5B), there is only one transmembrane helix in CHA1. As the consequence, the N-terminus of CHA1 tends to be in the extracellular domain (Fig. 5A). While another software PHYRE2, predicted two transmembrane helixes before the small loop with high confidence (Fig. 5C). Hence, the missing transmembrane helix (es) from the structure prediction tools may just be mis-prediction due to the lack of gene database of algae species. Since when trying other H^+-ATPases from algae species like *Klebsormidium flassidum* or *Chlamydomonas reinhardtii* with the same analysis tools, there were also either 8/9 or 10 transmembrane helixes being predicted. More background knowledge of DNA sequences and protein structures of the Charophyte and Chlorophyte algae would improve the accuracy and credibility of the prediction, and would give more information about the structure -function relationship of P3A H^+-ATPases.

Regulation domain.

Analysis of the C terminal (R-domain) shows, as expected from the P3A H^+-ATPases evolution perspective (Pedersen et al. 2012), that the well conserved Region I, II and penultimate threonine (pT) for land plants, are missing in *CHA1* (Fig. 6), which is consisted with the suggestion by Okumura and his coworkers that pT H^+-ATPase most likely appeared in bryophyte (Okumura et al., 2012). The alignment of the C-terminal domain with P3A H^+-ATPases from the Chlorophyta, Charophyta and some protists showed little homology, and no clear domain pattern could be found (Fig. S4). Considering the diversity of the living environment of the algae and protists (fresh water/salinity water,

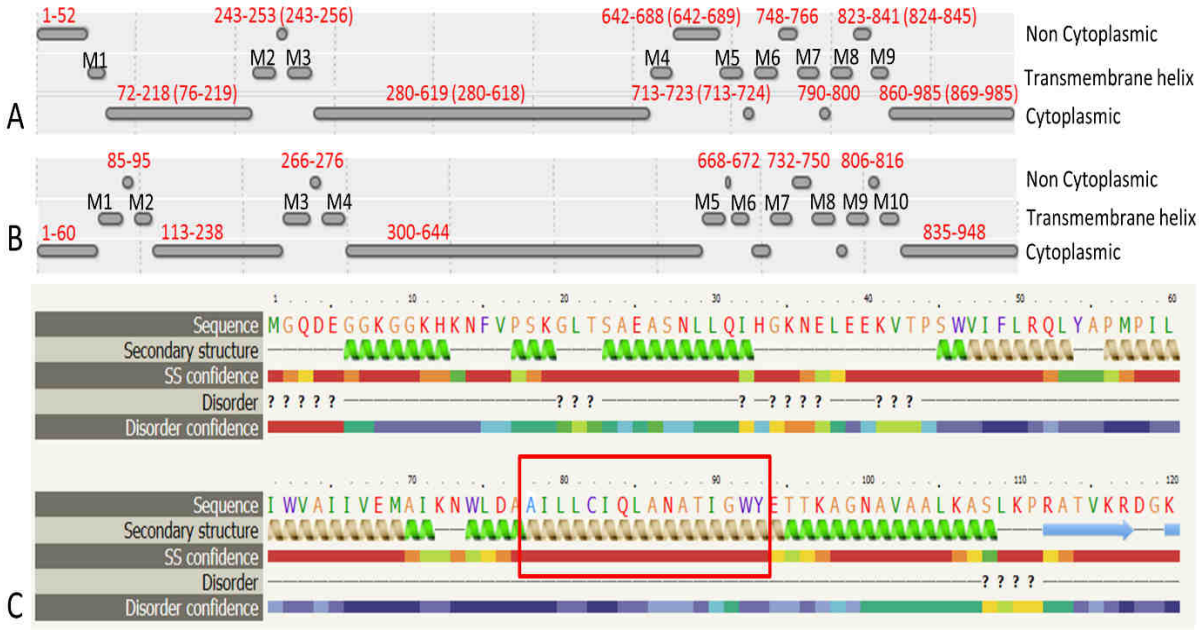

Figure 5. Predicted transmembrane segments of CHA1 from different tools. (A) Result of CHA1 from Interpro, including the prediction from Phobius and TMHMM server v.2.0 (the numbers are in brackets when they are different from Phobius). (B) Result of AtAHA2 from Interpro, including the prediction from Phobius and TMHMM server v.2.0 (the numbers are in brackets when they are different from Phobius). (C) First two transmembrane helixes of CHA1 predicted from PHYRE2, the controversial M2 (predicted as transmembrane helix by PHYRE2 but not by Interpro) is high-lighted in red box.

with/without light, etc.) the H^+-ATPase would be regulated under different conditions by different regulators for the different species and circumstances. Further analysis by e.g. mutation studies could be carried out to understand the regulation mechanism of H^+-ATPases in *Chara* and other algae which could survive in certain extreme environments, which might shed new light into plant tolerance mechanisms.

Up-hill transport capacity.

It is well-established that PM H^+-ATPases are able to build a high electrical membrane potential difference (PD) across the fungal and plant plasma membranes up to -300mV and -200mV, respectively. Based on the crystal structure of *Arabidopsis thalianan* AHA2, as proposed for the plant PM H^+-ATPases, there are three residues in the center transporting unit which are essential for building up such a steep electrochemical gradient: Asp684 (on M6), the centrally located proton acceptor/donor; Asn106 (on M2), closely juxtaposed to the protonated Asp684, the two creates a hydrogen bonding functioning as

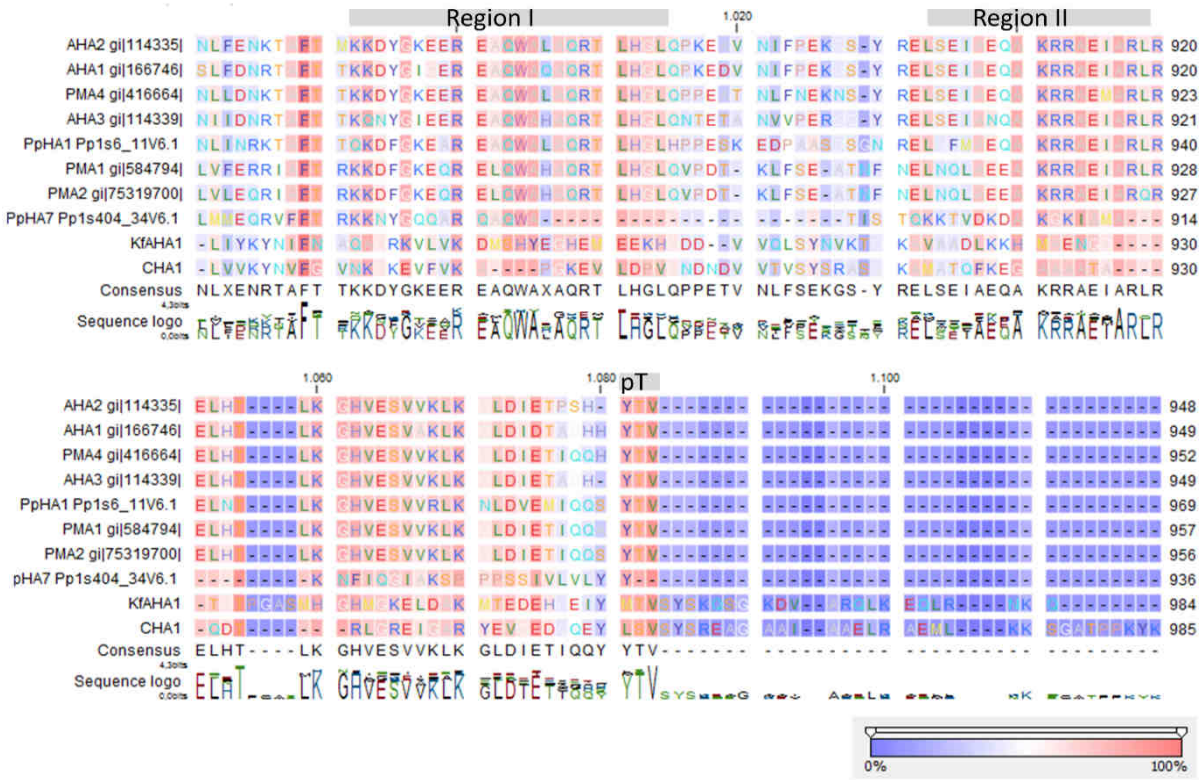

Fig 6. C-terminal alignment of CHA1 with other PM H^+-ATPases. Color bar below indicates the conservation degrees of the alignments. Gray bars above indicate the conserved C-terminal regions in land plants PM H^+-ATPases.

the "gate keeper" along the transport pathway; positively charged Arg55 (on M5), placed at the exit of the transport pathway, acting as a "positive plug", neutralizing the deprotonated negatively charged Asp684, preventing the reflux of proton (Pedersen et al., 2007; Buch-Pedersen et al., 2009).

In the steady state, at neutral pH and in light, with the proton pump in control, certain Characean algae (*Chara, Nitella*) cells could also reach a membrane potential of -200mV or even lower (Lucas, 1982; Beilby and Casanova, 2014). From these values it seems that the PM H^+-ATPases of these algae also have a strong capacity to pump the protons in the up-hill direction, building up a huge chemical-electrical gradient. The alignment of amino acid sequences of CHA1 and other P3A H^+-ATPases, shows an asparagine in position 87 of CHA1 corresponding to the gate-keeper residue Asn106 in the transmembrane segment M2 (Fig. 7A). Interestingly, the "positive plug" Arg655 in M5 is neutralized by a hydrophilic glutamine in CHA1 (Gln629), and this glutamine seems to be the dominant residue among the other algae species at this position (Fig. 7B). Instead, an arginine shows up at 6 residues in front of the same transmembrane segment M5, as Arg623 in *Chara*, which seems also quite conserved among the

PM H^+-ATPases of the algae species, just as the Gln629 (CHA1). At the same position in yeast *Saccharomyces cerevisiae* PMA1 there is also an arginine (Arg695). Early research found that in yeast this positive charged

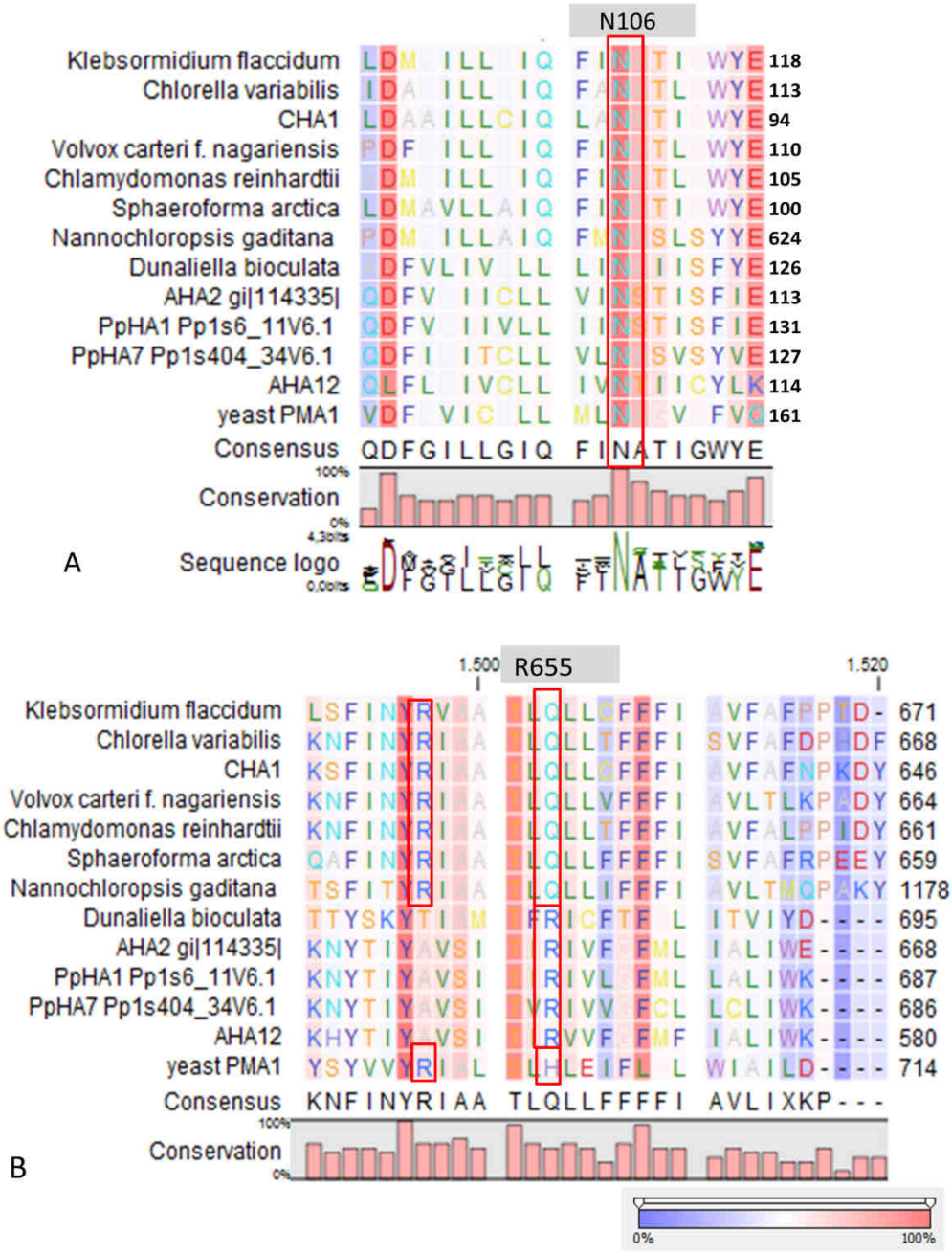

Fig 7. Alignment of transmembrane segments M2 and M5 (based on AtAHA2) of CHA1 and other P3A H^+-ATPases. (A) Alignment of M2. (B) Alignment of M5. Gray boxes indicate the residue and number in AtAHA2. Red boxes indicate certain conserved residues which may evolved with the proton up-hill transport ability. Color bar below indicates the conservation degrees of the alignments.

Arg695 in M5 together with the negative charged Asp730 in M6, formed a salt bridge linking M5 and M6, turning out to be important for the structure stability of PMA1 (Gupta et al., 1998). The positive charged His701 in yeast PMA1, which aligned with positive charged Arg655 in AtAHA2, also has an essential role in the protein folding and location functioning, which is dominant lethal when mutated (Dutra et al., 1998). This fact makes it difficult to check whether the His701 (PMA1) would have the "plug effect" in yeast, resulting a higher PD (-300mV).

Thus, in certain amount of algae PM H^+-ATPases, a positive charged residue in the middle of M5 is missing comparing to yeast and land plants, yet there is a quite conserved arginine at 6 residues in front same as in yeast PMA1. Question would be whether this arginine in algae functions as a "salt bridge" to maintain the protein structure or a "positive plug" to support the up-hill proton transport. The well-studied electro-physiology characters of *Chara* combining mutation study of CHA1 might give some structure and functional hints to this new pattern of R*****Q in M5 among these algae species.

Expression of wild-type and truncated CHA1 in null mutant Yeast strain

To confirm that the isolated *CHA1* gene functions as a proton pump and to further investigate the function of the C-terminal as an auto-inhibition domain, the full length CHA1 gene (985 amino acid, indicated as wt in the figures) and the C-terminal step-wise deletion mutants ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, or ΔC887 (Fig. 8A), were sub-cloned into the yeast expression plasmid and transformed into YAK2 null mutant strain for the yeast complementation assay.

Results showed that on galactose medium, yeast YAK2 strains could survive well when both the *PMA1* and *CHA1* genes were expressed (Fig. 8B). When culturing on glucose medium, the expression of only CHA1 was not sufficient to support the yeast growth (Fig. 8C). As (parts of) the C-terminus may act as an auto-inhibitory domain, the absence of survival of the yeast YAK2 strain with only a complete CHA1 expressed may be due to auto-inhibitory-induced inactivity of the H^+-ATPase. To verify this hypothesis, 7 different lengths of C-terminal truncations were also tested under the same condition (ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, ΔC887). Two tested mutants (ΔC908 and ΔC898)

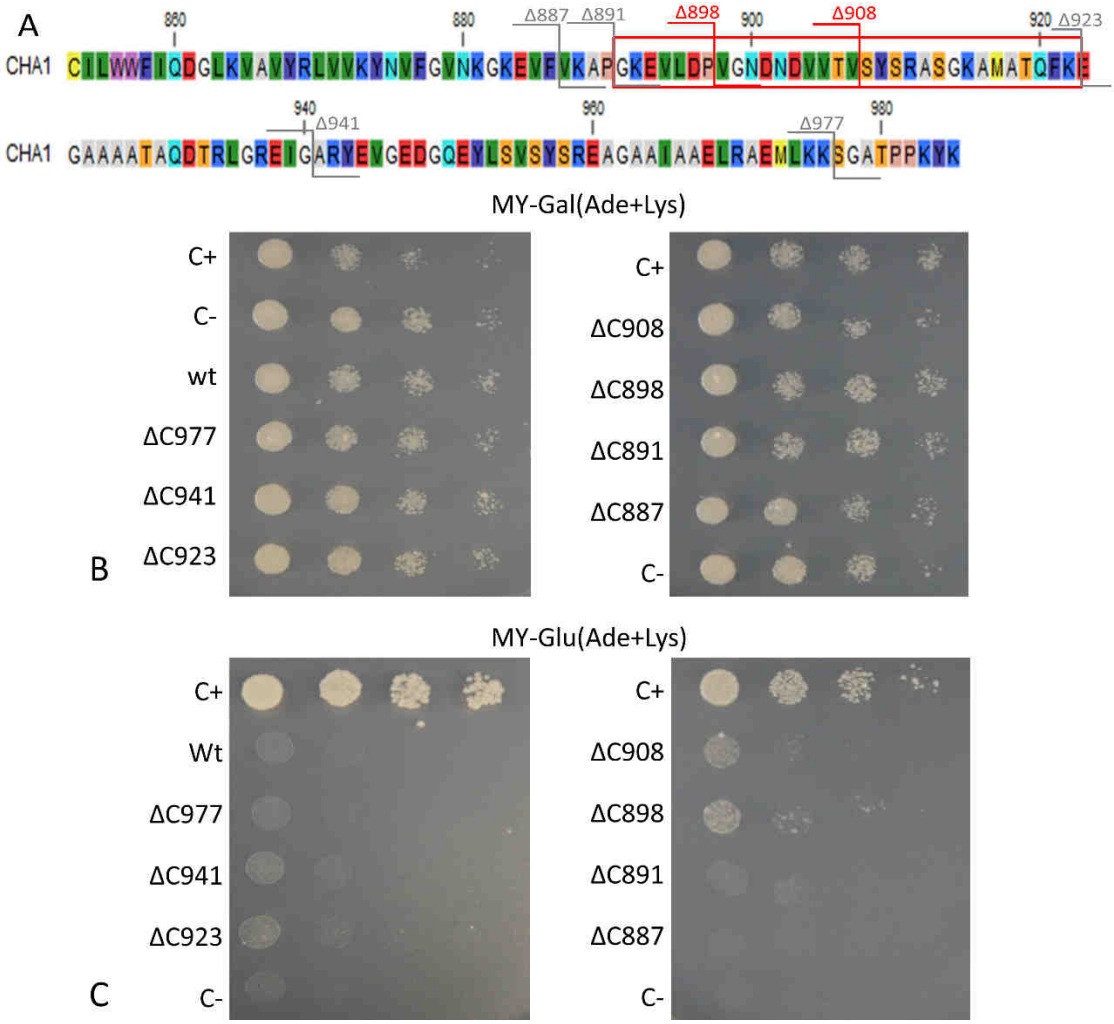

Figure 8. (A) C-terminus of CHA1. Red numbers and cutting lines indicate the positions in CHA1 from where C-terminal deletions where made that rescue the null mutant yeast strain. Gray numbers and cutting lines indicate the truncation sites of CHA1 that failed to rescue the null-mutant yeast strain. The red box indicates the potential regulation domain. Different residues are marked with different background colors. (B, C) Complementation assay of wild-type and 7 C-terminally truncated versions of CHA1 (ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, ΔC887). Transformants were serially diluted and dropped onto solid selection medium (MY medium addition with adenine, lysine) with either galactose (Gal) or glucose (Glu). (B) Yeast YAK2 strains grow on Gal-medium expressing both yeast PMA1 (under the control of Gal1 promotor) and heterologous CHA1 (under the control of yeast PMA1 promotor, indicated wt), C-terminal truncated genes (under the control of yeast PMA1 promotor, indicated ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, and ΔC887, respectively) together with positive (C+) and negative (C-) control. (C) Same yeast strains as in B (indicated wt, ΔC977, ΔC941, ΔC923, ΔC908, ΔC898, ΔC891, and ΔC887, respectively) grown on Glu-medium without galactose), only expressing heterologous CHA1 and truncated versions under control of the yeast PMA1 promotor. Positive (C+) and negative (C-) control are included as well.

showed the capacity to support the growth of yeast, though at a lower level as compared to the positive control with *Nicotiana plumbaginifolia* proton pump *pma2* gene (C+) (Fig. 8C). The truncation of the last 87 amino acids (ΔC898) conferred the highest rescue ability to the *CHA1* gene (Fig. 8C).

Compared to the higher plant proton pumps expressed in yeast systems, CHA1 presented a similar complementation assay result as Arabidopsis AHA2 (Palmgren and Christensen, 1993). Also, here the wildtype gene barely maintains the growth of the yeast, while the mutants of certain length C-terminal deletion seem to boost the activity of the pump and are able to partially support the growth of the null mutant yeast (Fig. 8). In conclusion, CHA1 can function as a proton pump in yeast provided that part of the C-terminus is removed. This suggests that the C-terminal of CHA1 (in particular, between Gly891 and Glu923) harbors a regulatory (auto-inhibition) domain. Comparing the C-terminal domain of CHA1 with the C-terminal of fungi and higher plants, no similar functional domains could be found (Fig. S2). Also, the plant H^+-ATPases classical 14-3-3 binding motif could not be detected. This opens the question of how CHA1 is regulated through the C-terminal, which remains an interesting target for further studies.

CHA1 localization in yeast (N-terminal GFP fusion)

It was shown for Arabidopsis AHA1-3 expression in the yeast system that, although all the plant proton pumps were successfully synthesized in this heterologous system, some failed to enter the secretory pathway and thus to be properly targeted at the PM. This seemed to be the most likely cause of unsuccessful complementation (Villalba et al., 1992; Palmgren et al., 1993). Since a similar situation may be true for CHA1, we investigated this possibility by expressing an N-terminal GFP-CHA1 fusion in the yeast system.

Based on the analysis of three independent transformations, we conclude that GFP-CHA1 expressed in the yeast BY4743 shows strong signals on plasma membrane and on some cytoplasmic membrane system. In contrast to the visible continuous circle observed for the PM-localized yeast PMA1 (Mason et al., 2006), GFP-CHA1 showed a punctured circle representing PM-localized fusion protein, and a strong perinuclear signal, most likely representing ER-localized fusion protein (Fig. 9A). The GFP-CHA1 versions with C-terminal deletions ΔC898 (which can support the growth of yeast) showed the same localizations as the wildtype CHA1 (Fig. 9B), whereas the non-rescue version

ΔC887 showed the PM signal in a few cells, while most cells showed a speckled cytoplasmic GFP signal (Fig. 9C).

We further tested the N-terminal truncations of CHA1 based on the predicted transmembrane segments as in figure 5, truncation of the first 46 amino acid at N-terminal cytoplasmic domain (ΔN46) and truncation of the first 55 amino acid (ΔN55) including the whole N-terminal cytoplasmic domain and part of first transmembrane helix (M1). ΔN46 showed the same pattern as the wildtype CHA1 gene, while further truncation (incomplete M1) clearly has a negative influence on the protein targeting or stability, thus a cytoplasmic staining pattern as with ΔC887 could also be seen with ΔN55 (Fig. 9D and E).

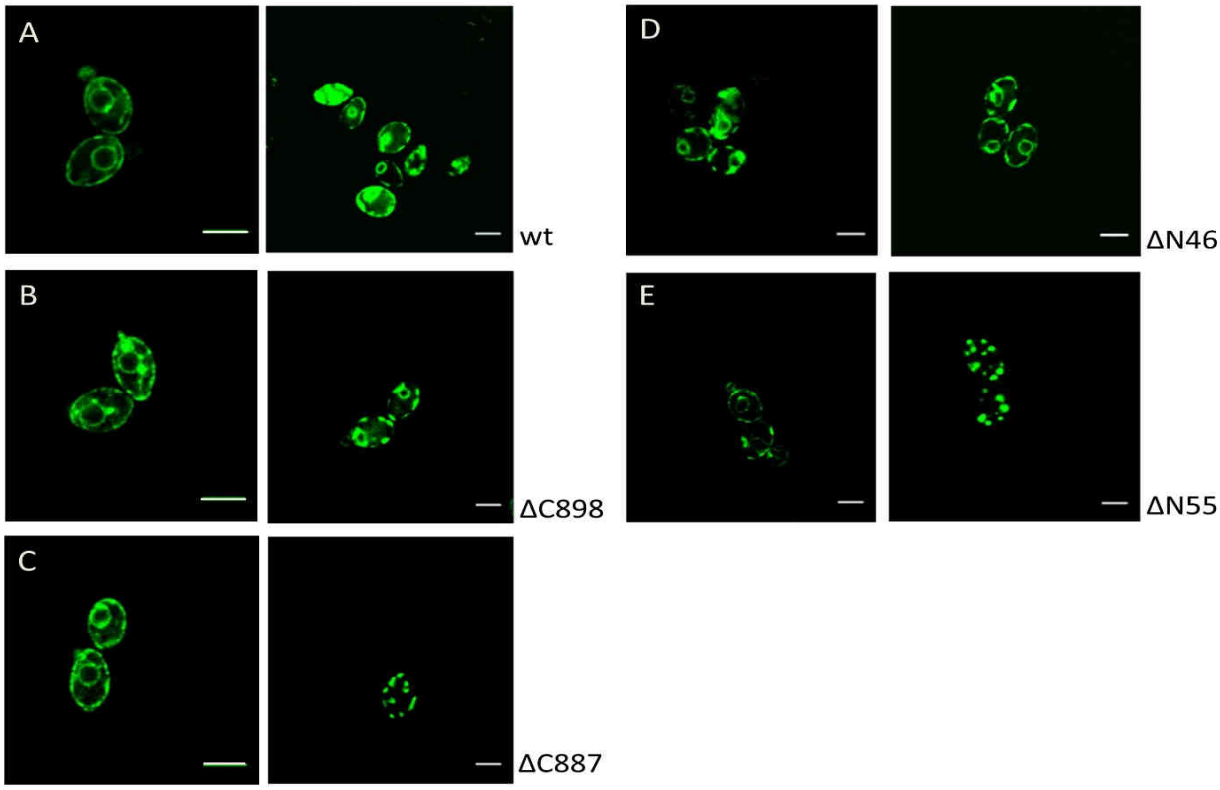

Figure 9. Subcellular localization of N-terminal GFP-CHA1 fusion protein and its N/C-terminal truncated versions in yeast BY4743 strains. Two identical localization samples of each line are presented from 3 independent transformations. (A) Wild-type YFP-CHA1 shows uneven PM and perinuclear localization. (B) The C-terminal truncated versionΔC898-CHA1 also shows uneven-PM and perinuclear localization. (C) C-terminal truncated version ΔC8887-CHA1 shows either uneven-PM, perinuclear localization or a punctate cytoplasmic-pattern. (D) N-terminal truncation of ΔN46-CHA1 shows the same uneven-PM and perinuclear localization. (E) N-terminal truncation of ΔN55-CHA1 shows a weak PM signal in a small portion of cells, while most of the cells shows punctate cytoplasmic pattern. Scale bars, 5μm.

In conclusion, CHA1 can be synthesized and is likely transported to the yeast plasma membrane, but it is not able to support the yeast growth. With a C-terminal deletion of 87 amino acids, CHA1ΔC898 is transported to the PM and is also sufficiently active in the yeast cells to partially compensate for the loss of the yeast proton pumps. This indicates that the C-terminal domain of CHA1 functions as an (auto)inhibitor of the pump activity, at least in the yeast system, and this inhibition cannot be eliminated by the yeast regulation system. Biochemical experiments are necessary to identify the enzymatic properties of the CHA1 with/without C-terminal truncations, such as the transport kinetics, pH profile, regulation mechanisms. Also, it seems that the integrity of the first transmembrane helix and ~20-30 amino acids after the last transmembrane helix are essential for the proper targeting and stability of the protein. Any sabotage may cause it to become trapped in cytoplasmic bodies (Mason et al, 2006 & 2014).

CHA1 localization in plant protoplast (N-terminal fusion with YFP)

To study the expression and sub-cellular localization of CHA1 in plants, an N-terminal YFP-CHA1 fusion was expressed in *Arabidopsis thaliana* Col-0 protoplasts from the viral *35S* promoter (*p35S::YFP-CHA1*). In three independent transformation experiments YFP-CHA1 showed strong cytoplasmic localization, with a relatively weaker signal on the plasma membrane (Fig. 10A). Mutant fusion proteins with a truncated C-terminus (ΔC887, ΔC898, ΔC941) showed the same localization as the wild-type –YFP-CHA1 protein (Fig. 10B, C, D).

Even though the plasma membrane localization signals were not so stable or strong in the protoplasts, they indicated that at least part of the produced YFP-CHA1 protein is correctly secreted to the PM. The reason for the strong internal signal is unclear, but it suggests that either biosynthetic secretion is inefficient, or that the fusion protein is readily internalized.

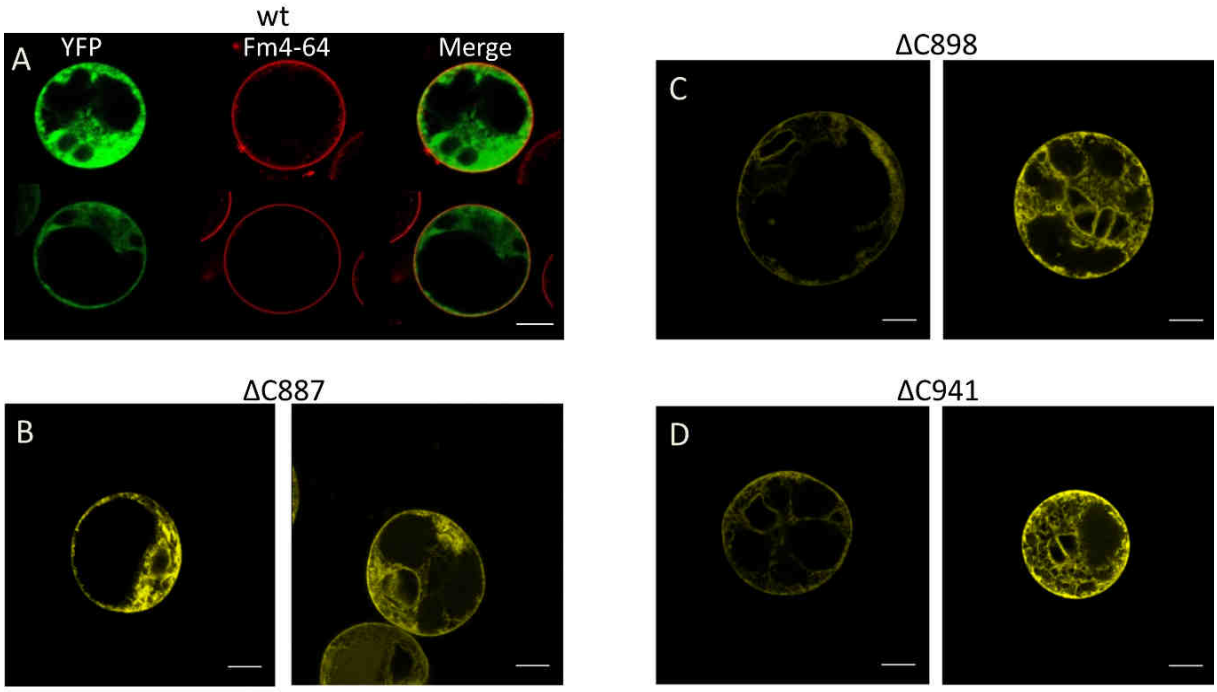

Figure 10. Localization of N-terminal YFP-CHA1 fusion and C-terminally truncated mutant verions in *Arabidopsis thaliana* Col-0 protoplasts. (A) two representative cells expressing N-terminal YFP fusion with wildtype CHA1 in green (artificial color, indicated YFP), Fm4-64 plasma membrane dye in red (indicated Fm4-64) and the merged picture of both (indicated Merge). (B) Two representative cells expressing N-terminal YFP fusion with C-terminal truncated CHA1-ΔC887, with both PM and ER localization. (C) Two representative cells expressing N-terminal YFP fusion with C-terminal truncated CHA1-ΔC898, with both PM and ER localization. (D) Two representative cells expressing N-terminal YFP fusion with C-terminal truncated CHA1-ΔC941, with both PM and ER localization. Scale bars: 10μm.

Summary

A potential PM H^+-ATPase (CHA1) was isolated from *Chara braunii*. Sequence analysis indicated it as a P3A ATPase, with the diversity in the regulation domain and the proton transport cavity, which showed a new perspective in the PM H^+-ATPases evolution pattern. Functional and mutational studies need to be carry out to confirm its biogenesis characters.

By complementation in yeast we could show that CHA1 has proton pump functionality, as it can partially rescue the proton pump-lacking yeast strain. This activity could only be found in a C-terminal truncated version of the protein, although both the wild-type and the truncated versions are targeted to the plasma membrane. This was shown in an assay using N-terminal GFP fusion to the protein. This suggests that CHA1 regulation is prone to a C-terminal localized auto-inhibitory process, that can be circumvented by deletion.

Although further truncation at the C-terminal of CHA1 disrupts the plasma membrane localization, which is a prerequisite for a functional pump. Also, in Arabidopsis protoplasts the wild-type CHA1 protein and the C-terminal deleted proteins are expressed in the plasma membrane. However, the details of the regulation mechanism of CHA1 still remain unclear. More biochemical experiments with the wild type, single point mutations and N/C-terminal truncations should be carried out *in vitro* to profile the enzymatic properties of the pump. Expressing fluorescently-labelled CHA1 in *Chara* internodal cells would be essential to reveal the possibly role of CHA1 in the mechanism of the pH banding pattern in *Chara* species: different level of pump activation or different densities at the plasma membrane bands.

Acknowledgement

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Table 1 primers used for gene isolation

Primer name	5'-3' DNA Sequence
1405_F0	ATGGGGCAGGAGGAAGAGCGTAAGGGC
1405_R0	TCACTTGTGCTTGCGCGGCGGCGGC
4956_F0	ATGCCTCTCAAGAGTAACGGCG
4956_R0	TTACTTGGCTCGCGTATCCATCGAGCG
181b_F0	ATGGGGCACGAAGACGGGGGTAAGGG
181b_R0	TCACTTCTTCTTCGCCTGGTAGAGCGC
PPs F1	GCGGGAATGAACGTGCTGTGC
PPs R1	TGGAAGATGCACCTGGCCAC
181b_CT1-T1	GACTTCCTGACTCTCTTC
181b_CT1-T2	CGATGGGTTGGTTCTGGA
181b_CT1-T3	CTCATCTCTCTCGGCATCT
181b_CT2-T1	GACTTCCTGACTCTCTTC
181b_CT2-T2	CGATGGGTTGGTTCTGGA
181b_CT2-T3	CTCATCTCTCTCGGCATCT
181b_NT-T1	CCTTGCCATCCCTCTTTA
181b_NT-T2	GCATTACCTGCCTTCGTC
181b_NT-T3	GATAAGGATGGGCATAGG
AD1	NTCGASTWTSGWGTT
AD2	NGTCGASWGANAWGAA
AD3	WGTGNAGWANCANAGA
181_b F1	CGACCTCTGCGAGAACAAGCGG
181_b F2	CAAGGAGAGGTGGAGGCGACC
181_b F3	GGCAAGGTGCAGACCATCG
181_b F4	GAAGGTGACCCCCAGTTGG
181_b F5	GGGATCCACCTGCGTGCAAGG
181_b F6	GGATGCTCGGTGGGTATGACCGG
181_b R2	CTGGATAACCATCTTGTTCAGCG
181_b RP3	GAGATGCCGAGAGAGATGA
181_b CTR	CTACTTGTACTTGGGCGGCGTT

Table 2 primers used for plasmids construction

Primer name	5'-3' DNA Sequence	Purpose
GF_CHA1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGCAGGATGAGGGGGGTA	pART7(35S)YFP-CHA1
GR_CHA1	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTTGTACTTGGGCGGCGT	
GR_ ΔC887	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGAACACCTCCTTGCCCTT	
GR_ ΔC898	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATGGGTCAAGCACCTCCTT	
GR_ ΔC941	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGCCGATTTCCCTTCCCAG	
CHA1_F_SpeI	GACTAGTATGGGGCAGGATGAGGGGGGTAAG	pUG34-GFP-CHA1
ΔN46_F_SpeI	GACTAGTATGGTCATCTTCTTGCGGCAG	
ΔN55_F_SpeI	GACTAGTATGCCTATGCCCATCCTTATCT	
CHA1_R_SalI	GCGTCGACCTACTTGTACTTGGGCGGCGTTGCG	
ΔC887_R_SalI	GCGTCGACGAACACCTCCTTGCCCTT	
ΔC898_R_SalI	GCGTCGACTGGGTCAAGCACCTCCTT	
CHA1_F_PstI	GCTGCAGATGGGGCAGGATGAGGGGGGTAAG	2μp(PMA1)CHA1
CHA1_R_HindIII	CCAAGCTTCTACTTGTACTTGGGCGGCGT	
ΔC887_R_HindIII	CCAAGCTTCTAGAACACCTCCTTGCCCTT	
ΔC891_R_SalI	GCGTCGACCTAAGGAGCCTTCACGAACAC	
ΔC898_R_HindIII	CCAAGCTTCTATGGGTCAAGCACCTCCTT	
ΔC908_R_SalI	GCGTCGACCTAGACGGTCACCACATCGTT	
ΔC923_R_SalI	GCGTCGACCTACTTGAACTGCGTAGCCAT	
ΔC941_R_HindIII	CCAAGCTTCTAGCCGATTTCCCTTCCCAG	
ΔC977_R_ SalI	GCGTCGACCTACTTCTTCAACATCTCGGCCCT	

References

Baunsgaard, L., Fuglsang, A.T., Jahn, T., Korthout, H.A.A.J., de Boer, A.H., Palmgren, M.G. (1998). The 14-3-3 proteins associate with the plant plasma membrane H^+-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. *Plant J.,* 13 (5): 661-671.

Beilby, M.J. and Casanova, M.T. (2014). The Physiology of Characean Cells. Springer, Berlin, Heidelberg.

Berecki, G. (2001). *Chara coralline*: an alternative model for ion channel research. PhD thesis, Leiden University, the Netherlands.

Buch-Pedersen, M.J., Venema, K., Serrano, R., Palmgren, M.G. (2000). Abolishment of Proton Pumping and Accumulation in the *E*1P Conformational State of a Plant Plasma Membrane H^+-ATPase by Substitution of a Conserved Aspartyl Residue in Transmembrane Segment 6. *J. Biol. Chem.,* 275 (50): 39167-39173.

Buch-Pedersen, M.J. and Palmgren, M.G. (2003). Conserved Asp684 in transmembrane segment M6 of the plant plasma membrane P-type proton pump AHA2 is a molecular determinant of proton translocation. *J. Biol. Chem.,* 278 (20): 17845-17851.

Buch-Pedersen, M.J., Pedersen, B.P., Veierskov, B., Nissen, P., Palmgren, M.G. (2009). Protons and how they are transported by proton pumps. *Eur. J. Physiol.,* 457:573-579.

Bulychev, A.A., Cherkashin, A.A., Rubin, A.B., Vredenberg, W.J., Zykov, V.S., Muller, S.C. (2001). Comparative study on photosynthetic activity of chloroplasts in acid and alkaline zones of *Chara corallina. Bioelectrochemistry*, 53: 225-232.

De Kerchove d'Exaerde, A., Supply, P., Dufour, J., Bogaerts, P., Thines, D., Goffeau, A., Boutry, M. (1995). Functional complementation fo a null mutation of the yeast *Saccharomyces cerevisiae* plasma membrane H^+-ATPase by a plant H^+-ATPase gene. *J. Biol. Chem.,* 270 (40): 23828-23837.

Duby, G. and Boutry, M. (2009). The plant plasma membrane proton pump ATPase: a highly regulated P-type ATPase with multiple physiological roles. *Eur. J. Physiol.*, 457: 645-655.

Dutra, M.B., Ambesi, A., Slayman, C.W. (1998). Structure-function relationships in membrane segment 5 of the yeast Pma1 H^+-ATPase. *J. Biol. Chem.,* 273 (28): 17411-17417.

Ekberg, K., Pedersen, B.P., Sorensen, D.M., Nielsen, A.K., Veierskov, B., Nissen, P., Palmgren, M.G., Buch-Pedersen, M.J. (2010). Structural identification of cation binding pockets in the plasma membrane proton pump. *PNAS*, 107 (50): 21400-21405.

Ekberg, K., Wielandt, A.G., Buch-Pedersen, M.J., Palmgren, M.G. (2013). A conserved asparagine in a P-type proton pump is required for efficient gating of protons. *J. Biol. Chem.,* 288 (14): 9610-9618.

Fisahn, J. and Lucas, W.J. (1995). Spatial ortanization of transport domains and subdomain formation in the plasma membrane of *Chara corallina*. *J. Membr. Biol.*, 147: 275-281.

Foissner, I. and Wasteneys, G.O. (2014). Characean internodal cells as a model system for the study of cell organization. *Int. Rev. Cell Mol. Biol.*, 311: 307-364.

Fuglsang, A.T., Visconti, S., Drumm, K., Jahn, T., Stensballe, A., Mattei, B., et al. (1999). Binding of 14-3-3 protein to the plasma membrane H^+-ATPase AHA2 involves the three C-terminal residues Tyr^{946}-Thr-Val and reguires phosphorylation of Thr^{947}. *J. Biol. Chem.*, 274 (51): 36774-36780.

Gleave, A.P. (1992). A verstile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.*, 20: 1203-1207.

Gupta, S.S., De Witt, N.D., Allen, K.E., Slayman, C.W. (1998). Evidence for a salt bridge between transmembrane segments 5 and 6 of the yeast plasma-membrane H+-ATPase. *J. Biol. Chem.*, 273 (51): 34328-34334.

Hohm, T., Dermarsy, E., Quan, C., Petrolati, LA., Preuten, T., Vernoux, T., Bergmann, S., Frankhauser, C. (2014). Plasma membrane H^+-ATPase regulation is required for auxin gradient formation preceding phototropic growth. *Mol. Syst. Boil.*, 10: 751.

Hori, K., Maruyama, F., Fujisawa, T., Togashi, T., Ymamoto, N., Seo, M., Sato, S., et al. (2014). *Klebsormidium flaccidium* genome reveals primary factors for plant terrestrial adaptation. *Nature Comm.*, 5: 3978.

Kuhlbrandt, W. (2004). Biology, structure and mechanism of P-type ATPases. *Nature*, 5: 282-295.

Lucas, W.J. and Smith, F.A. (1973). The formation of alkaline and acid regions at the surface of *Chara corallina* cells. *J. Exp. Bot.*, 24: 1-14.

Lucas, W.J. (1979). Alkaline band formation in *Chara corallina*: Due to OH^- efflux or H^+ influx? *Plant Physiol.*, 63: 248-254.

Lucas, W.J. (1982) Mechanism of acquisition of exogenous bicarbonate by internodal cells of *Chara corallina*. *Planta*, 156:181-192.

Luo, H., Morsomme, P., Boutry, M. (1999). The two major types of plant plasma membrane H^+-ATPases show different enzymatic properties and confer differential pH sensitivity of yeast growth. *Plant Physiol.*, 119: 627-634.

Masaki Okumura, Koji Takahashi, Shin-ichiro Inoue & Toshinori Kinoshita. (2012). Evolutionary appearance of the plasma membrane H^+-ATPase containing a penultimate threonine in the bryophyte. *Plant Signal Behav.*, 7 (8): 979-982.

Mason, A.B., Allen, K.E., Slayman, C.W. (2006). Effects of C-terminal truncations on trafficking of the yeast plasma membrane H^+-ATPase. *J. Biol. Chem.*, 281(33): 23887-23898.

Mason, A.B., Allen, K.E., Slayman, C.W. (2014). C-terminal truncations of the *Saccharomyces cerevisiae* PMA1 H^+-ATPase have major impacts on protein conformation, trafficking, quality control, and function. *Eukaryot. Cell*, 13 (1): 43-52.

Moriau, L., Bogaerts, P., Jonniaux, J., Boutry, M. (1993). Identification and characterization of a second plasma membrane H^+-ATPase gene subfamily in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.*, 21: 955-963.

Morsomme, P., Slayman, C.W., Goffeau, A. (2000). Mutagenic study of the structure, function and biogenesis of the plasma membrane H^+-ATPase. *Biochim. Biophys. Acta*, 1469: 133-157.

Morth, J.P., Pedersen, B.P., Buch-Pedersen, M.J., Andersen, J.P., Vilsen, B., Palmgren, M.G., Nissen, P. (2011). A structural overview of the plasma membrane Na^+, K^+-ATPase and H^+-ATPase ion pumps. *Nature*, 12:60-70.

Oecking, C. and Hagemann, K. (1999). Association of 14-3-3 proteins with the C-terminal autoinhibitory domain of the plant plasma-membrane H^+-ATPase generates a fusicoccin-binding complex. *Planta*, 207(3): 480-482.

Okumura, M., Inoue, S., Takahashi, K., Ishizaki, K., Kohchi, T., Kinoshita, T. (2012a). Characterization of the plasma membrane H^+-ATPase in the liverwort *Marchantia polymorpha*. *Plant Physiol.*, 159: 826-834.

Okumura, M., Takahashi, K., Inoue, S., Kinoshita, T. (2012b). Evolutionary appearance of the plasma membrane H^+-ATPase containing a penultimate threonine in the bryophyte. *Plant Signal. Behav.*, 7(8): 979-982.

Palmgren, M.G., Sommarin, M., Serrano, R., Larsson, C. (1991). Identification of an autioinhibitiory domain in the C-terminal region of the plant plasma membrane H^+-ATPases. *J. Biol. Chem.*, 266 (30): 20470-20475.

Palmgren, M.G. (2001). Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 52: 817-845.

Pedersen, B.P., Buch-Pedersen, M.J., Morth, J.P., Palmgren, M.G., Nissen, P. (2007). Crystal structure of the plasma membrane proton pump. *Nature*, 450: 1111-1115

Pedersen, C.N.S., Axelsen, K.B., Harper, J.F., Palmgren, M.G. (2012). Evolution of plant P-type ATPases. *Front. Plant Sci.*, 3:31

Portillo, F. (2000). Regulation of plasma membrane H^+-ATPase in fungi and plants. *Biochim. Biophys. Acta*, 1469: 31-42.

Regenberg, B., Villalba, J.M., Lanfermeijer, F.C., Palmgren, M.G. (1995). C-terminal deletion analysis of plant plasma membrane H^+-ATPase: yeast as a model system for solute transport across the plant plasma membrane. *The Plant Cell*, 7: 1655-1666.

Sakalis, P.A. (2013). Visualizing virulence proteins and their translocation into the host during *Agobacterium*-meiated transformation. PhD thesis, Leiden University, the Netherlands.

Serrano, R. (1989). Structure and function of plasma membrane ATPase. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 40: 61-94.

Shimmen, T., Mimura, T., Kikuyama, M., Tazawa, M. (1994). Characean cells as a tool for studying electrophysiological characteristics of plant cells. *Cell Struct. Funct.*, 19: 263-278.

Shimmen, T. and Wakabayashi, A. (2008). Involvement of membrane potential in alkaline band formation by internodal cells of *Chara corallina*. *Plant Cell Physiol.*, 49(10): 1614-1620.

Speth, C., Jaspert, N., Marcon, C., Oecking, C. (1997). Regulation of the plant plasma membrane H^+-ATPase by its C-terminal domain: what do we know for sure? *Eur. J. Cell Biol.* ,89: 145-151.

Sze, H., Li, X., Palmgren, M.G. (1999). Energization of plant cell membranes by H^+-Pumping ATPases: regulation and biosynthesis. *The Plant Cell*, 11: 677-689.

Tazawa, M. and Shimmen, T. (2001) How characean cells have contributed to the progress of plant membrane biophysics. *Aust. J. Plant Physiol.*, 28: 523–539.

Villalba, J.M., Palmgren, M.G., Berberian, G.E., Ferguson, C., Serrano, R. (1992). Functional expression of plant plasma membrane H^+-ATPase in yeast endoplasmic reticulum. *J. Biol. Chem.*, 267 (17): 12341-12349.

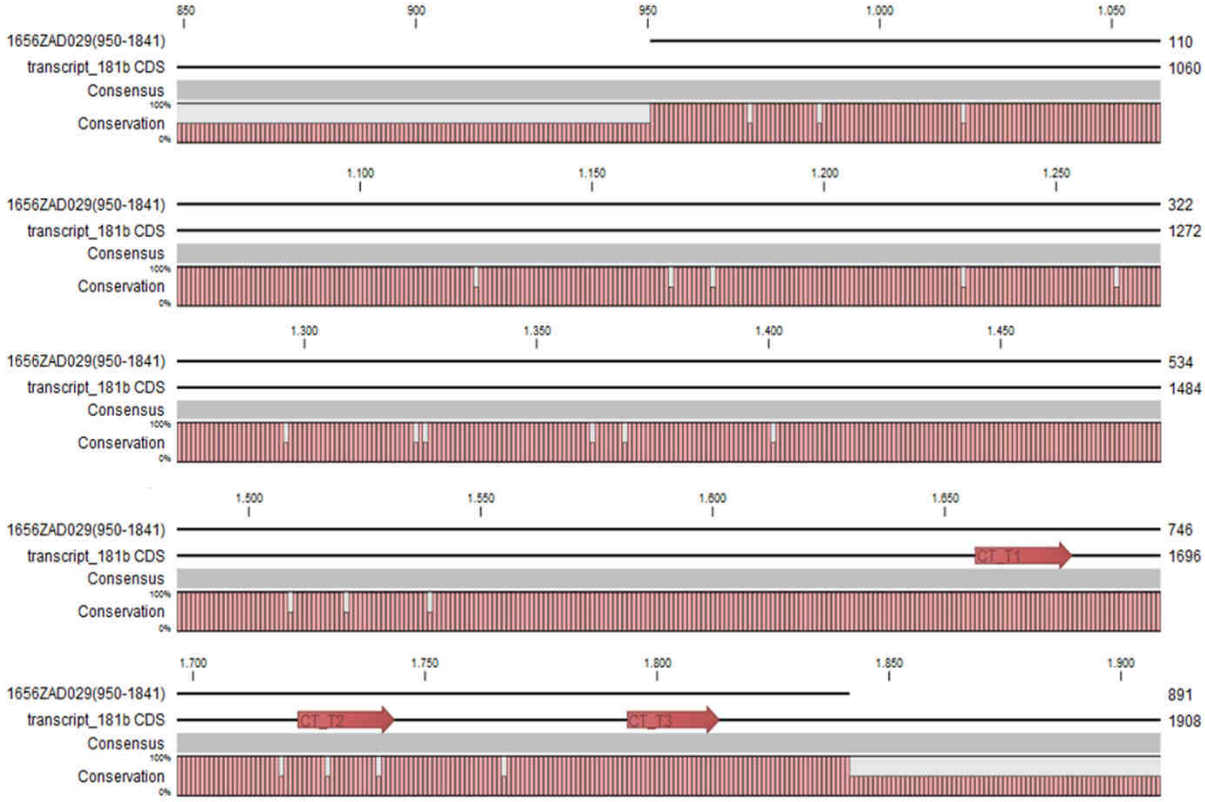
Wielandt, A.G., Pedersen, J.T., Falhof, J., Kemmer, G.C., Lund, A., Ekberg, K., Fuglsang, A.T., et al. (2015). Specific activation of the plant P-type plasma membrane H^+-ATPase by lysophospholipids depends on the autoinhibitory N- and C- terminal domains. *J. Biol. Chem.*, 290 (26): 16281-16291.

Wolf, A.H., Slayman, C.W., Gradmann, D. (1995). Primary structure of the plasma membrane H^+-ATPase from the halotolerant alga *Dunaliella bioculata*. *Plant Mol. Biol.*, 28: 657-666.

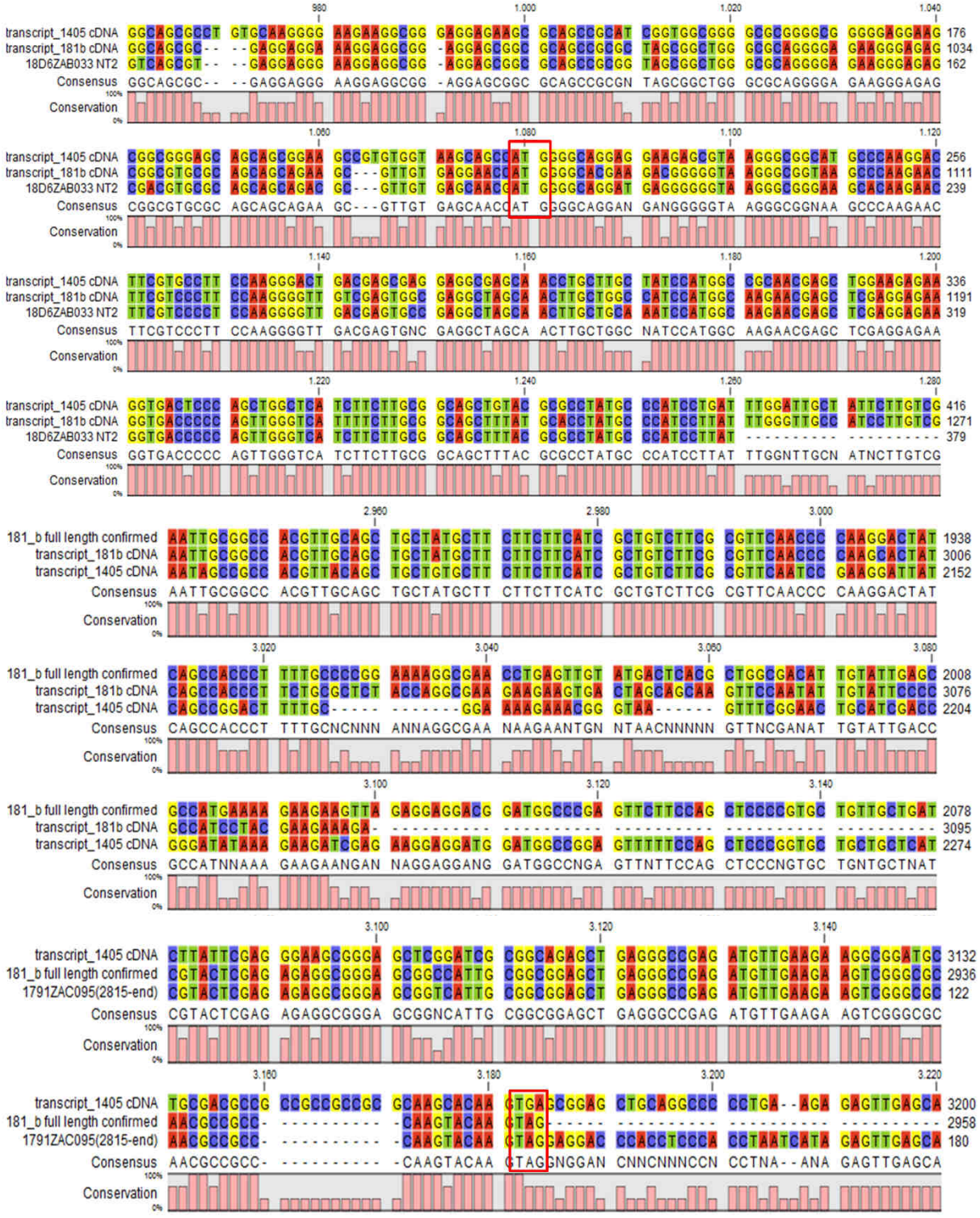
Zhang, S. and van Duijn, B. (2014). Cellular auxin transport in algae. *Plants*, 3, 58–69.

Zhang, S., de Boer, A.H., van Duijn, B. (2016). Auxin effects on ion transport in *Chara corallina*. *J. Plant Phyiol.*, 193: 37-44.

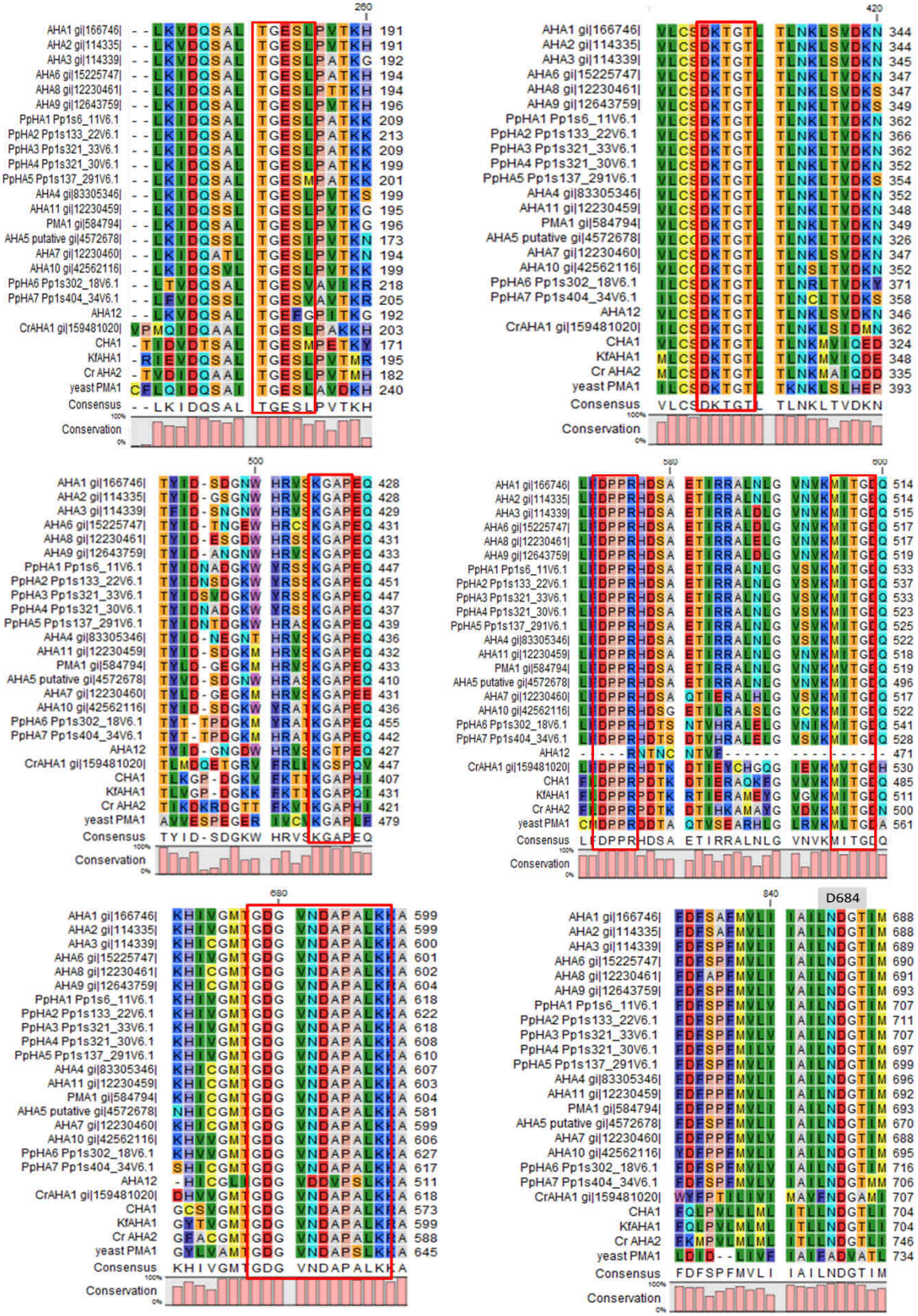
Zhang, X. (2016). Functional analysis of *Agrobacterium tumefaciens* virulence protein VirD5. PhD thesis. Leiden University. Leiden, the Netherlands.



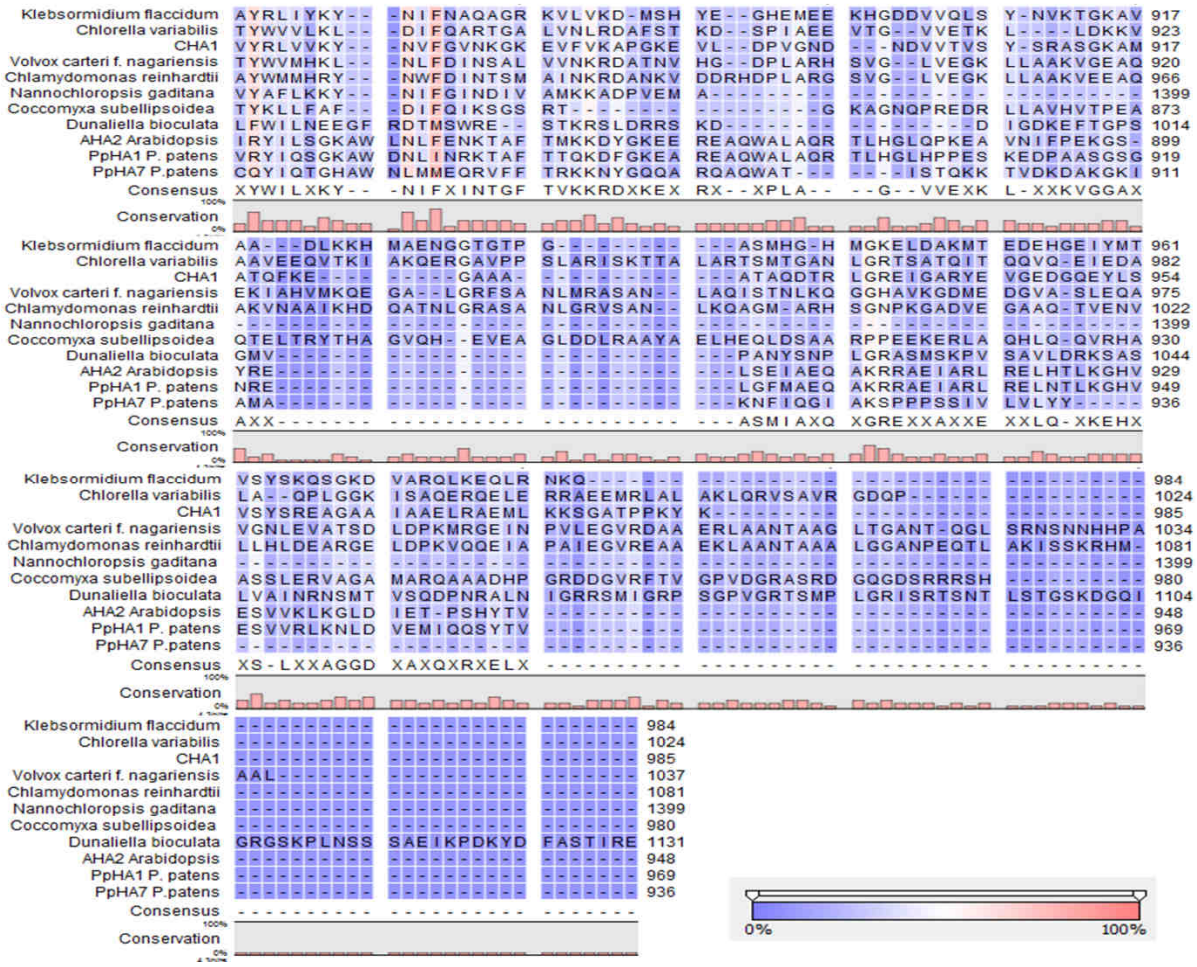
Supplementary figure 1. Alignment of transcript_181b CDS with PCR fragment of the conserved part (with primers PPs F1 and PPs R1). With red arrows indicate the primers designed for C-terminal tail PCR.



Supplementary figure 2. Details of alignment of PCR assembling result with transcript_181b and transcript_1405. Start codon and stop codon are high-light in red box.



Supplementary figure 3. CHA1 alignment with other PM H^+-ATPases. The 6 specific conserved domains for P type ATPases in red boxes. H^+ acceptor/donor Asp684 (AtAHA2 numbering) indicated on top of the alignment in gray box.



Supplementary Figure 4. Alignment of PM H^+-ATPAses from other algae and protists at the C-termial. Color bar below indicates the conservation degrees of the alignments.