The Chara plasma membrane system : an ancestral model for plasma membrane transport in plant cells

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Chapter 1

General introduction

Algae

Algae are a diverse group of aquatic photosynthetic organisms. They are not closely related with each other in an evolutionary perspective, and they could be unicellular or multicellular, microscopic or giant, but are not highly differentiated as the plants (Barsanti and Gualtieri, 2014). The taxonomy of algae is yet contentious and undergoes rapid changes as new molecular information is discovered. Nevertheless, based on the different chloroplast pigments (e.g. chlorophylls, carotenoids and phycobiliproteins), algae can be divided into three main groups, green, red, and brown. They can be found almost everywhere in the world within sea water or fresh water systems, providing food for the other aquatic lives and contributing to a great amount of the oxygen on earth. With the development of science and technology, algae reveal an unparalleled potential and enormous value in food and energy production, environment management, as well as pharmaceutical and industrial usage.

Green algae

There are two great clades of green algea, the Chlorophyte and Charophyte. The Chlorophytes are found both in marine and freshwater environment, while the Charophytes are exclusively living in freshwater. The Charophytes are considered to be the closest lineages of land plants (Embryophyte), which consist of six distinct classes based on the most recent phylogenetic opinion: three early divergent classes including Mesotigmatophyceae, Chlorokybophyceae and Klebsormidiophyceae, and three late divergent classes including Charophyceae, Coleochaetophyceae and Zygnematophyceae (Delwiche and Cooper, 2015; Zhong et al., 2015; Domozych et al., 2016).

Chara

Chara is a genus of multicellular Charophyte green algae belonging to the class of Charophyceae, in the family of Characeae. It usually forms dense meadows at the lower level of littoral zones of oligotrophic and mesotrophic water bodies (Scribaila and Alix, 2010). The thallus of *Chara* is essentially filamentous, but highly resembles land plants and other submerged plants, with a long photosynthetic stem-like up-ground axes (consisting of internodal and nodal cells, end by end), whorls of leaf-like branchlets growing around the nodal cells, and colorless root-like rhizoids anchoring in the soil (Beilby and Casanova, 2014). The most remarkable feature in the morphology of *Chara* is the elongated cylindrical internodal cell, which can reach over 10 cm in length and 1 mm or more in diameter (Braun et al., 2007). The axis of internodal cells of most *Chara* species is covered by a cortex layer of small, linearly aligned cells. In contrast to this, a few species are ecorticate (without cortex layer cells), e.g. *Chara corallina*, *Chara australis* and *Chara braunii*. These species are commonly used in physiological and cell biological studies or are used as model system for different other interests, e.g. auxin polar transport, cellular organization (Boot et al., 2012; Beilby and Casanova, 2014; Beilby, 2016).

pH banding formation along *Chara* internodal cells

Under the stimulation by light, ecorticate *Chara* internodal cells (both axis and branch) can form a distinguished pH banding pattern along the long axis, with small sharp alkaline (pH 8.5-9.5) regions and bigger, more uniform, acid (pH around 5.5) regions (Lucas and Smith, 1973). In general, correlated to the pH banding, it is reported that the chloroplasts in the acid regions are larger with a higher quantum yield and efficiency of carbon fixation (Price et al., 1985; Bulychev et al., 2001). The quantity and size of the charasomes (convoluted plasma membrane domains, a special membrane structure only found in *Chara* algae) and mitochondria are also dramatically bigger in the acidic regions as compared to the alkaline regions (Franceschi and Lucas, 1980; Schmolzer et al., 2011; Foissner et al., 2014). Furthermore, the cell cytoskeleton, in particular the microtubule network, is organized differently among the bandings (Wasteneys and Williamson, 1992). Last but not least, the cell elongation is mainly restricted to the acid regions while the cell wall at the alkaline regions is thicker (Metraux et al., 1980; Popper and Fry, 2003). This inhomogeneity on the one hand, was a kind of disadvantage for the electrophysiological studies carried out with this large cell system but on the other hand, it allowed for pattern formation as a new object of study using *Chara* as a model system (Beilby and Bisson, 2012). Based on a number of studies, the current model describing the banding pattern is shown in figure 1. The network that links the different processes and structures can be described as:

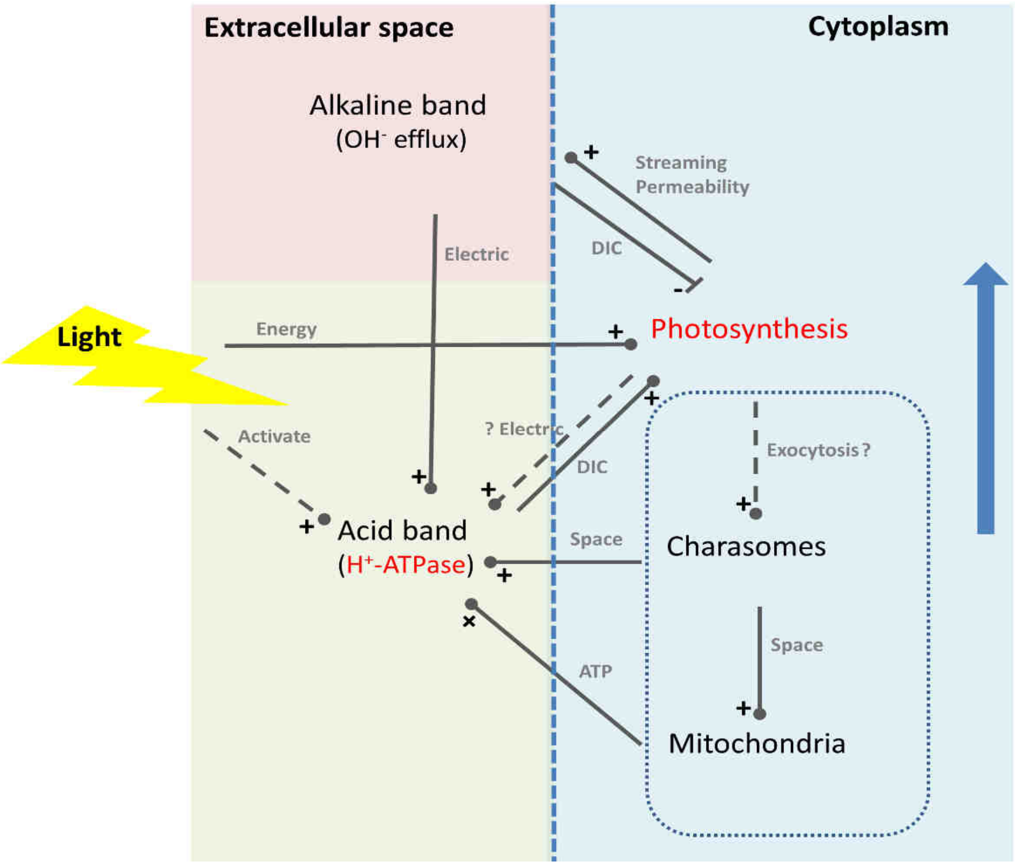

Figure 1. Components (cell structures, metabolic processes, etc.) which are involved in *Chara* pH banding phenomenon induced by light, and the possible relationship among all the components. Blue background indicates the cytoplasm, with the blue arrow indicating the direction of cytoplasmic streaming. The dashed box indicates a slow regulation system, consisting of mitochondria and charasomes. The dashed effect indicators refer to stimulations by unclear mechanisms.

1. *Chara* PM H^+-ATPases are triggered after illumination (above a threshold of light intensity) and start to pump out of the cell a great amount of H^+ through the cell membrane (Beilby and Bisson, 2012; Foissner and Wasteneys, 2014). Although light-induced H^+-ATPase activity in *Chara* is taken as a default fact, the regulation mechanism by light and the signal pathways behind it are unclear. Limited studies showed evidence for the regulation of the H^+-ATPases by ATP levels, photosynthetic electron flow, etc. (Tazawa et al., 1979; Tsutsui et al., 2001; Marten et al., 2010).
2. The resulting acidified microenvironment facilitates the uptake of dissolved inorganic carbon (DIC) from the environment, by both the diffusion of CO_2 and cotransport of HCO_3^- with H^+. The improved DIC uptake at the acid band increases the rate of photosynthesis at this location (Lucas et al., 1983; Beilby and Bisson, 2012).

3. The cytoplasmic streaming in the *Chara* internodal cell has been proposed as an important participant in the long-distance regulation and generation of spatial patterns of the photosynthesis system (Bulychev and Komarova, 2014). The by-products of photosynthesis, OH^- and H_2O_2 (under excessive irradiance), accumulate around the chloroplasts and are carried downstream by the cytoplasmic streaming. This facilitates the opening of H^+/OH^- permeable channels (resulting in the arising of an alkaline band/patch) by elevation of the cytoplasmic pH and further shifting of the cytoplasmic redox balance (Dodonova and Bulychev, 2011; Beilby and Bisson, 2012; Eremin et al., 2013). The pH raise in the alkaline band is suggested in turn to reduce the amount of membrane permeable CO_2 at this location, thus enhances the sensitivity of non-photochemical quenching (NPQ) to photosynthetic flux densities (PFD) and further promotes a stronger NPQ at the alkaline region (Krupenina and Bulychev, 2007; Eremin et al., 2013). As NPQ is an effective and harmless way to get rid of the excess light energy to minimize potential photo-damage at high light intensities (Kanazawa and Kramaer, 2002; Krupenina and Bulychev, 2007), therefore, the formation of alkaline bands is very likely to function as a self-regulating, balancing protection mechanism in response to fluctuating light and other environmental stimuli (Krupenina and Bulychev, 2007; Bulychev and komarova, 2014). This is supported by the finding that more bands appear with increasing light intensities (Lucas, 1975), and that no photo-inhibition was observed for *Chara* under intensive irradiation (Vieira Jr. and Necchi Jr., 2003; Schaible et al., 2012).
4. The elevated permeability for H^+/OH^- at the alkaline band would increase the membrane conductance and provide an extra load for the operation of H^+-ATPase in the adjoining acid regions, thus enhancing both the passive flux in the alkaline band and active H^+ extrusion in the acid band (Eremin et al., 2013; Bulychev and Komarova, 2014). This self-enhancing circulation, may offer an explanation for the phenomenon observed by Lucas (1975) who showed that the stabilized bands persist after the light intensity was reduced to a level below the threshold.
5. Besides the above fast regulation mechanisms which mainly rely on the electro-chemical fluxes and signals, there is a slower response in the form of the subcellular reorganization. This reorganization is the dynamic formation of charasomes at the acid bands that is driven by the

photosynthesis, and which is also positively feeding back to enhance the banding pattern (Schmoelzer et al., 2011; Foissner et al., 2015). The formation and gathering of charasomes are not necessary for the band formation, but the appealing of high density of charasomes at the acidic bands is found to be photosynthesis- and pH banding-dependent, which further leads to a stronger acidification due to the high densities of H^+-ATPases and mitochondria (providing energy for H^+-ATPases) in these convoluted areas (Foissner et al., 2015). Up to date, it is known that charasomes are formed by exocytosis of the trans-Golgi network (TGN) vesicles and by local inhibition of endocytosis. The charasomes degrade in the darkness by clathrin-dependent endocytosis (Foissner et al., 2015; Hoepflinger et al., 2017). However, the signals responsible for charasomes formation and degradation upon light stimulation are still unknown.

Plant hormones

The life of animals and plants is highly regulated by a system of signal molecules, which are called hormones. In plants, there are five major types of hormones (also known as phytohormones), including auxin, gibberellin, cytokinin, ethylene, and abscisic acid. Together or independently, these hormones are in charge of plant cell development, differentiation, tropism, reproduction, death and so on. Some of the phytohormones, such as auxin, are also found in algae, showing similar functions. But the knowledge related to the hormones in algae is rather scarce comparing to what has been studied referring to plants.

Auxin

Auxin is the first discovered and most studied plant hormone. It acts as the major regulator throughout the development of the whole plant. There are five endogenous auxins in plants, all with an aromatic ring and a carboxylic acid group. The most abundant and basic form of auxin is indole-3-acetic acid (IAA) (Fig. 2A) which functions as the predominant endogenous auxin. Due to the chemical lability of IAA in aqueous solution, there several synthetic auxin analogs are commonly used as substitutes in scientific research and commercial usage, including 1-napthaleneacetic acid (NAA) (Fig. 2B) and 2,4-dichlorophenoxyacetic acid (2,4-D). At the cellular level, auxin is known as the key element (trigger) in the classical "acid growth" theory, which involves the activity of the plasma membrane H^+-ATPases (Hohm et al., 2014; Falhof et al., 2016). At levels of the plant, auxin controls the spatial patterns of embryophyte growth and development, responses to environmental stimuli like gravity and light (gravitropism and phototropism) through the establishment of auxin concentration maxima and gradients (Petrasek and Friml, 2009).

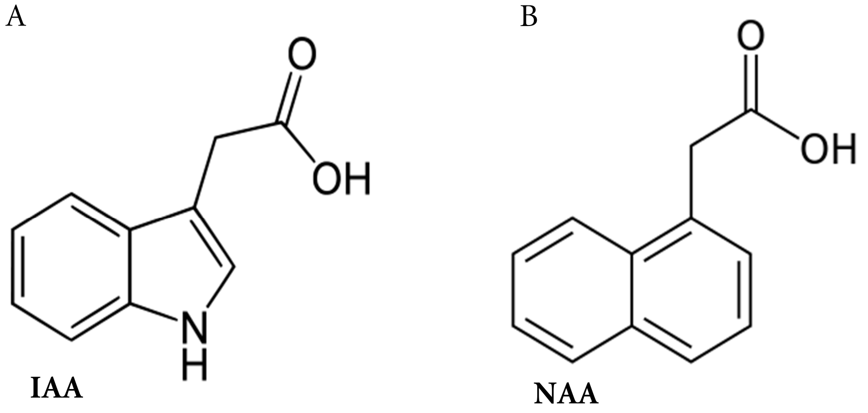

Figure 2. Chemical structure of two main auxin isoforms. (A) the main endogenous auxin. (B) a commonly used synthetic analog.

Auxin in Charophyte algae

Numerous studies of auxin have been carried out in plants (land plants in particular), while only in the recent decades, with the increasing availability of gene information of algae and interests in the evolutionary perspective, the study of auxin in green algae starts to cut a striking figure (Beilby, 2016; Harrison, 2016). Recent research demonstrated that a similar morphology strategy as in land plants could be seen in *Chara* algae. In this respect auxin regulated processes, such as the auxin regulated polar growth of rhizoids (Klämbt et al., 1992), the apical dominance (Clabeaux and Bisson, 2009), polar auxin transport through the internodal cells (Boot et al., 2012), and polarized accumulation of PIN2-like proteins during spermatogenesis (Zabka et al., 2016), were reported for *Chara* algae. Analysis of expressed sequence tag libraries of some Charophyte algae revealed the presence of some key proteins involved in the auxin signaling pathway, such as AUXIN/INDOLE-3-ACETIC ACID proteins and PIN-FORMED-LIKE proteins (de Smet et al., 2011).

Hence, there is a tempting hypothesis that the classical auxin machinery in plant might also be present (or partially present) in the plant-like *Chara* algae. Auxin related processes may be involved in the band formation model shown in figure 1. In this respect we can regard known features of auxin in plants:

1. Auxin can regulate the cell elongation by activating plasma membrane H$^+$-ATPase under different stimuli, e.g. light triggered phototropism;
2. Auxin can induce exocytosis and rapid synthesis of a high-turnover pool of plasma membrane (Hager et al., 1991);
3. Auxin induces local activation of plasma membrane H$^+$-ATPase which may cause a pH difference inside and outside of the cell, which further interferes with the auxin transport and gradient (Hohm et al., 2014).

An alternative model for *Chara* band formation without auxin

The above hypothesis is based on the known mechanisms in land plants, yet with information regarding to *Chara* algae, another alternative model (without direct involvement of auxin) seems to fit better. As proposed in figure 3, plasma membrane H$^+$-ATPase is activated by light through phototropin photoreceptors (Marten et al., 2010; Hohm et al., 2014). While phototropins further regulate the vesicle trafficking by the Golgi apparatus, which happens to be the origin of charasomes, instead of the endoplasmic reticulum originated exocytosis induced by auxin (Hager et al., 1991; Kong and Nagatani, 2008). Certainly, more genomic information and functional experiments are required to elucidate all elements involving in this hypothesis.

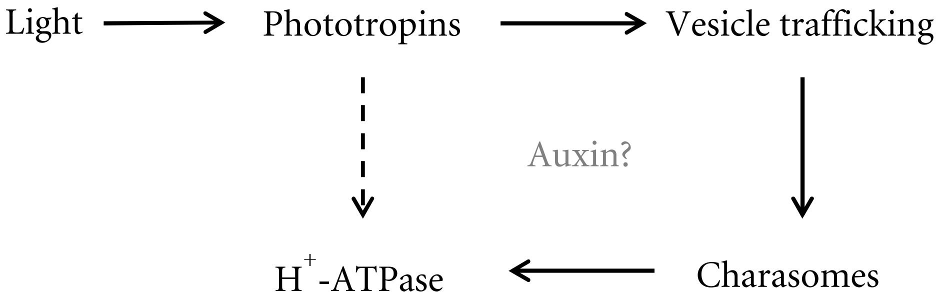

Figure 3. Hypothetic model of mechanism behind the *Chara* pH banding phenomenon. Solid arrow lines indicate that the regulation mechanisms behind have relative evidences in *Chara*, while the dash arrow line indicates potential relationship in *Chara* regarding to other non-closely related species.

P-type ATPases

P-type ATPases are a large, ubiquitous family of molecular pumps, characterized by phosphorylation (P) intermediating and ATP-hydrolysis driving. The family has a core of three cytoplasmic domains consisting of a nucleotide-binding (N) domain, an actuator (A) domain, and a phosphorylation (P) domain with a conserved sequence motif of DKTGTLT (referring to the reversibly phosphorylated aspartic acid (D)) (Kuhlbrandt, 2004). The typical catalytic cycle of the P-type ATPases involves at least two main conformations (E_1 and E_2). The affinity for ATP and the ions to be transported is high at E1. The hydrolysis of ATP phosphorylates the ATPase and change the conformation from E1 to E2. E2 is the low affinity state for ATP and the transported ions. In consequence, the inside entrance of the ion transport tunnel closes and the outside exit opens, and is followed by the release of the ions. Dephosphorylation leads to the reverting of the enzyme to the E1 conformation again (Palmgren and Harper, 1999; Morth et al., 2011). Based on the ion specificities, the P-type ATPases can be divided into 5 subfamilies (P1-P5/I-V) and further divided into subclasses (A, B, C and so on) (Morth et al., 2011; Pedersen et al., 2012). The P-type ATPases are involved in different transport processes, which are indispensable in many fundamental cellular functions. For example, the Na^+/K^+ ATPase (P2C ATPase) was the first discovered P-type ATPase which exists in the plasma membrane of all animal cells. It is responsible for the non-symmetric distribution of the sodium and potassium ions across the animal cell plasma membrane. This forms the basis for the resting membrane potential in these cells of -30 mV to -70 mV. It plays, therefore, also a unique role in transmembrane transport of other molecules, neuron excitation, signal transduction, etc. (Morth et al., 2011).

The P-type H^+-ATPases, as the equivalent to the animal Na^+/K^+ ATPase, are defined as the P3A P-type ATPases, are mainly existing in the plasma membrane (PM) of plants, algae and fungi (also found in protists and prokaryotes). As the primary transporter in plants, PM H^+-ATPases creates an electro-chemical gradient for protons which can drive secondary transport processes through the plasma membrane. As such, it facilitates nutrient uptake, cell expansion and other essential metabolic processes. In contrast to the Na^+/K^+ ATPase in animal cells, the PM H^+-ATPase directly contributes, via the electrogenic nature of the pump, a large part of membrane potentials in plants (up to -250 mV) and fungi (up to -300 mV) (Buch-Pedersen et al., 2009; Haruta et al., 2015).

PM H^+-ATPases regulation

PM H^+-ATPases are involved in a diversity of physiological processes, which could only be achieved by a sophisticated regulation system at the transcriptional, translational and enzymatic levels (Portillo, 2000; Arango et al., 2003). At the transcriptional level, there are many different environmental factors that are able to trigger the expression of the PM H^+-ATPases. These environmental factors are highly species-specific (e.g. glucose and extracellular pH to yeast, hormones and light to plants) and developmental stage-related (e.g. growth conditions of yeast, and development/ aging of plants) (Portillo, 2000). For fast responses of PM H^+-ATPases, the major regulation takes place at the post-translational level and mainly through phosphorylation (Haruta et al., 2015; Falhof et al., 2016). In plants, there are approximately 100 residues at the C-terminal (also named the regulation (R) domain), including three main modules as inhibition region I, inhibition region II and penultimate threonine (pT), acting together as an auto-inhibition domain by interfering with the catalytic domains (Palmgren et al., 1991). The phosphorylation at the penultimate threonine creates a binding site for 14-3-3 proteins (Fuglsang et al., 1999). The binding of 14-3-3 proteins releases the inhibition from the C-terminal and hence activates the proton pump (Jahn et al., 1997). Besides this well-known key switch, the phosphorylation of other residues at the C-terminal can tune up or tune down the pump activity (Duby et al., 2009; Speth et al., 2010; Piette et al., 2011; Rudashevskaya et al., 2012). On the other side there are around 10 amino acid residues in the N-terminal assisting the C-terminal in the H^+-ATPases regulation (Ekberg et al., 2010; Rudashevskaya et al., 2012). In yeast, the R-domain is only about 40 residues with little homology to the equivalent plant sequence. It has been reported that in yeast the main regulation mechanism is through the phosphorylation of two tandemly positioned residues (Serine and Threonine) in the C-terminal, which does not need the involvement of 14-3-3 proteins (Portillo, 2000; Kuhlbrandt, 2004).

PM H^+-ATPases in algae

As compared to the well-studied land plants and fungi, the PM H^+-ATPases in algae are still relatively untouched and provide many opportunities for further investigations. Part of the absence of algae PM H^+-ATPase details is due to the lack of gene sequence information and proper tools for the molecular manipulation. Up to now evidences indicate that the highly conserved penultimate threonine in all vascular plants most likely appeared with the emergence of plants to the terrestrial environment (Okumura et al., 2012a, b). There coexists non pT H^+-ATPases in the most basal lineage of extant land plants, the liverwort *Marchantia polymorpha,* and the moss *Physcomitrella patens.*

In these systems, pT H^+-ATPases are still the main pump and in majority, leaving the non pT H^+-ATPases barely studied (Okumura et al., 2012b; Pedersen et al., 2012). While in the green algae, so far, no pT H^+-ATPases have been found (Okumura et al., 2012a, b). Thus, how the non-pT H^+-ATPases function in algae as the key player and how they are regulated, are still remaining undefined.

Together with gaining new regulation modules in the H^+-ATPases during the evolution from algae to land plants, there is an opposite trend of losing Na^+ export ATPases (Pedersen et al., 2012). It is known that Na^+/K^+ ATPase and H^+-ATPase are exclusively existing in animal cells and (vascular) plant cells, respectively. However, the co-existence of Na^+/K^+ ATPase and H^+-ATPase was found in the chlorophyte marine algae *Ostreococcus tauri* and the terrestrial algae *Chlamydomonas reinhardtii* (Pedersen et al., 2012). Thus, there is another interesting topic to be figured out with the evolutionary of PM H^+-ATPases in algae: whether the Na^+-ATPases were lost at a branch point during the evolution of the streptophyta lineage, while H^+-ATPase progressively replace the Na^+-ATPases as the main transporter (Palmgren, 2001; Pedersen et al., 2012).

Outline of this thesis

In the past, people have used *Chara* grown in the wild for their experiments. However, to guarantee a more constant supply of homogeneous research material it is good to culture *Chara* in the lab. Unfortunately, this is not easy, and in **Chapter 2** we have summarized the *Chara* culture in our lab, the lessons and experiences gained in the past few years, as a base for further research with *Chara*.

In **Chapter 3**, we compared the cellular auxin transport in *Chara* cells with that in classical land plants models, proposed the potential model for auxin polar transport through *Chara* internodal and nodal cells. With the hypothetic model, we could list out the similarities and differences between land plants and “plant-like” *Chara*, set out the potential interesting target for further studies.

In **Chapter 4**, we investigated one of the auxin regulation functions in *Chara* cells- the effects of auxin on cell membrane potential and transmembrane ion fluxes (in specific, K^+ and H^+ fluxes). Since the electrical-physical status and dynamics (especially the pH and permeability) of the plasma membrane in turn would influence the auxin transmembrane traffic.

Results in **Chapter 4** indicated that, different from the land plants cells, auxin couldn't directly regulate the H^+-ATPase activity in *Chara* cells. Since there was little knowledge about the H^+-ATPases in algae, in **Chapter 5**, we used RNA based next generation sequence information to isolate a H^+-ATPase from *Chara*. Comparison of the amino acid sequence of this proton pump with those in flowering plants detected a different C-terminal cytoplasmic domain, which suggested that this *Chara* transporter is differently regulated compared to its land plant orthologs.

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