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To my family

致我亲爱的家人

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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:

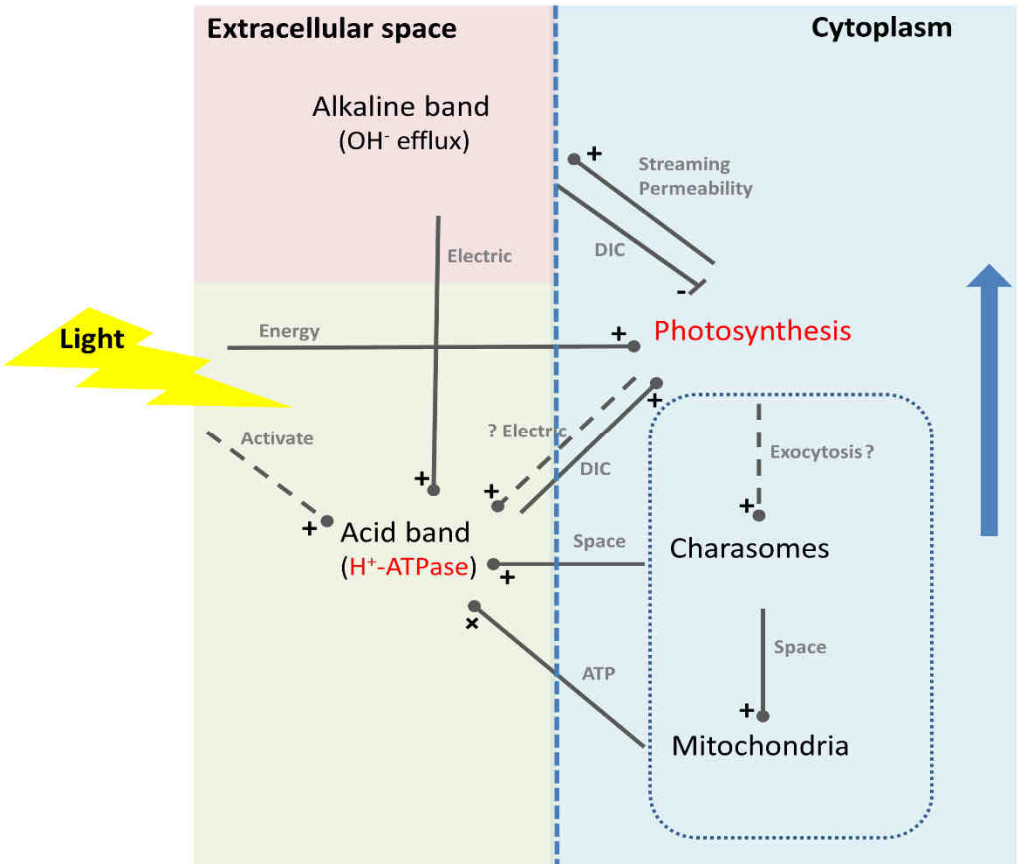

Figure 1. Components (cell structures, metabolic processes, etc.) which are involved in *Chara* pH banding phenomenon induced by light, and the possible relationship among all the components. Blue background indicates the cytoplasm, with the blue arrow indicating the direction of cytoplasmic streaming. 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).

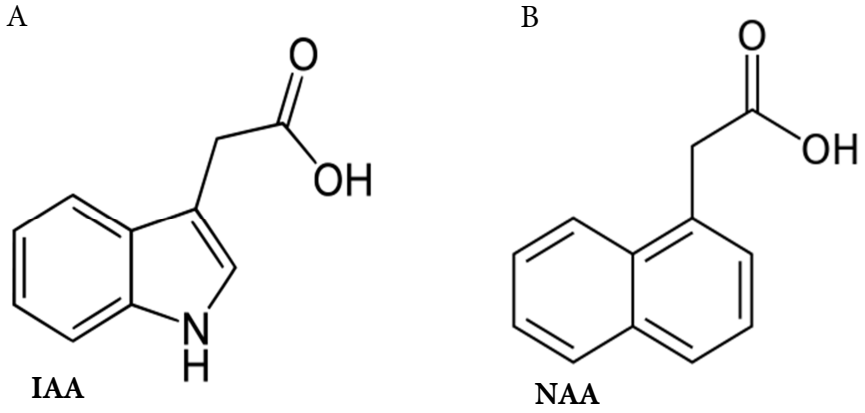

Figure 2. Chemical structure of two main auxin isoforms. (A) the main endogenous auxin. (B) a commonly used synthetic analog.

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.

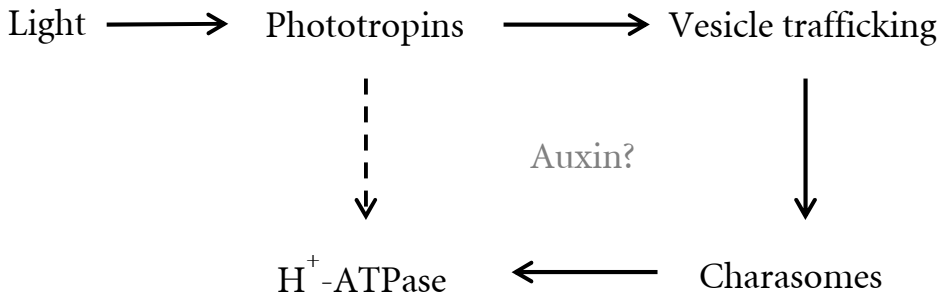

Figure 3. Hypothetic model of mechanism behind the *Chara* pH banding phenomenon. Solid arrow lines indicate that the regulation mechanisms behind have relative evidences in *Chara*, while the dash arrow line indicates potential relationship in *Chara* regarding to other non-closely related species.

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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Chapter 2

The culture of *Chara sp.* for research: does and don'ts

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Abstract

The *Chara* algae are popular world-wide as a regulator and indicator for environmental (fresh water system) management, as well as a research subject and model system in different labs for decades. With the rapid development of using *Chara sp.* as a model system to study plant development, cellular biology, hormone and signal systems, etc., there is an urgent requirement for a more consistent and efficient material supply. Thus, we propose and discuss here some efforts of trying to culture *Chara sp.* for a long period in a standard lab condition, including culture settings (e.g. light, temperature) and procedures (planting and harvesting).

Introduction

Chara algae are fresh water, multicellular, green macro-algae closely related to the land plants. They commonly form dense meadows in oligo- and moderately eutrophic waters. In ecological systems, the Charophytes are considered as the "gold standard" in littoral areas, indicating and regulating healthy, clear-water ecosystems (Ibelings et al., 2007; Richter and Gross, 2013; Beilby and Casanova, 2014). The dominance of *Chara* only in clear, non-turbid water system makes them a good indicator for water pollution status (Ibelings et al., 2007; Singh et al., 2013a). With the *Chara* dominance, they can effectively takeup, storage and immobilize the macronutrients especially phosphorus, acting as nutrient sinks and sediment stabilizers to maintain the water clarity (Kufel and Kufel, 2002; Bakker et al., 2010; Blindow et al., 2014). By lowering the nutrient concentration, *Chara* can be used for water management against cyanobacteria, phytoplankton and invasive aquatic plants, helping with the re-oligotrophication of the water body (Ibelings et al., 2007; Hidding et al., 2010; Richter and Gross, 2013). Besides, they are also proposed for phytoremediation applications for decrease of trace metal from the industry (Laffont-Schwob et al., 2015; Poklonov, 2016).

Apart from the great interests of *Chara* in ecology studies, the *Chara sp.* also got special attention in physiological and cell biological studies, especially in the fields of membrane transport, cell motility and electrical signalling, because of their unique internodal-cell geometry (Shimmen et al., 1994; Shimmen and Lucas, 2003; Foissner et al., 2015). Besides an interest in the algae themselves they also provide a unique model system for investigation of especially more general cell physiological processes and their importance as an intermediate in the evolutionary developments from water to land plants (Shimmen et al., 1994; Boot et al., 2012; Zhang and van Duijn, 2014). The major advantages of *Chara* algae as a model system for these types of studies are:

1. *Chara sp.* have giant single cell internodal cells. These cells can reach a length of more than 10 cm with a diameter of around 1mm (Braun et al., 2007; Foissner & Wasteneys, 2014). Most *Chara* species have internodal cells that are surrounded by a cortex of small cortical cells (Fig. 1A). However, certain species of *Chara* have ecorticate (without cortex) large internodal cells (Fig. 1B), such as *Chara australis*, *Chara corallina* and *Chara braunii* (Beilby and Casanova, 2014), which allows for direct observation, measurements and manipulation for cell biological and

physiological studies (Winter et al., 1987; Shimmen et al., 1994). For instance, the cells can be manipulated by centrifugation, perfusion to create different membrane systems for electrical measurements, which set the base and gave a huge boost for the modern plant electrophysiology (Beilby, 2016).

2. *Chara sp.* are closely related to the land plants. As one of the closest ancestor of land plants, *Chara* has evolved a plant-like morphology, with a similar yet simpler morphological and cell composition structure (Casanova 2007). The study of similarities and differences in the metabolism, hormone regulations, cellular processes, makes *Chara* cells a good model system for studies relevant to the land plants, e.g. tropism and polarity growth, wound-healing mechanism, cyto-architecture and development (Braun et al., 2007; Boot et al., 2012; Foissner and Wasteneys, 2012& 2014). Last but not least, the study of *Chara* at the evolutionary perspective, could fill the gap between the Chlorophyta and Streprophta offering a better map at the genomic level, e.g. the mechanism of effective nutrient up-taking and higher salinity tolerant of Charophyceae algae could shed light on the modern agriculture investment (Pedersen et al., 2012; Domozych et al., 2016).

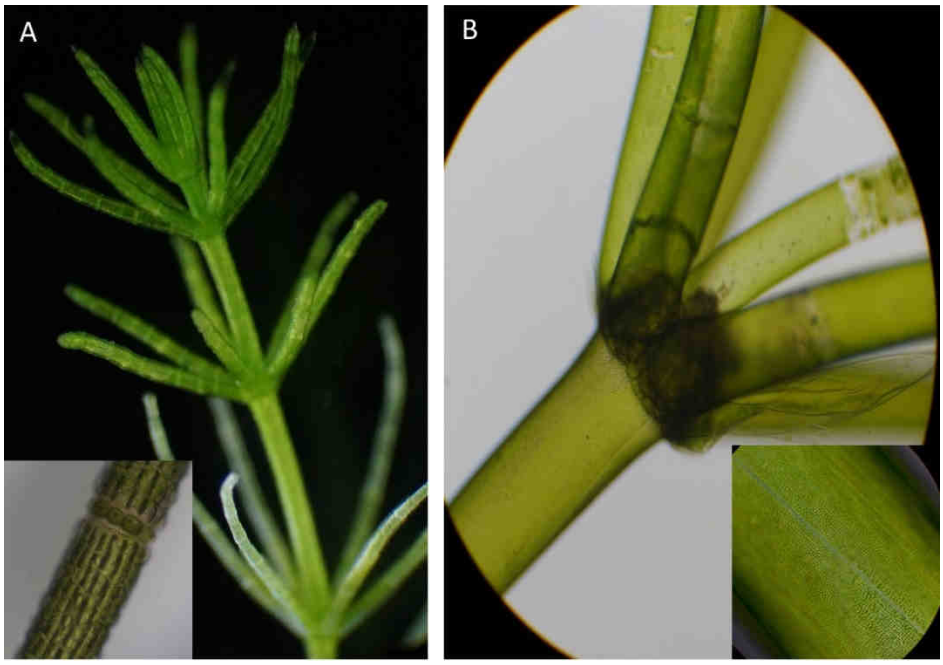

Figure 1. Cortical and ecorticate *Chara* species. (A) *Chara vulgaris* (cortical specie), inserted picture shows the cortical cell layer covering the center internodal cell. (B) *Chara corallina* (ecorticate specie), inserted picture shows the visible chloroplasts layer underneath the cell membrane.

Besides the clear advantages of the *Chara* cells as a model system there are some aspects that put limitations to the use of *Chara* in research. One of the most prominent of these limitations refers to the availability of algae material and (standardized) culture of *Chara*. The difficult aspects in respect to algae material availability can be summarized as:

- Unreliable and unpredictable availability and production in nature. *Chara* is relatively widespread in natural fresh waters, but most of the *Chara* species have an annual life cycle in their wild habitats, which makes the sample collection highly unpredictable and seasonal dependent.
- Absence of information on standard (optimal) growth conditions and growth protocols. The growth and morphology of *Chara* are strongly affected by the environmental conditions. In this respect light conditions (intensity, day-night cycle), water chemistry (nutrients, pH etc.) and soil composition are most prominent in influencing the basic conditions.
- Difficulties and uncertainties in the precise taxonomy and determination of *Chara* species. The methods applied in *Chara* taxonomy are undergoing a continues revolution from rough specimen observing to molecular experimental approaches during the past decades. The genetic distances among "species" in algae is in general larger than in animals or land plants.

The above factors may cause inconsistencies between different independent experiments, due to for instance different culture conditions of different "varieties" resulting in the use of algae with different properties. In addition, the aspects of the taxonomy difficulties may result in confliction or confusing experimental results from labs all over the world using different *Chara* species under identical names.

For starting experiments with *Chara* cells it is easiest to collect the algae material directly from nature and keep them alive in the lab in an aquarium for couple of days or weeks. But a stabilized and standard lab culture for a longer period is still difficult to achieve.

In our research over several years, we have tried to culture the most commonly used *Chara* species in the physiological research in the lab under different conditions. These are *Chara corallina* as the major species and some other species such as *Chara braunii* and *Chara australis*. In these trials, based on culture condition variations and the (scarce, as concerned the description of culture conditions) literature, we were able, after lots of failures, to culture *Chara*

species and keep them in good condition in the artificial environment for more than two years. In this paper we describe to preferred conditions for standardized *Chara sp.* culture, as well as the major pitfalls and conditions to be avoided.

The culturing of *Chara sp.*

In general, *Chara* algae can be cultured in aquaria, water tanks etc. of different sizes. In the culturing the factors of importance to be discussed are:

- Soil structure and composition and soil coverage
- Water quality
- Water volume and level
- Water streaming and mechanical stresses
- Light
- Temperature

Soil and soil coverings

Different soil sources are described in the literature referring to the culture of *Chara*, e.g. pond mud, forest soil, clay, soil & sand mixture (Smith and west, 1969; Weifer and Spanswick, 1978; Klima and Foissner, 2008; Kataev et al., 2012). It is reported that, small granule size substrate (425-710 μm) was shown to benefit the growth of both shoots and rhizoids of *Chara hispida* and *Nitella flexilis* (Andrews et al, 1984a).

Forest soil is among the most popular substrates used for the *Chara* culture (Berecki et al., 1999; Lew, 2015). The advantages of forest soil include that it is easy to collect, and full of nutrients to support the growth of *Chara.* The disadvantages of forest soil are that the composition and structure are undefined and that it not only differs from region to region but also can be very different locally on collection sites and over time. Biological potting and seeding soil without fertilizer can be used as an alternative to forest soil, but also these soils can be very diverse in composition and structure. Therefore, a lot of trial and error goes into testing for the right soil for growing *Chara.* Yet another soil for *Chara* growth in aquaria can be so called aquarium soil with or without nutrients (e.g. Tetra Plant, Complete Substrate). These aquaria soils are better defined, clean and tested for aquarium plant growth.

In relationship to hygiene and cleanliness, forest and potting soil are potentially the biggest source for contaminations. To avoid contaminations, the soil should be at least double autoclaved (Lew, 2015), which greatly reduces the chance of contamination by fungi, bacteria and secondary algae.

We grew *Chara* on many types of soil, but the best results were on either forest soil or (and) aquarium soil. Forest soil was collected from temperate broadleaf and mixed forests (Leidse Hout Park, Leiden, the Netherlands) by removing the top layer (2-5 cm) and collecting the soil underneath to a depth of about10 cm. The forest soil was autoclaved two times before use and aquarium soil was used directly from the package. A layer of less than (about) 3 cm thick forest or aquarium soil was placed on the bottom of the aquarium and covered by 1 cm or more aquarium grit on top. This extra top layer prevents the disturbance of the soil and provides a hard base for the rhizoids to anchor. Both systems can support the healthy growth of *Chara* for a long period (at least two years).

Recommendation: carefully sterilize the soil source collected from the nature to reduce the risk of contamination. Standard commercial aquarium soil and grit for pond plant (e.g. Velda, Tetra Plant) would be a recommended alternative.

Water composition

In natural habitats, *Chara* is often lush in clean and rather hard water (Garcia and Chivas, 2006; Beilby and Casanova, 2014). In literature in many cases artificial pond water (APW) containing 0.1 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM NaCl (pH about 6.0) was used as a supplement to the nutrients from the soil or used alone for pre-experimental culturing (Smith and West, 1969; Klima and Foissner, 2008). It is recommended to use demineralised (Demi) water instead of tap water. As a standard medium the APW we used, has been proven to be sufficient for the healthy growth of *Chara*. In experiments we tested richer, more nutrients containing media like the Broyer and Barr medium (Lew, 2015) with the same soil conditions. In these experiments, no obvious difference was observed with the growth of *Chara* as compared to growth in APW.

So, we maintained APW as the preferred medium. For a full mature culture, medium was replaced once a month or even less. The old medium was removed by a siphon tube from the bottom of the tank and new medium was added slowly at the other side of tank along the wall to minimize mechanical disturbances (change 1/3-1/2 medium and extra caution with new cultures). The

siphon effect is also helpful in removing the planktonic micro-algae sticking at the bottom or along the tank wall.

Recommendations: 1. low nutrient medium with demi-water; 2. regular (but not too often) exchange of medium to reduce the growth of phytoplankton and cyanobacteria.

Volume and water level

The size of the aquaria and containers used for *Chara* culture are in first instance given by practical considerations. In our experiments two different sets were used for the *Chara* culture. To start new cultures from cuttings (4-6 cells), either by horizontal way or vertical way (see other chapter below), small tanks (24x15x15 cm) were used. 2-3 small tanks were placed into a big tank (60x29x26 cm). The larger tank was covered with Plexi-glass on top to reduce the evaporation. The small tanks were filled to 5 cm (in the beginning) with APW and the big tank was also partly (fully when cultures have grown) filled with APW to create high humidity to reduce the evaporation. Refreshing of the water was done by first removing and then replenishing medium from/to the big tank. The use of this system reduces the mechanical stress due to turbulence and it minimizes the possible secondary algae contamination. Water level could be lower (3-4cm above the top of *Chara* thalli) at the start of a new culture (horizontal way of planting, see below) and fill up gradually during the establishment, until the big tank was almost full.

With large and mature *Chara* cultures, it is possible to transplant them into a big tank with the same substrate and APW medium.

Recommendation: adjust the water level with the growth of *Chara.*

Movement and mechanical stress

In different experiments it was found that turbidity and physical disturbance are rather important negative effectors of the establishment of Charophytes acid-alkaline zones along the internodes, and wave damage was considered one of the top causes of mortality (Casanova and Brock, 1999; Blindow, 1992; Schwarz et al., 2002; van Nes et al., 2002). The *Chara sp.* without cortex (ecorticate) are more fragile to mechanical disturbance than the corticated ones. Apart from the direct damage, turbulence may disperse sediment resulting in a turbid

environment and increased risk of epiphytic and micro-organism contaminants to settle on the *Chara* cells (Kairesalo, 1987).

Recommendation: once the cultures were settled, it is recommended to avoid rough transport of the tanks and be very careful while replacing the medium.

Light and day-night cycle

In the natural habitat, *Chara sp.* are found preferentially in clear, transparent water systems. In these water systems they could be found either in the shallow beds with a few centimetres depth (under shadow) or deeper than 10 meters (Andrews et al., 1984; de Winton et al., 1991; Garcia and Chivas, 2006). This suggests that *Chara sp.* have a good capacity to adjust to and grow under different light conditions. In most studied cases, Charophyte meadows formed the deepest vegetation of the lakes and growth was beyond the depth limit of vascular plants, e.g. *Chara corallina* meadows were found at 10-15 meters and *Chara braunii* at 9 meters under water in New Zealand (de Winton et al.,1991; Schwarz et al., 2002). In addition, the Charophytes tend to form low cover growths in shallow water while high-intense meadows with longer shoots as established below 7 meters depth (de Winton et al., 1991; Asaeda et al. 2007). These all indicate that light intensity has effects on the growth and morphology of *Chara*, and that *Chara sp.* have low light requirements, which has been confirmed by reports that low light intensities of 1-10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ can support *Chara* growth and development (van den Berg, 1999; Bulychev et al., 2013).

Based on these indication in our laboratory cultures, different light intensities have been tested (2-250 $\mu mol \cdot m^{-2} \cdot s^{-1}$). According to our experiences, through different stages of *Chara* culture (planting, recovering, flushing), dimmed light worked fine to support the growth of *Chara*. In particular, dimmed light is highly recommended to start a new *Chara* culture, also since it can effectively suppress the unwanted competition from planktonic algae. To achieve that, the sides of the tanks were covered with black paper to reduce the light from the sides that would reach the bottom part of the tank and a layer of white filter paper was used to cover the top of the tank to create some shade. Though there was no sign of harmful or photo-inhibition effect from a direct strong irradiation once the *Chara* culture reached the lush and dominant condition. To a certain extent, we detected that *Chara* internodal cells showed a darker green

colour under stronger light conditions which confirmed earlier observations (Asaeda et al., 2007).

Different day/night light cycles have been reported among labs, ranging from 12-18 hours for day light (Lucas, 1975; Weifer and Spanswick, 1978; Andrews et al., 1984; Klima and Foissner, 2008; Hidding et al., 2010). 16/8h (light/dark) is applied in our lab.

Recommendation: low radiation in general is in favour of *Chara* dominance over secondary (disturbing) algae.

Temperature

Charophytes can be annual (e.g. *Chara muelleri*) or perennial (e.g. *Chara australis*). With the perennial ones, a wide temperature range for culture can be applied (Casanova and Brock, 1996). Similar to the irradiation level, different temperatures have influence on thalli development and morphology. At lower temperature (5^0- 10^0C), *Chara* thalli tend to show more apical dominance with smaller side branches; while at higher temperature (15^0- 22^0C), they grow faster and develop more and larger side branches (Andrews et al., 1984). Reports also showed that, between 10^0-25^0C, with increasing temperature, the dark respiration rate of *Chara* increases but the highest photosynthetic rates differed among species (Vieira Jr and Necchi Jr, 2003).

Different temperatures (10^0-25^0C) have been used among different research groups for different purpose (Andrews et al., 1984; Mimura and Shimmen, 1994; Klima and Foissner, 2008). For our cultures, we wanted to maintain a high growth rate of *Chara*, and yet supress the growth of secondary plankton algae. So, we avoid the higher temperature of 25^0C, which is more favourable for the quick growth of phytoplankton. Both 18^0C, 22^0C were used and these temperatures can well support the *Chara* growth and healthy oogonia and antheridia were developed during the culture.

Recommendation: lower temperature (18^0- 22^0C) can help to supress the fast-growing secondary algae in favour of *Chara* dominance.

Starting a new culture

To begin a new *Chara* culture, it is very important to start with healthy *Chara* explants. Healthy *Chara* look fresh green and have strong cells which have a high turgor pressure. The physiological status of the *Chara* explants can be easily checked by the presence of a high velocity (50-100 $\mu m \cdot s^{-1}$) of cytoplasmic streaming under a microscope (Kikuyama et al., 1996; Kataev et al., 2012). In general, new cultures are started by using plants from another culture or from nature.

One method to start a new culture is to cut out 4-6 internodal cells, preferably with side branches still on them, from whole thalli. These internodal cells are kept in the similar orientation (all original top parts in the same direction) and are placed (together) horizontally on the soil and subsequently a thin cover of soil is put on top of the cells, leaving the original top part unburied (Lew, 2015). When sufficient explants are available, it is also possible to start a culture by planting complete thalli with rhizoids in a vertical way, in a similar way that plantlets are planted in soil. Big thalli with bright green colour and a high planting density are proven to be an advantage to start a culture. Avoiding mechanical turbidity and dimming the light could encourage the development and anchoring of the rhizoids and support the *Chara* thalli to build up its dominancy.

The density of *Chara* explants being planted is also important for start a new culture. In natural habitat, *Chara* usually grows in meadows/patches, and they form dense vegetation, with high biomass per unit area (Blindow, 1992; van den Berg et al., 1998). Field data also showed that a high early season Charophyte biomass could decrease the probability of algal blooms later in summer (Bakker et al., 2010). And by establishing a proper population, *Chara* can moderate the surrounding environment and cope better with the unfavorable disturbances (Kufel and Ozimek, 1994).

In consistence, in our experience, high density gives better growth both to start a new culture or maintain a stable culture, providing that high density could build up a stronger resistance to non-ideal physical conditions (light/temperature), and a lower chance of epiphytic contamination.

Recommendation: plant *Chara* in clusters (in our experiments the best results were achieved with explants as big as we could obtain).

Diseases/ problems

Competitors /phytoplankton

In natural habitats, *Chara* species are found in lakes that are usually defined as remarkably clear. The dominant position of *Chara* over the phytoplankton is often indicated as strong allelopathic effects of *Chara sp.*, which have been investigated for the past few decades (Berger and Schagerl, 2003 &2004; Gross, 2003; Gross et al., 2007). Some natural compounds were isolated from *Chara sp.* that showed pronounced photosynthesis inhibiting effects (Anthoni at el., 1980; Wium-Andersen et al., 1982; Berger and Schagerl, 2003&2004). However, the allelopathy in situ is still under debate, and the release of allelopathical compounds may also be species specific (Forsberg et al., 1990; Berger and Schagerl, 2004). Likely many more other impactors can be involved in their relationships, yet not well studied (van Donk and van de Bund, 2002). The advantage of an artificial lab culture, under the low irradiation and nutrition level, is that the competition from phytoplankton is rather negligible. Regular replacing the culture medium and wiping clean the side-walls of the tank during the medium replacement could keep this balance well maintained.

Epiphytic growth/ cyanobacteria

The epiphyte community is usually referred to as the mixture of microalgae, bacteria, fungi, inorganic particles and detritus, attaching to the surface of submerged aquatic vegetation (Vis et al., 2006). It has a close negative relationship with the light and CO_2 availability to the growth of macrophyte (Kairesalo, 1987). In the lab culture, epiphytic growth (mainly with cyanobacteria) on *Chara* thalli is usually the top risk to jeopardize the whole culture system (Fig. 2). The dimmed light condition can help with the suppression of phytoplankton growth but not the growth of cyanobacteria, since they have a high capacity of shade-tolerance (Scheffer et al., 1997). Though, cyanobacteria are rather sensitive to temperature and the dominance usually occurs when water temperature is higher than 20^0C (Havens, 2008; Bakker et al., 2010). Thus, the use of a lower temperature, that is still feasible for *Chara* growth and development, like 18^0C or a bit lower is, therefore, in favour to prevent the over growth by cyanobacteria. In general, decreasing the nutrient concentrations can also reduce epiphytic problems to a certain extent. Introducing herbivores (e.g. snails or Daphnids) into the culture was also recommended by other

researchers (Lew, 2015; Bakker et al., 2010) to control epiphytes and secondary algae.

We tried to introduce zebrafish into the culture system, but the result did not turn out well. On the one hand, zebrafish tend to not only eat epiphytic algae but also feed on *Chara* thalli, which lead to some mechanical damage to the *Chara* thalli. On the other hand, the excreta of zebrafish enrich the medium and further encourage the proliferation of cyanobacteria and fungi. The research of fish effects *in situ* also confirmed a poor performance of Charophytes caused by fish exposures (Winton et al., 2002).

In case of some *Chara* thalli gets entangled by the cyanobacteria, immediate removal of the lesions and replacing the medium could reduce the chance of further contamination.

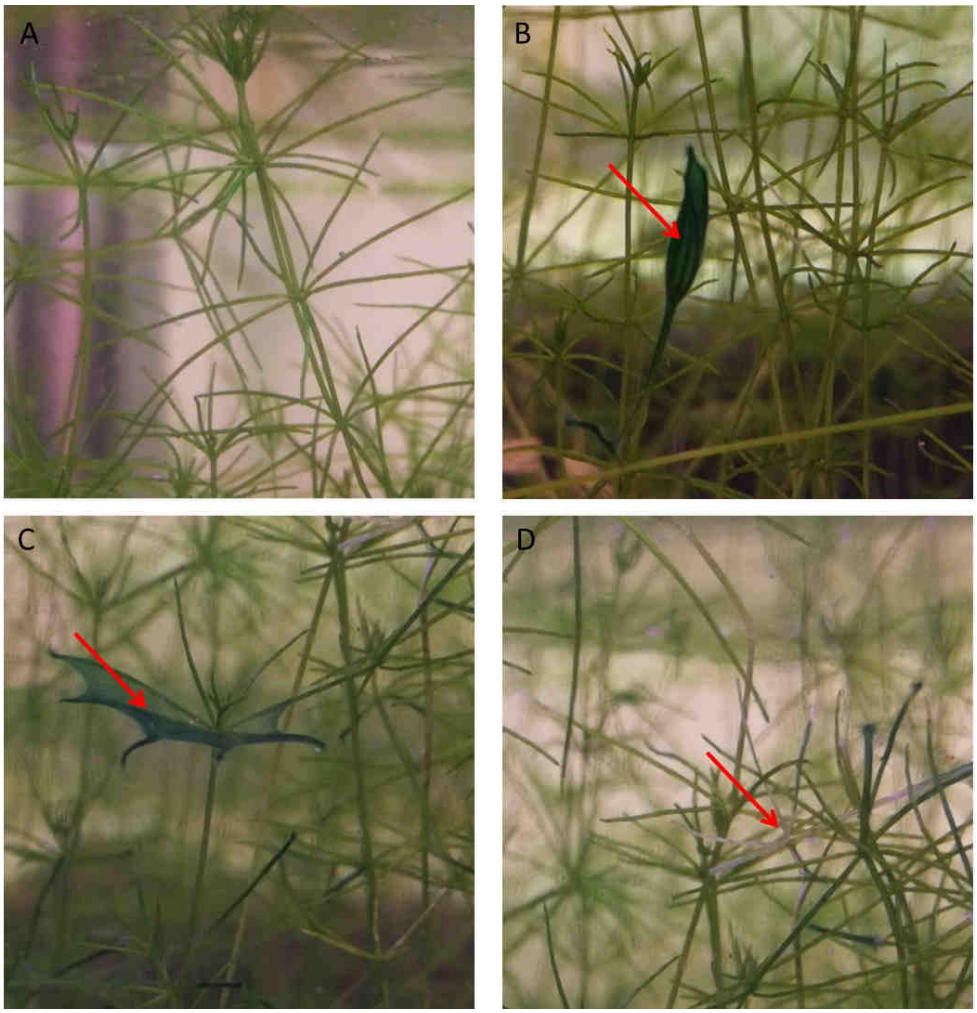

Figure 2. *Chara corallina* lab culture pictures. (A) healthy *Chara corallina* culture. (B&C) contaminated by cyanobacteria (red arrows). (D) transparent dead cells (red arrow).

Hygiene

Even though the *Chara* cultures are non-axenic, it is still important to be aware of the hygienic issues. While handling the *Chara* thalli, harvesting or the exchange of the medium, be sure to wash hands with soap, and remove the soap completely with tap water. Clean the tools (tweezers and scissors) with 70% ethanol and wait till the complete evaporation.

Handling

Shipping

Since the availability of most *Chara* species is (very) limited in most countries, researchers have to rely often on explants of laboratories in other parts of the world. These explants need to be shipped. For the shipment, tubes 2/3 filled with liquid (lake water/ APW), or plastic bags with wet filter paper inside around the explants were two mostly used methods. They both worked fine, though for the latter one it is important to use a box for shipping to avoid any physical pressure during the shipment. The physical condition of the *Chara* thalli before shipment is of great importance and only robust, bright green plant material should be used for shipment. Last but not the least, whole thalli with shoots and rhizomes together can keep the explants in much better conditions than the cuttings.

Harvesting

Referring to the hygiene, harvest the thalli only with clean hands and clean tools (section of Hygiene). Use scissors to cut the internodal cells or tweezers to pull out the whole thalli depending on the requirements. Fresh harvests could be rinsed by demi water and collected in APW solution for further usage.

Conclusions and recommendations

Chara could be an ideal model system because of the giant single cells and because it is assumed that the Characeae are an important link in the development from water to land plants. In the past many interesting results are produced from experiments with Chara.

What is typical for a model system is that experiments could be repeated in every laboratory wherever, provided that a standard protocol has been used.

Unfortunately, in 2017 we are far from having fully developed such a protocol for *Chara* as a model system. We are just on the way. We are still dependent on plant material that has been collected fresh in the field every time again. It is not always possible to find (ecorticate) *Chara* in the neighbourhood of a laboratory because *Chara* doesn't occur (*Chara corallina*, *Chara australis*, *Chara hispida* in the Netherlands) or is very rare (*Chara braunii* in the Netherlands). So, in many cases, we are dependent on the goodwill of colleagues elsewhere. It is absolutely necessary to build up an international network, which we did.

Recommendation: try to get a certain numbr of plants (of one particular species and collected at the same spot) at the disposal. Having reached this result comparison of different culture conditions would be a challenge.

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Chapter 3

Cellular Auxin Transport in Algae

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Abstract

The phytohormone auxin is one of the main directors of plant growth and development. In higher plants auxin is generated in apical plant parts and transported from cell-to-cell in a polar fashion. Auxin is present in all plant phyla, and the existence of polar auxin transport (PAT) is well established in land plants. Algae are a group of relatively simple, autotrophic, photosynthetic organisms that share many features with land plants. In particular, Charophyceae (a taxon of green algae) are closest ancestors of land plants. In the study of auxin function, transport and its evolution, the algae form an interesting research target. Recently, proof for polar auxin transport in *Chara* species was published and auxin related research in algae gained more attention. In this review we discuss auxin transport in algae with respect to land plants and suggest directions for future studies.

Introduction

Auxins are a class of plant hormones with morphogen-like characteristics that regulate the rate of cell division, cell elongation and expansion, ethylene biosynthesis, apical dominance, organ differentiation and some other essential processes in plant growth (Salisbury and Ross, 1992; Raven et al., 1999).

In the late 19th century, Charles Darwin investigated "the power of movement in plants" (Darwin and Darwin, 1881). However, it took nearly a century before, in 1926, Fritz Went opened a new field of study by successfully isolating "the plant growth substance" auxin (Went, 1974; Taiz and Zeiger, 1991). Later he introduced the avena curvature test enabling many experimenters to be active in auxin research (Went and Thimann, 1937; Went, 1939). Now it is known that auxin is locally synthesized in (land) plants, mainly in shoot tissues, from where it is distributed unevenly throughout the whole plant (Taiz and Zeiger, 1991). Concentration differences of auxin can cause different responses in plant development. During the early 20th century, the traditional "cut-off and stuck-on" tests and later the use of ^{14}C-indoleacetic acid showed that auxin is distributed through the plant by energy-requiring polar transport, with three important characteristics: chemically highly specific, oxygen-requiring and pH dependent (Jacobs, 1979).

The most abundant naturally occurring auxin is indole-3-acetic acid (IAA) which shows characteristic "polar transport" throughout the whole plant (Bartel, 1997; Friml and Palme, 2002; Friml, 2003; Lau et al., 2009). Mid 1970s, based on the known properties of auxin movement (saturable, energy- and protein synthesis- dependent and unidirectional), the "chemiosmotic hypothesis" was formulated as a classic model to explain the mechanism of polar auxin transport (PAT) (Friml, 2003; Vieten et al., 2007). According to this model, IAA can easily enter the cell cytoplasm in an undissociated lipophilic form (IAAH) when in a slightly acidic extracellular environment (pH 5.5). While in the cytosol, with a neutral pH of about 7, most of the IAA will be dissociated into anions (IAA^-) and, therefore, trapped inside the cell. To aid the exit of IAA^- active auxin anion efflux carriers were proposed, and the asymmetric distribution of such carriers was postulated to attribute to the directionality of auxin transport (Petrasek and Friml, 2009). This hypothesis later has been proven to be suitable to describe in general auxin transport in land plants (Vieten et al., 2007).

Although auxins are shown to be present in early-diverged lineages plants such as mosses, liverworts and algae (van Overbeek, 1940; Ergun et al., 2002), research on auxin transport and signaling remains mainly focused on seed plants, predominantly in the model plant *Arabidopsis thaliana* (Cooke et al., 2002). However, algae share many specialized characteristics with the land plants while evolutionally earlier and of simpler structures. Hence, they may provide unique features that may help to unravel (new) auxin working mechanisms, auxin transport characteristics and functions. In this paper, we look into auxin as a plant hormone in algae, and specially focus on auxin transport.

The roles of Auxin in algae

Algae belong to a very large and diverse group of simple, typically autotrophic organisms. Increasingly data appears that all land plants (embryophytes) diverged from ancestral Charophycean algae, a class of green algae, about 400-500 million years ago, table1 (Niklas, 2000; Proseus and Boyer, 2006). Table 1 also shows that the red algae and brown algae are more distant from the streptophytes. In this respect, green algae especially members in the class of Charophyceae are promising candidates to study evolutionary aspects, (new) functionalities and cellular physiology of auxin.

Bioassays, high-performance liquid chromatography, mass spectrometry and some other physicochemical analysis, together with other circumstantial evidences, proof the existence of auxin or auxin-like compounds in many species of algae (Niemann and Dorffling, 1980; Cooke et al., 2002; Tarakhovskaya et al., 2007). The measured concentrations in these studies are highly variable. The presence and action of auxins have been shown both in unicellular and multi-cellular algae (Kenneth and Kurt, 1959; Jacobs et al., 1985; Nowak et al., 1988; Cooke et al., 2002; Lau et al., 2009). For example, in red algae (e.g. *Grateloupia dichotoma*, *Gracilaria vermiculophylla*, *Agardhiella subulata*) and green algae (e.g. *Chlorella pyrenoidosa*, *Micrasterias thomasiana*) auxin stimulates cell division and cell enlargement (Wood and Berliner, 1979; Vance, 1987; Bradley and Cheney, 1990; Yokaya and Handro, 1996; Garcia-Jimenez et al., 1998; Yokaya et al., 1999) and affects the development and growth of rhizoids as well (Provasoli and Carlucci, 1974; Klambt et al., 1992; Basu et al., 2002). Although the full-value of plant hormone systems in algae is still under debate, the aforementioned studies about auxins on algal growth and development indicate that its functions likely

correspond to its activity in higher land plants (Bradley, 1991; Evans and Trewavas, 1991; Tarakhovskaya et al., 2007). As studies so far concentrated on land plant corresponding functions other, new and unexpected, roles for auxins in algae may have been overlooked and remain to be discovered.

Table 1. Partial classification of plants and brown algae

KINGDOM	DIVISION	CLASS	ORDER
Plantae	Chlorophyta (green algae)		
	Streptophyta	**Charophyceae** (green algae)	Zygnematales, Coleochaetales, Charales, Desmidiales, Klebsormidiales, Mesostigmatales
		Embryophyceae (land plants: mosses, lycophytes, ferns and horsetails, seed plants)	
	Rhodophyta (red algae)		
Chromalveolata	Heterokontophyta	Phaeophyceae (brown algae)	

Transport of auxin in algae

Auxin acts both as hormone and morphogen. The role of auxin in apical dominance can be regarded as a classical example of hormonal integration based on hormone distribution in land plants. Similar phenomena have also been described in various seaweeds, suggesting that in algae similar auxin distribution systems and carriers supporting PAT may be present (Buggeln, 1981; Bradley, 1991).

The events in auxin signaling as established in seed plants do not only involve the sensing of auxins at the level of the target cells and their responses but also

auxin biosynthesis and metabolism, intracellular compartmentalization, and directional transport through cells facilitated by specific transporters (Smet et al., 2011; Feraru et al., 2012).

Auxin transport in seed plants is characterized by its polarity, directionality, distance, and transporting cells (Lomax et al., 1995; Cooke et al., 2002). Polar auxin transport (PAT) is facilitated by influx carriers (AUX1/LAX proteins) and efflux carriers (PIN proteins), together with some other transport proteins (ABCB/PGP transporter family) (Friml and Palme, 2002; Vieten et al., 2007). The asymmetric distribution of efflux-carriers, mainly related to the plasma-membrane-localized PINs, aids to the gradient of auxin concentration through the whole plant, while the differences of auxin concentration in turn regulate the number and location of efflux-carriers (Morris, 2000; Paciorek et al., 2005; Petrasek and Friml, 2009; Friml, 2010). The endoplasmic reticulum (ER)-localized PIN proteins (like PIN5, PIN8) are thought to charge the intracellular compartmentalization of auxin and homeostasis, in cooperation with members of the recently-found auxin carrier family PIN-LIKEs (PILS) (Mravec et al., 2009; Barbez et al., 2012; Sauer et al., 2013).

To understand this whole system better, splitting it into several parts and searching them back in the evolutionarily earlier organisms of green or brown algae lineages will be helpful.

In view of some of the apparent morphological similarities between algae and land plants one might ask if the ability of auxin polar transport is required for such differential development? Studies in the large coenocytic (multi-cellular structures without cross walls) green alga *Caulerpa paspaloides* show different results. Although these algae show characteristics like a leaf-like assimilator which grows up, rhizoid clusters that grow down, and a rhizome that grows horizontally, auxin displays a uniform and non-polar distribution, which might be caused by diffusion and cytoplasmic streaming (Jacobs, 1979; Brennan and Jacobs, 1980; Dibb-Fuller and Morris, 1992). This suggests that auxin polar transport and auxin gradients do not participate in, at least, the later development and maintenance of these three different organs. In multi-cellular algae the existence of PAT has also been investigated. In *Chara*, a branched, multi-cellular green alga, a specific carrier system is suggested to mediate the transmembrane auxin transport (Dibb-Fuller and Morris, 1992). This carrier lacked the inhibition by 1-N-Naphthylphthalamic acid (NPA, a phytotropin) which is typical for inhibiting efflux carriers in land plants (Dibb-Fuller and

Morris, 1992). In the same study, such specific auxin carriers were not found in the simple unicellular green alga *Chlorella vulgaris* (Dibb-Fuller and Morris, 1992). On the other hand, Klämbt and coworkers reported that in growing rhizoids of *Chara*, auxin efflux showed NPA-sensitive activation (Klämbt et al., 1992). These contradictory results are thought to be due to some additional effects of NPA, unrelated to its ability to increase intracellular IAA levels by blocking IAA efflux (Cooke et al., 2002). Recent experiments, showing NPA sensitive polar transport of radioactive labeled auxin, provide more direct proof of the presence of PAT in *Chara* and suggest the presence of specific auxin efflux carriers as in land plants (Boot et al., 2012). In addition, PAT has been shown to exist in brown algae species like *Fucus distichus* and *Ectocarpus siliculosus* (Basu et al., 2002; Sun et al., 2004).

Comparing studies in unicellular (micro or coenocytic) green algae with multi-cellular green algae (*Chara*), Dibb-Fuller and Morris proposed that "the appearance of specific auxin carrier systems in the *Chloroplyta* may have been functionally associated with the evolution of multi-cellularity, rather than with the evolution of a plant body which is characterized by distinctly different morphological regions" (Dibb-Fuller and Morris, 1992). This conclusion seems in accordance with the early hypothesis raised by others, that after the development of multicellular organisms, simple diffusion of IAA would not be efficiency enough, the movement across cell membranes is required for polar transport of IAA (Goldsmith, 1977; Jacobs, 1979; Brennan and Jacobs, 1980). Although the relatively lipid-soluble IAA could account for very slow diffusive transport through membranes, for more effective longer distance transport and gradients in tissue, membrane bound auxin transporters and channels are required.

Since PAT in seed plants is largely dependent on the asymmetric distribution of PIN proteins, the PIN proteins seem a key in the investigation of PAT mechanisms. Though the PILS carrier family is quite conserved throughout the evolution of plants and can be found from unicellular algae to highly developed seed plants, the PIN exporter families at the plasma membrane are comparatively young in evolutionarily perspective (Smet et al., 2011; Viaene et al., 2013). The plasma membrane-localized PIN proteins are thought to exist only in land plants, while the endoplasmic reticulum (ER)-localized PIN proteins (like PIN5, PIN8 in *Arabidopsis*) are evolutionarily older, and can be traced back to an origin in Streptophyta algae (Viaene et al., 2013).

By using a basic local alignment search tool (BLAST), partial PIN protein sequences can be identified in several species of green algae like *Spirogyra*, *Penium*, and the evolutionarily even earlier lineage *Klebsormidiophyceae* (Leliaert et al., 2012; Viaene et al., 2013). However, the above results are based on the expressed sequence tag (EST) database, since the nuclear genomes sequences database is barely described in the multicellular green algae of the Streptophyta group. The scant annotated genomes are almost all from the unicellular algae of the Chlorophyta group, and there is not yet evidence of the existence of PIN protein sequences (Smet et al., 2011; Viaene et al., 2013). With the growing information on nuclear genomes of multicellular green algae groups, a clearer picture is soon expected on the presence of PIN proteins.

Although evidence for functional PIN proteins supporting PAT in algae is (still) scarce this does not rule out the (functional) existence of PAT in algae. In the brown algae *Ectocarpus siliculosus* PAT has been shown while this species lacks any PIN homolog (Bail et al., 2010). The possibility of other types of auxin (polar) transporter mechanisms rather than PIN proteins cannot be excluded. Besides, the presence of plasmodesmata or other active (e.g. vesicle based) transport systems may exist. Researches have shown that there exists a special plasma membrane invagination-structure named charasomes in *Chara* species, but not in *Nitella* species. Interestingly, PAT also shows up in *Chara corallina,* but not in *Nitella (unpublished data)* (Franceschi and Lucas, 1980; Lucas and Franceschi, 1981; Schmolzer et al., 2011; Boot et al., 2012; Boot et al., 2013). Charasomes are thought to be closely related to the ability of endocytosis in *Chara* and the origin of vesicles (Franceschi and Lucas, 1980). Although even if it is possible for auxin to be transported in vesicles, the driving force of these vesicles in these algae species remains a mystery: the block of cytostreaming by cytochalasin H could not stop the auxin transport in Chara while the normal microtubule system seems not fast enough to reach the observed speed of auxin transport. This suggests the existence of an unknown amplification mechanism (Boot et al., 2012; Raven, 2013). Taking everything into consideration, it is still a challenge to identify possible PIN protein independent polar auxin transport mechanisms that may have emerged in the early period of evolution and may or may not have been eliminated during the evolution of land plants. Algae form an attractive model to investigate these potentially early systems and learn more about the basic properties of auxin transport and auxin function.

At present, our knowledge on the role of auxin and its transport in algae is still very limited. However, we are still able to draw some conclusions based on the available studies. With regard to (polar) auxin transport, Figure 1 illustrates a summary and comparison between land plants and multi-cellular algae, like *Chara corallina*. In both models PAT is present based on auxin influx carriers and NPA sensitive efflux carriers, and a chemiosmotic mechanism. Although the presence of NPA sensitive PAT in *Chara* in combination with our understanding of PAT in land plants justifies this hypothetical model, it must be emphasized that the hypothetical presence of these specific transport proteins in algae is only based on indirect evidence. In addition, the membrane potential difference across the plasmalemma should also be taken into consideration as this plays a significant role in the chemiosmotic mechanism, and complicates the auxin transport model (Rubby and Sheldrake 1973; Raven, 1975; Fisahn et al., 1992). Compared with our knowledge the model system of seed plants--Arabidopsis, studies on algae are still facing many challenges, especially with regard to the still insufficient genetic background database and possibilities for application of molecular biology tools.

Algae as model system

Research in the field of auxin and algae were mainly on the brown algae when related to the function of auxin, while the green algae were more favorable in studies related to signaling and transport. In view of modern phylogenetics, the division Charophyta contains the closest living relatives to the land plants. In this division, the order Charales has been widely accepted as a sister-relationship to the land plants over the past century, which was based also on the morphological point since the Charales develop the most complicated, land-plant-like body structures as compare to all the other orders (Karol et al., 2001). Now the phenomenon of PAT is observed in *Chara*, a genus of Charales, showing that, after the discovery of PAT in moss species (*Physcomitrella patens*) (Sun et al., 2004; Fujita and Hasebe, 2009), these green algae species are so far the earliest life forms showing PAT in the non-land plants (Dibb-Fuller and Morris, 1992). Recent research data show that Zynematales and Coleochaetales are closer to the land plants than Charales (Finet et al., 2010; Wodniok et al., 2011; Timme et al., 2012) and it has been proposed that the grade of organismal complexity of Charales may have evolved independently. It remains to be discovered whether the Charales share a similar auxin polar transport mechanism with the land

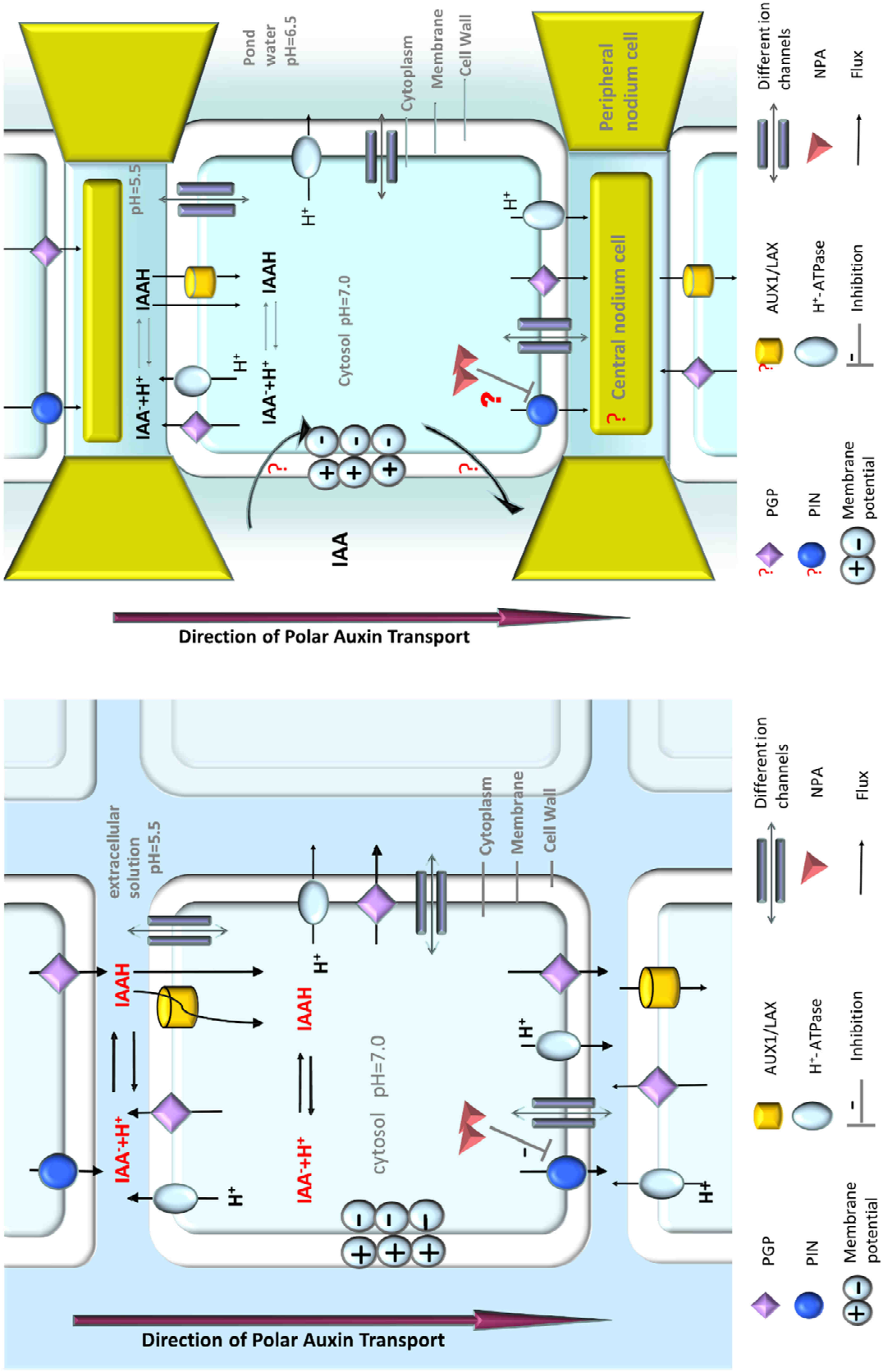

Figure 1. (A) Auxin transport in seed plants; (B) The hypothetical auxin transport system in *Chara*.

plants or have evolved a different mechanism of PAT. Therefore, from an evolutionary point of view, auxin related research aiming at early mechanisms and functions should be focused on these organisms. Within the order of *Charales* species from the genera *Chara* have shown a great potential for the future studies in auxin signaling and transport. Species of *Chara* have the giant internodal cells that provide an ideal and unique model for polar auxin transport research at the single cell level while they do show up the ability of polar transport of auxin. To some extent, these cells are superior to the model system *Arabidopsis thaliana*, such as for cell biological cell physiology studies of auxin transport and signaling at the single cell level as advanced microscopy, electrophysiology, and transport studies that can be applied to the intact cell under natural conditions.

Using algae in auxin research requires some precautions and approaches that are less relevant in studies on land plant. For instance, the use of non-sterile algae materials could lead to false conclusions as bacteria or secondary algae may produce auxin-like substances or break down endogenous algal auxin. Some special cautions are also necessary in choice and conditioning of cells for auxin transport studies, especially since the function of auxin in algae remains unclear. In our recent experience, we noticed that in some situations the *Chara* internodes may not show the PAT phenomenon (data not show). This could be an example of auxin feedback regulation on the efflux carriers. The reasons and details need a further study. Also, in higher plants such effects have been reported. Decapitation of growing shoots can result in the loss of polar auxin transport in segments from internodes subtending the apex (Morris and Johnson, 1990).

Besides the possibility that "apical dominance" may also be part of the algae development strategies, there are some other unique characteristics of algae that require attention. Unicellular algae have big surface area in connection with the environment, and also multi-cellular algae cells have a strong exposure to the environment, in contrast to cells in land plants. Despite this strong exposure to the environment algae are able to control environmental parameters around the cells. The internodal cells of *Chara corallina*, for example, can build up several alkaline bands separated by acid bands along the longitudinal axis to facilitate the uptake of inorganic carbon (Lucas, 1979&1980; Shimmen and Wakabayashi, 2008). This band-formation ability is quite strong and shows that the cells can manipulate the extracellular micro-environment. Due to this acid-alkaline

banding pattern, application of the classical "chemiosmotic mechanisms" for auxin transport should only be applied with great care (Fig. 1), as pH balance conditions may vary strongly and abruptly along the cell surface length.

Conclusions

Genetic research and development of molecular biological tools, such as genetic transformation, as well as tools for studying the developmental biology for *Chara* and *Nitella* species is still in its infancy. However, based on our detailed knowledge of auxin function, signaling and transport in land plants, combined molecular and cell physiological research in *Chara* and *Nitella* may open a new page on our understanding of auxin's central role in plant growth and development.

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Chapter 4

Auxin effects on ion transport in *Chara corallina*

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Abstract

The plant hormone auxin has been widely studied with regard to synthesis, transport, signaling and functions among the land plants while there is still a lack of knowledge about the possible role for auxin regulation mechanisms in algae with "plant-like" structures. Here we use the alga *Chara corallina* as a model to study aspects of auxin signaling. In this respect we measured auxin on membrane potential changes and different ion fluxes (K^+, H^+) through the plasma membrane. Results showed that auxin, mainly IAA, could hyperpolarize the membrane potential of *C. corallina* internodal cells. Ion flux measurements showed that the auxin-induced membrane potential change may be based on the change of K^+ permeability and/or channel activity rather than through the activation of proton pumps as known in land plants.

Introduction

The plant hormone auxin, IAA, plays an essential role in plant growth and development. With respect to the physiological aspects of this plant hormone we can distinguish the (polar) auxin transport related processes and the (cellular) signaling related processes. Compared to our knowledge of auxin action in (red, brown, green) algae species, the regulation, signaling and working mechanism of auxin is much clearer for the land plant species (Lau et al., 2009; Wright and Nemhauser, 2015; Enders and Strader, 2015; Di et al., 2015). In land plants, auxin is produced in apical parts of the plants and is transported to specific directions with the help of different auxin transporters among which the so-called PIN efflux transporters (Morris, 2000; Viaene et al., 2013; Adamowski and Friml,2015; Qin and Dong, 2015). At the cellular level, active auxin levels can also be regulated by biosynthesis, storage and conjugation with other molecules (Vernoux et al., 2010; Kramer and Ackelsberg, 2015). Research into auxin related cellular signaling showed different physiological responses of plant cells upon auxin stimulation (Berleth et al., 2004). In these responses membrane hyperpolarization, activation of the plasma membrane H^+-ATPases and potassium channels are well established (Ephritikhine et al., 1982; Felle et al., 1991; Van Duijn and Heimovaara-Dijkstra, 1994; Philippar et al., 2004; Christian et al., 2006; Takahashi et al., 2012; Xu et al., 2012; Osakabe et al., 2013).

As it was already shown in the 1950s or even earlier, that algae species naturally synthesize auxin and that the growth of most of marine/fresh water, unicellular/multicellular algae can be regulated by auxin, (van Overbeek, 1940; Cooke et al., 2002; Tarakhovskaya et al., 2007) it is believed that auxin, as an important growth regulator, dates back to a very early stage of plant evolution. Despite these findings basic knowledge of the role, transport and cellular signaling of auxin in algae is very limited. With respect to polar auxin transport (PAT), as a unique aspect of auxin's role in plant growth and development, it is known that in higher plants the responsible auxin carriers can be divided into two groups, auxin-uptake carriers and auxin-efflux carriers. The carriers can be easily distinguished by their different sensitivities to different inhibitors such as NPA (1-N-naphthylphthalamic acid), a specific PAT inhibitor. In algae these auxin transport or carrier systems may be present as well (Dibb-Fuller and Morris, 1992; De Smet et al., 2011; Boot et al., 2012; Feraru et al., 2012). Some evolutionary less developed plants share similar body structures as the higher land plants, suggesting that auxin- like polar transport and gradients may play a

role in the development and growth. Indeed, in mosses the existence of polar auxin transport was reported (Fujita and Hasebe, 2009; Viaene et al., 2014), as well as in the cells of the multicellular algae *Chara corallina* (Boot et al., 2012). As a multicellular green alga, *Chara* has a differentiated plant body-like structure, which is also thought to be one of the closest relatives to the land plants (Qiu and Palmer, 1999; Wodniok et al., 2011; Timme et al., 2012; Zhang and van Duijn, 2014). Understanding the role, transport and physiology of auxin in these algae may reveal the evolution of auxin signaling and its functioning in plant evolution. In addition, this may indicate to us yet unknown aspects of auxin in higher plants. It is understandable that with the development of a differentiated, multicellular plant body, a better regulated auxin transmembrane transport pathway is needed to facilitate the morphogenic-signal function of auxin. Based on the studies up to date, there are still debates on whether *Chara* has similar influx and/or efflux auxin transporters, and whether these transporters have similar inhibition sites binding to phototropins. Although polar auxin transport is well established for *Chara* cells it does not automatically imply that auxin acts as a plant hormone in these algae, that auxin signaling occurs and that it plays similar roles in development as in land plants (Lau et al., 2009; De Smet et al., 2011; Zhang and van Duijn, 2014).

To gain a better understanding of the role of auxin and auxin transport in *Chara* cells, we aimed to investigate the well-known membrane potential and ion transport responses of higher plant cells to stimulation with exogenous auxin in *Chara* internodal cells.

Materials and Method

Algae

Chara corallina was cultured indoors at room temperature in aquaria filled with artificial pond water (APW) containing 0.1mM KCl, 0.1mM $CaCl_2$ and 0.1mM NaCl (pH about 6.0), and forest soil as described earlier (Berecki et al., 1999) under 8/16 light/dark conditions. Nutrients from the forest soil diffuse into the water to support growth of the algae. For the experiments fully grown algae were selected for their internodal cells to be used in measurements.

Membrane potential measurements

Changes of membrane potential were measured using the K-anesthesia method (Shimmen et al., 1976). Single internodal cells with node cells on both ends were separated from the plants and put in a chamber with two pools isolated by Vaseline with the volume of 3- 4ml each (Fig. 1). One pool (A) was filled with APW, the other one (B) was filled with 55 mM KCl dissolved in APW (unless stated otherwise). Electrical potential differences were measured by insertion of Ag/AgCl electrodes in the pools connected to an amplifier Model 750 (W.P. Instruments) in current clamp mode. Results were recorded and analyzed using the software package Clampex7 (Axon Instruments). The measurements took place inside a closed Faraday cage on a vibration free table to avoid disturbances from the environment, and to suppress the band formation ability of *Chara* cells, which may cause unexpected influences on the measurement, there was no extra light supplemented inside the shaded unit.

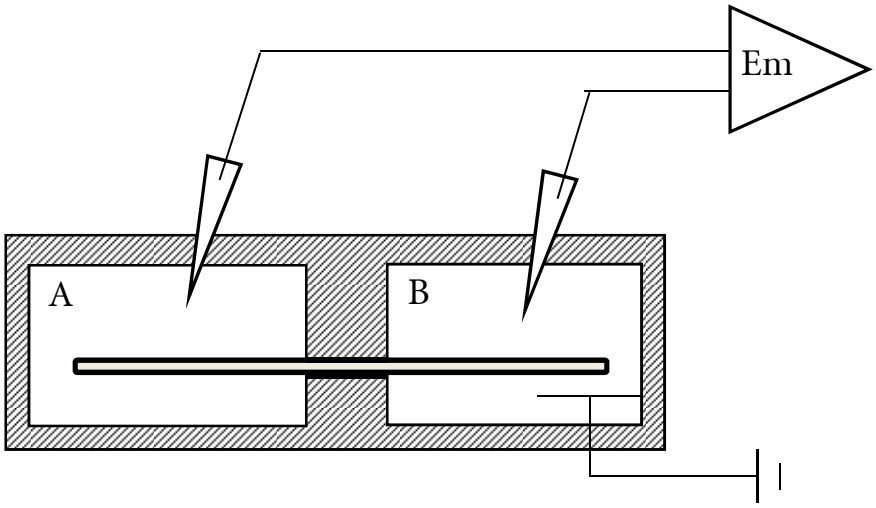

Figure 1. Membrane potential measurement set-up according to Shimmen et al. (1976). Single internodal cells with node cells on both ends were separated from the plants and put in a chamber with two pools isolated by vaseline. Pool A was filled with APW, pool B was filled with 55 mM KCl dissolved in APW (unless stated otherwise). Electrical potential difference between the two pools is measured by insertion of an Ag/AgCl electrode in each pool connected to a current clamp amplifier.

The effect of pH differences was tested with two or three levels for different concentrations of K^+ in the solution (0.1 mM, 1 mM, 10 mM and 55 mM).

Influence of three different types of auxin (IAA, 1-NAA, 2-NAA) on the cell membrane potential was tested for different concentrations (10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5} M). IAA and the other two homologs were dissolved with ethanol at the

concentration of 10^{-2}M and stored. Before use the stock was diluted with APW to final concentrations of 10^{-8}M to 10^{-5}M. To ensure that addition of auxin did not alter the pH, the pH of auxin solution was adjusted to the original APW pH. The addition of auxin was done carefully along the cell at the opposite side of the electrodes to avoid high peak concentration of auxin near the cells due to lack of dilution, as well as to avoid mechanical stimulation of the cell (Shimmen, 1997).

Ion flux measurements

Net fluxes of H^+ and K^+ were measured noninvasively using scanning ion-selective electrode technique. We used the ASET system (Automated Scanning Electrode Technique) from Science Wares Inc. The principle of this method and instrument are detailed in Jones et al. (1995), Shabala et al. (1997) and, Li et al. (2010).

Probes were fabricated from tributylchlorosilane (Fluka 90796) silanized 1.5 mm x 1.17 mm thin wall capillaries (Harvard Apparatus) pulled on a Sutter P-1000 pipette puller to give a 10 μm tip opening. Probes were backfilled with either 100 mM KCl for K^+-measurements or 15 mM NaCl, 40 mM KH_2PO_4, pH 7,0 (with NaOH) for pH measurements. The tip was filled with 150-300 μm K^+ LIX (Fluka 60031) or H^+ LIX (Fluka 95297) respectively through brief submersion of the tip in LIX held in a capillary.

In all the measurements below, the position of the probe tips was alternated with a 20 μm step between 50-70 μm from the cell surface in a perpendicular direction. At each location, the ion concentration was measured for 10 s allowing the solution to settle after the motion of the probes. The concentration difference between the two locations was directly calculated and reported as the flux. The data was recorded by ASET and further analyzed using Excel (Microsoft Corporation).

The influences of IAA, fusicoccin (FC), pH and light on the net fluxes of H^+ and K^+ were tested.

Banding formation

Band formation solution (BFS) was used to detect the acid/alkaline bands of *Chara*. BFS was freshly prepared by adding 0.5 mM $NaHCO_3$ and 5 mg/100 ml phenol red to APW (color range of phenol red: yellow while pH below 6.8, red while pH above 8.2). pH was adjusted to around 6.5 for a lighter background.

Results and discussion

Membrane potential measurements

In higher plants membrane potential hyperpolarization is a well-established rapid response to application of auxin. In order to study the auxin responsiveness of *Chara* intermodal cells, the effect of auxin on the membrane potential was studied. The membrane potential was measured with the 'K-anesthesia' method (Shimmen et al., 1976). First, some basic responses of the membrane potential were measured, such as pH dependence and K^+ dependence, followed by applications of auxin.

pH and K+ dependence

The membrane potential of *Chara* cells has been shown to be both dependent on the intracellular and extracellular potassium concentration ratio and the ATP (and pH) dependent activity of proton transport (Shimmen et al., 1994; Tazawa and Shimmen, 2001). To test the membrane potential measurements and to be able to discriminate between auxin-induced membrane potential changes and pH-induced membrane potential changes we first studied the effect of APW with different pH values and K^+ concentrations on the membrane potential as measured with the 'K-anesthesia' method.

In the experiment the membrane potential was measured for varying K^+ concentrations in pool B while in pool A standard APW (with 0.1 mM K^+) was used. Figure 2 shows the relationship between the measured membrane potential with this method and the ^{10}Log of the ratio of the K^+ concentration in the two pools. The membrane potential shows to be strongly potassium dependent and follows a linear relationship with the ^{10}Log of the potassium concentration ratio between the pools. Similar strong potassium dependency was found in earlier studies (Shimmen and Tazawa, 1977; Shimmen, 2001).

With regard to the sensitivity of the membrane potential to the extracellular pH it has been shown that this effect can be much stronger if the cells are in full light as compared to more dark conditions (Saito and Senda, 1973; Felle and Bentrup, 1976) which mainly is due to the presence of MgATP that provides the energy for the H^+-ATPase activity (Kaeamura et al., 1980). In the absence of ATP the membrane potential dependence on the extracellular pH is rather weak and completely absent if no MgATP is present.

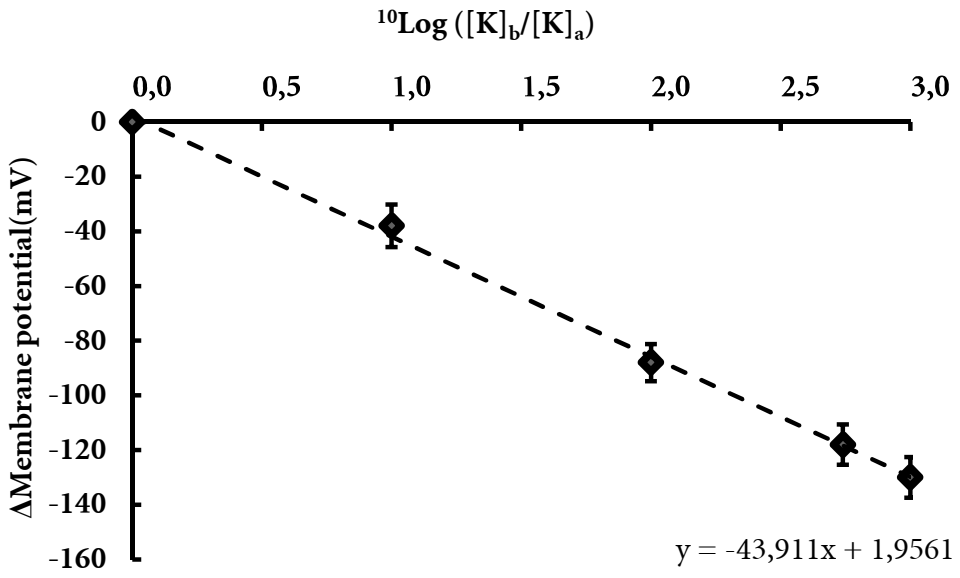

Figure 2. Membrane potential as measured for different potassium concentrations in pool B at a constant potassium concentration in pool A. Measured values represent the average of 6 independent measurements on different cells. Error bars are the standard error. The dashed line is a linear fit through the data points.

In our experiments the membrane potential was not strongly dependent on the extracellular pH, which may be attributed the rather low light conditions in the experimental set up. Maximal membrane potential changes were recorded for conditions with APW in both pools with an extracellular pH of about 5.2 in pool A and 7.3 in pool B of about –13.8 ± 4.1 mV (n=4). With increasing potassium concentration in pool B the effect of different pHs' in pool B on the membrane potential became much smaller. At 55 mM KCl in pool B with pH switching from 5.2 to 7.3 (pool A with standard APW at pH 5.2) the membrane potential change amounted only about 2.5 mV (n=2).

From our measurements we conclude that the membrane potential is both pH and K^+ concentration dependent. The concentration of K^+ in the bath solution has a significant influence on the membrane potential. For Increasing pH values we find more negative membrane potentials, but the changes caused by pH are mitigated in the presence of high concentrations of K^+. These findings are in agreement with earlier membrane potential measurements on *Chara* cells (Shimmen et al., 1994; Shimmen and Wakabayashi, 2008) and look similar to effects of pH and potassium on the membrane potential of higher plant cells (Lew, 1991; Katicheva et al., 2014).

Auxin dependence

Different concentrations of IAA, 1-NAA and 2-NAA in APW (pH 6.0) were added to pool A and the effects on the membrane potential were recorded and analyzed. From an IAA concentration of about 10^{-6} M and higher a change in membrane potential could be recorded (Fig. 3). After addition of IAA at sufficient concentration the potential changes rapidly (within a few seconds). The auxin analogues 1-NAA, 2-NAA were much less effective in inducing a membrane potential change. At a concentration of 1.25×10^{-5} M the average membrane potential change with 1-NAA was -2.7 ± 1.0 mV (n=6), and was for 2-NAA -7.7 ± 1.1 mV (n=6). This is much less than was recorded for IAA, which amounted -15.7 ± 1.2 mV (n=6) at the same concentration.

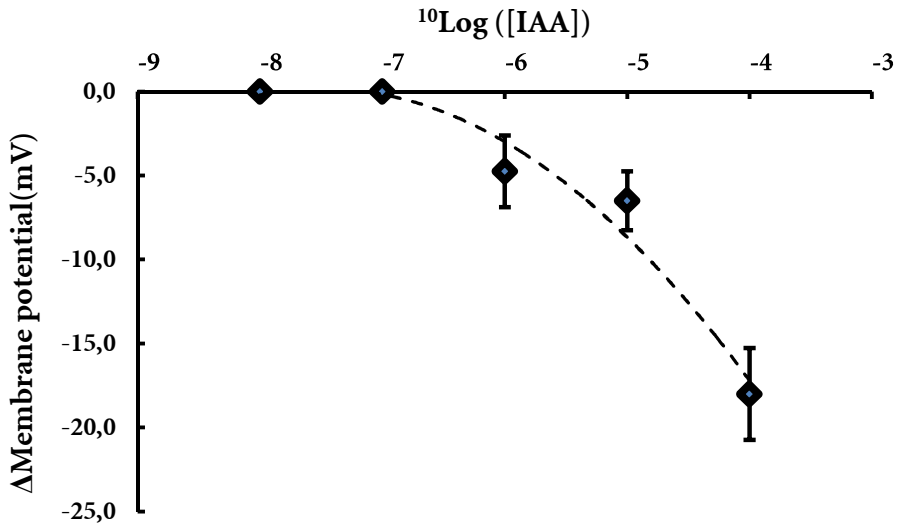

Figure 3. Membrane potential changes caused by different concentrations of IAA. Measured values represent the average of 5 independent measurements on different cells. Error bars are the standard error. The dashed line shows the trend.

Although all three tested auxins can induce a (small) membrane potential change, the effect of IAA is more obvious. The IAA concentration necessary to induce a significant membrane potential change can be regarded as comparable to auxin concentrations that are effective in higher plants with respect to induction of gene expression and differentiation (Tatematsu et al., 2004; Sun et al., 2013; Baster et al., 2013; Lee et al., 2009) as well as to auxin-induced membrane potential changes in higher plant cells which are typically reported to be effective in the range of 1 - 100 µM auxin (Van Duijn and Heimovaara-Dijkstra, 1994; Felle et al., 1991; Kirpichnikova et al., 2014; Ephritikine et al.,

1987; Barbier- Brygoo et al., 1989). The auxin-induced membrane potential changes in higher plant cells typically are in the order of -5 to -20 mV. These values and concentrations are well in agreement with the results we find in *Chara* intermodal cells.

Ion flux measurements

Auxin-induced membrane potential changes may be accompanied by different ion fluxes. Typically, proton and potassium fluxes are reported upon application of auxin to higher plant cells. As in land plants auxin is shown to be able to activate proton pumps directly as well as activate the synthesis of proton pump proteins (Hager et al., 1991; Hager, 2003; Takahashi et al., 2012), H^+ -fluxes may be expected upon auxin application to *Chara* cells. The proton pump also is a determinant in the membrane potential of *Chara* cells (see above and Tsutsui et al., 2001; Beilby, 2015). In addition, potassium fluxes are also reported upon auxin application to higher plant cells (Blatt et al., 1994; Claussen et al., 1996; Hager, 2003; Fuchs et al., 2006) and potassium is also an indispensable player in the resting membrane potential in *Chara* (see results above and Shimmen et al. ,1994).

In these experiments we chose H^+ and K^+ as our main target ions. The vibrating probe technique was used to measure real-time ion fluxes through *Chara* membranes.

Salt and light dependence

The responsiveness of the electrodes and the cells were tested by application of salt stress to the cells. Upon application of a salt shock the sensitivity of the H^+ and K^+ probes and the reactivity of the cells could be established. Two different NaCl concentrations were applied to the cells. In repetition 1 ml 100 mM NaCl-APW was added into 4 ml bath solution resulting in a final concentration of 25 mM NaCl followed by 2 ml 400mM NaCl-APW resulting in a concentration of 200 mM NaCl. This resulted with the low NaCl concentration (25 mM) stimulation in an immediate efflux of K^+ of a magnitude of about 50 $nmol \cdot m^{-2} \cdot s^{-1}$, and a slight change in the H^+ flux of about 4 $nmol \cdot m^{-2} \cdot s^{-1}$. When the high level (200 mM) NaCl stimulation was applied, there was an immediate activation of K^+ transport with a further activation of the proton flux (with a magnitude of 10-20 $nmol \cdot m^{-2} \cdot s^{-1}$), followed by several long lasting extreme peaks of K^+ efflux up to 600-700 $nmol \cdot m^{-2} \cdot s^{-1}$. An example of the responses in such an experiment

is shown in figure 4. From the responses on the addition of NaCl we conclude that both the cells and measurement system are functional for the measurement of K^+ and H^+ fluxes.

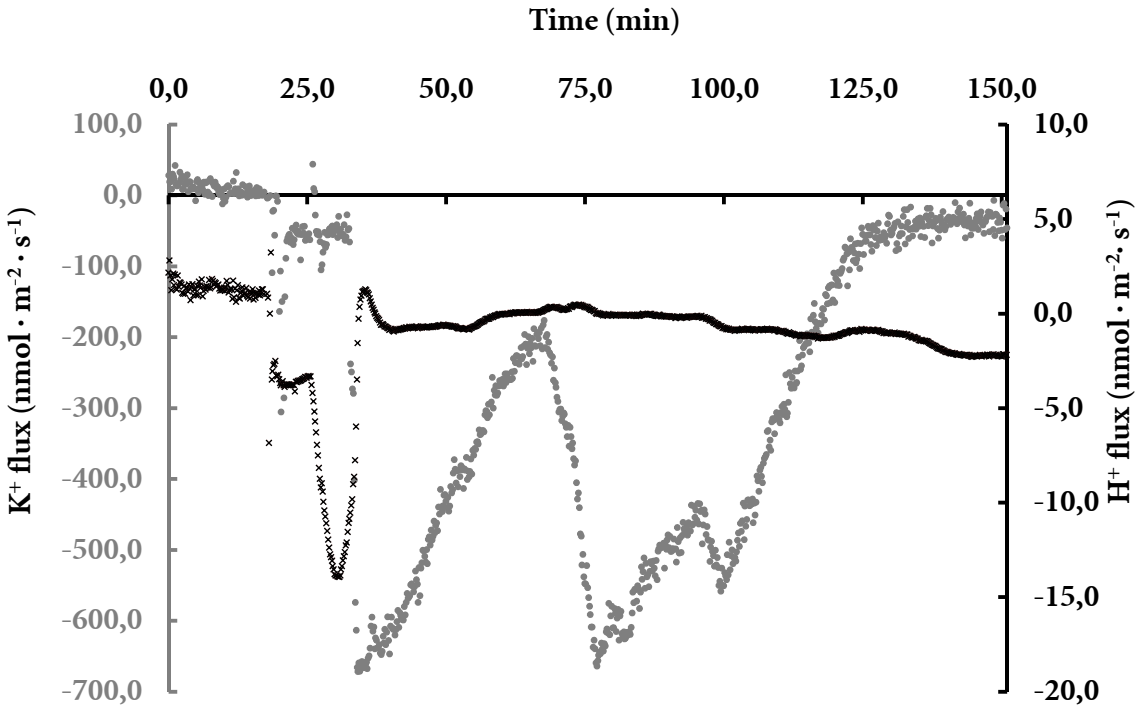

Figure 4. An example of salt stimulation. 200 mM NaCl stimulation was introduced at 20 min time point and the response was recorded for the next two hours. Gray dots indicate the real time recording of K^+ flux, black crosses indicate the real time recording of H^+ flux.

For measuring the H^+ and K^+ flux responses to auxin the occurrence of pH band formation in *Chara* intermodal cells (Fisahn et al., 1989) may complicate the analysis, as the responses may be different in the different pH zones along the cell. Therefore, in first instance we used the vibrating probe system to identify the pH banding and the accompanying fluxes along the *Chara* internodal cells.

In the formation of the pH banding of healthy *Chara* cells under light stimulation, the proton pumps are proposed to be the driving force (Schmolzer et al., 2011). During the experiments, we observed that around 80-90% of the surface area of *Chara* cells is indicated by phenol red to be acid (Fig. 5, insert). In our system the H^+ and K^+ selective probes were moved along the longitudinal axis of the *Chara* internodal cells over a certain distance to cover at least one acid band and one adjacent alkaline band. This resulted in H^+ and K^+ flux data for the different positions along the cell that could be linked to the acid and alkaline regions. The average fluxes from a representative cell in the stable state are shown in figure 5. Movement of the tip of the vibrating probe along the cell

length showed clear proton influx and efflux zones (Fig. 5), indicating the acid (proton efflux) and alkaline (proton influx) zones. Among these different zones, the probes also passed three chloroplast-free neutral line points (see Foissner et al., 2015 and P2, P7, P9 in figure 5). No evidence showed any link between the neutral line and the pH banding pattern, which matches the observation of former research (Foissner et al., 2015). The K^+ probes showed that the flux of K^+ was mainly present as a K^+ influx. Though there was no clear influx efflux pattern for K^+, a correlation between the H^+ and K^+ fluxes is present. K^+ influx was mainly present in the alkaline band (proton influx zone) and usually showed large variations in amplitude. Hence, the relatively large standard deviations of K^+ influx in the alkaline zones (Fig. 5). While in the acid band, K^+ uptake activity is much less, and only relatively small K^+ influxes were measured. In contrast to this with mechanical or chemical stimulations, K^+ efflux could also be recorded in the acid zone together with an enlarged H^+ efflux, as see in figure 4.

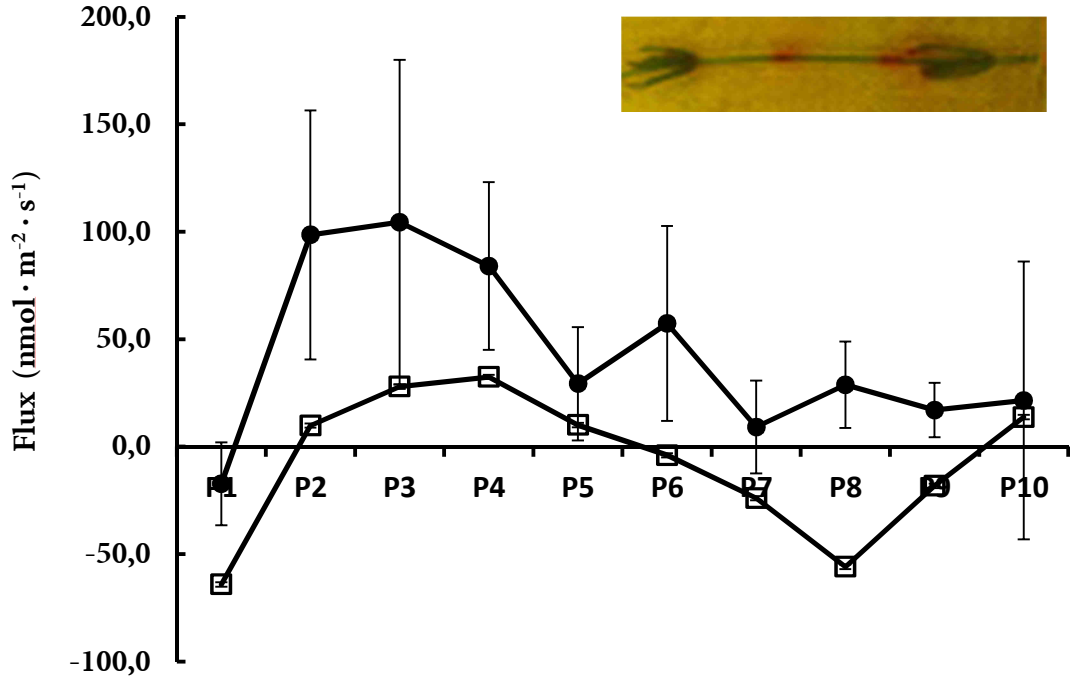

Figure 5. H^+ and K^+ fluxes for 10 different positions (P1-P10) along the longitudinal axis of the cell. Distance between each measuring point ranges between 1- 5mm. Every data point is an average of 5-10 min measurements of real-time flux with standard deviation. P2, P7, P9 are measurements done beside three continuously neutral lines points along the probe moving direction. P10 was measured near the cell node of the same moving direction. Positive flux in the figure represents an influx of positive ions. Solid circle indicates the K^+ flux. Open squares indicate the H^+ flux.

Light, known to be the trigger of the band formation, as another activator of proton pumps, was tested by switching on or off the microscope light. As expected, light can trigger an increase of the efflux or influx of protons dependent on the position along the axis of *Chara* cells. At the boundary of different bands, the proton flux showed fluctuations (unstable patterns) between influx and efflux during the test period (0.5-1 hour). Together with the proton flux, the flux of potassium also showed a strong response to the light, with more active influx in the alkaline band upon light stimulation. Switching off the light caused an immediate reduction of potassium influx or even triggered an efflux. An example is given in figure 6.

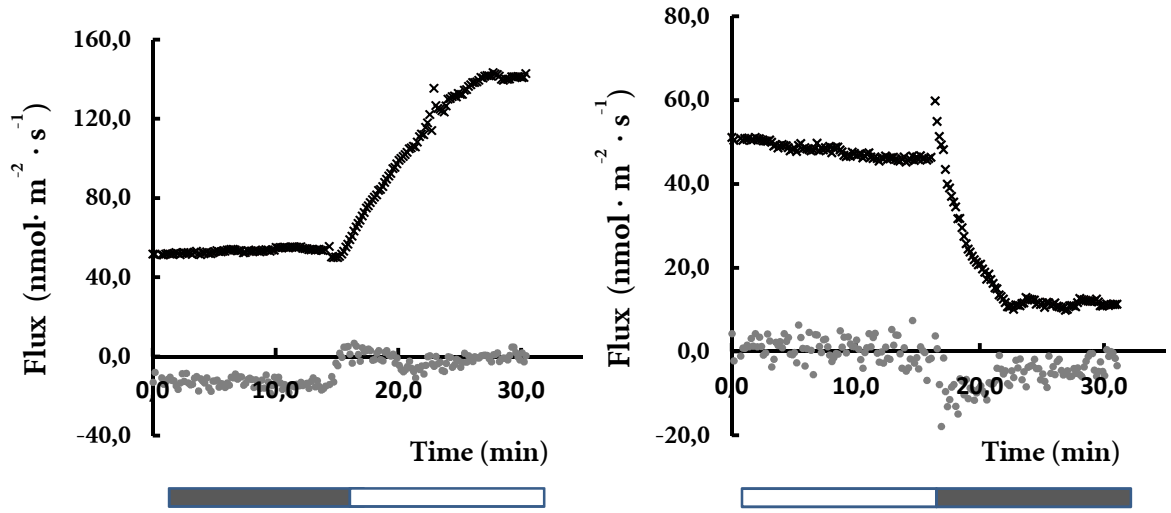

Figure 6. An example of light stimulation of the real-time H^+, K^+ fluxes. Gray dots indicate the real time recording of K^+ flux, black crosses indicate the real time recording of H^+ flux. Bars below indicates the light conditions. White bar shows the period with light on and dark bar shows the period with light off.

Auxin dependence

The effect of IAA on H^+ and K^+ fluxes was tested with the vibrating probe system. Since IAA is an organic weak acid a disturbance of the solution pH would be calculated as proton flux based on the principle of the measurement technique. Figure 7 shows an example of pH influence on the fluxes in the acid band. Upon the addition of 1 ml APW with pH 4.0 to 3 ml APW with pH 5.1 a peak H^+ flux is induced, while there are no related K^+-flux responses.

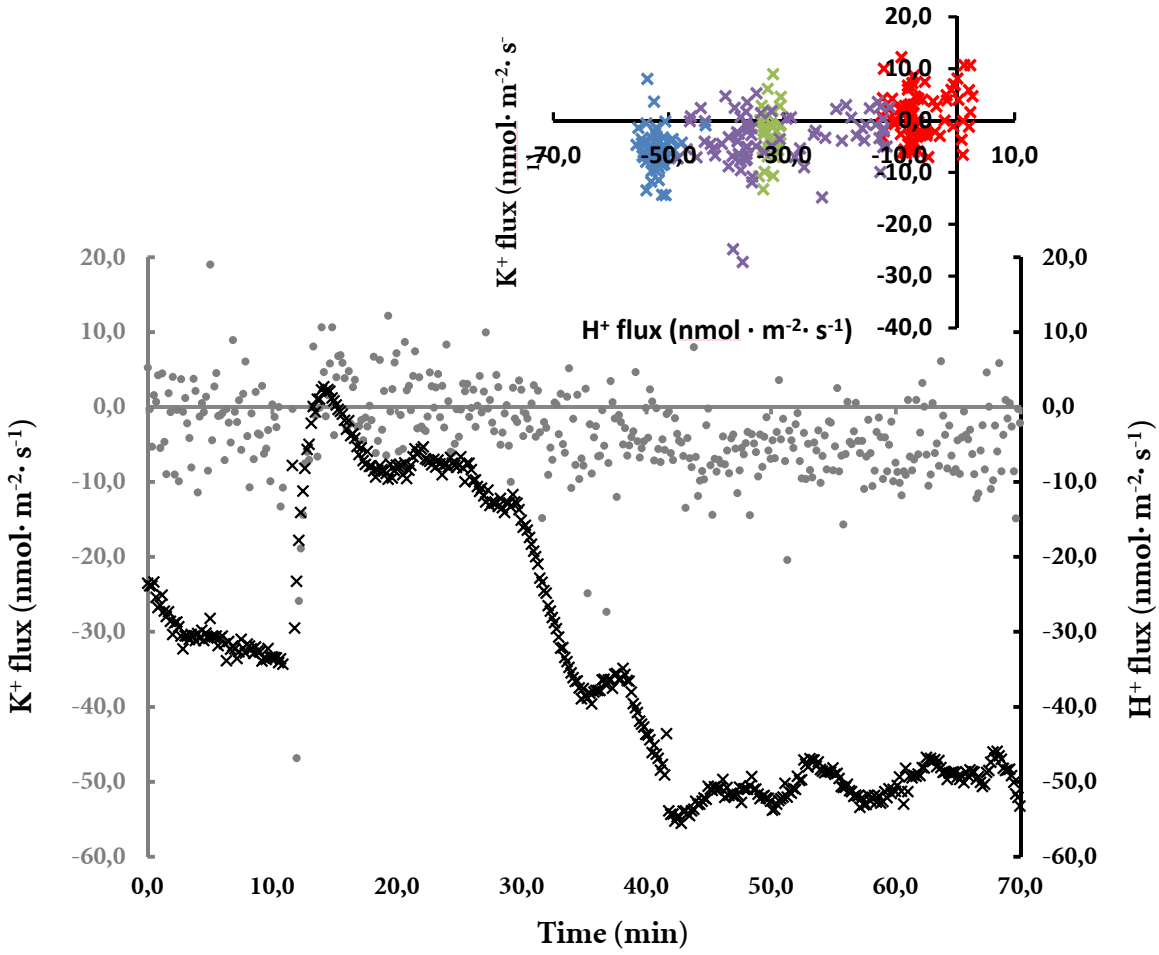

Figure 7. An example of pH stimulation. 1 ml pH 4.0 APW was added into bath solution (pH 5.0 APW) at 10 min. The response was recorded for the next hour. Gray dots indicate the real time recording of K^+ flux, black crosses indicate the real time recording of H^+ flux. The insert shows the relationship between H^+ and K^+ during a certain period. Green crosses represent the original stable state of the H^+, K^+ fluxes. Red crosses represent the first 15 min after the stimulation. Purple crosses represent the next 15 min and blue crosses represent the final stable state after the stimulation.

To separate the possible IAA influence on the PM proton pump from the acidification of the solution caused by the addition of IAA, the original bath pH without IAA was adjusted to the pH of the IAA solution (around pH 4.0) for the measurements of IAA-induced fluxes. In this situation no clear pH banding could be detected along the cell. H^+ and K^+ fluxes were measured continuously during slowly and evenly adding 1 ml 4×10^{-5}M IAA to 3 ml APW (final concentration of 10^{-5} M IAA). This addition showed no strong changes in H^+ flux (the small pH shifts are hard to avoid due to the unstable pH of the IAA

solution). However, clear IAA-induced K^+ fluxes (in the range of about 100 nmol·m^{-2}·s^{-1}) were seen in most of cases (5 out of 6). A typical example is shown in figure 8.

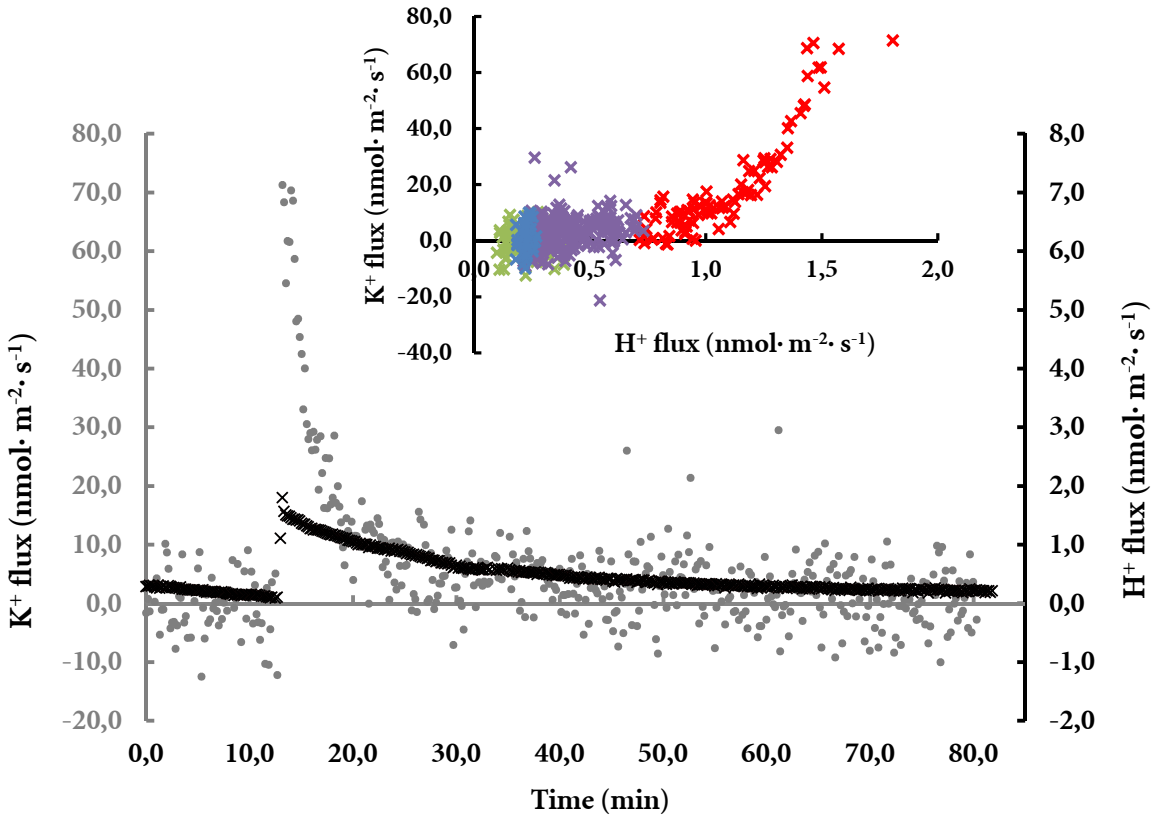

Figure 8. An example of the effect of IAA on ion-fluxes. IAA was introduced into the bath solution at 10 min. The response was recorded for the next hour. Gray dots indicate the real time recording of K^+ flux, black crosses indicate the real time recording of H^+ flux. The insert shows the relationship between H^+ and K^+ during a certain period. Green crosses represent the original stable state of the H^+, K^+ fluxes. Red crosses represent the first 15 min after the stimulation. Purple crosses represent the next 15 min and blue crosses represent the final stable state after the stimulation.

In *Chara* cells, PM H^+-ATPases are believed to be the driving force of the pH banding phenomenon and the main donator to the H^+ flux through the membrane. PM H^+-ATPases are well known to exist in fungi and higher plants and the regulation mechanisms has also been widely studied in yeast and many land plant species (Morsomme and Boutry, 2000). The C-terminus auto-inhibition domain functions as a main switch in activating the pump function. In yeast, the proton pump is activated by stepwise phosphorylation of two tandemly positioned residues, Ser-911 and Thr-912, at the C-terminus (Lecchi et al., 2007) while in land plants, a conserved penultimate threonine (pT-H^+-

ATPases) at the C-terminus is playing a key role in the regulation through phosphorylation (Wielandt et al., 2015). Based on the acid-growth theory, auxin has been proved of inducing the phosphorylation of penultimate threonine to create a binding domain with 14-3-3 proteins and further activating the PM H^+-ATPases in Arabidopsis (Takahashi et al., 2011). Nevertheless, in algea and lower plants the structure of the PM H^+-ATPases are still unknown. In moss the co-existence of a non-pT type PM H^+-ATPases has been confirmed (Okumura et al., 2012a) and there is so far no evidence of the pT-H^+-ATPase in algea species (with/without sequence information) including *Chara*.

The fungal toxic fusiccocin (FC), due to its ability to enhance the binding of 14-3-3 to the phosphorylated C-terminal domain of the PM H^+-ATPases in land plants, has been widely used in the research related to PM H^+-ATPases activities (Jahn et al., 1996). Effects of FC on the photosystem II (PSII) effective quantum yield and extracellular pH in the light-induced pH banding in *Chara* has been reported (Bulychev et al., 2005). However, as no proton fluxes were induced by IAA in the Chara internodes it may be expected that FC is also not effective. Indeed, the addition of different concentrations of FC did not resultin a change ofthe measured H^+ fluxes along the *Chara* internode cells (data not show). The apparent discrepancy between the reported FC-induced increased acidification in the banding pattern (Bulychev et al., 2005) and our results may be due to timing in experimentation (Bulychev et al., 2005 reported on an effect more than 1 h after FC application) and the age of the plant material (fully grown in our experiments) may play a role as well. Our results are definitely not enough to rule out the involvement of phosphorylation and 14-3-3 protein in the regulation of *Chara* PM H^+-ATPases. Yet the available sequence data indicates quite a variety in the PM H^+-ATPases C-terminus among different species of algea (Okumura et al., 2012b). This variety would also embrace the possibility of other new regulation mechanisms.

Conclusions

Based on the above data, it seems that the plant hormone auxin does induce cellular physiological responses in *Chara* cells, such as membrane potential changes and K^+ fluxes, that are also seen in higher plants.

On the other hand, the well documented (Rober-Kleber et al. 2003; Takahashi et al., 2012) induction of H^+-ATPase activity by auxin in higher plants was not

found in the *Chara* cells we used. The age and developmental stage of the cells may be a relevant factor in this. Young, developing cells may show the induction of proton fluxes by IAA. Future research on the relationship between IAA and *Chara* development is necessary to resolve this possibility

Though *Chara* cells have been used as an interesting research target for almost half a century, the lack of gene sequences information has slowed down the understanding progress. Further research would be interesting and necessary to find out the characteristics of these possible new type of proton pumps and further understand the auxin regulation mechanism in these plant-like algea systems.

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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

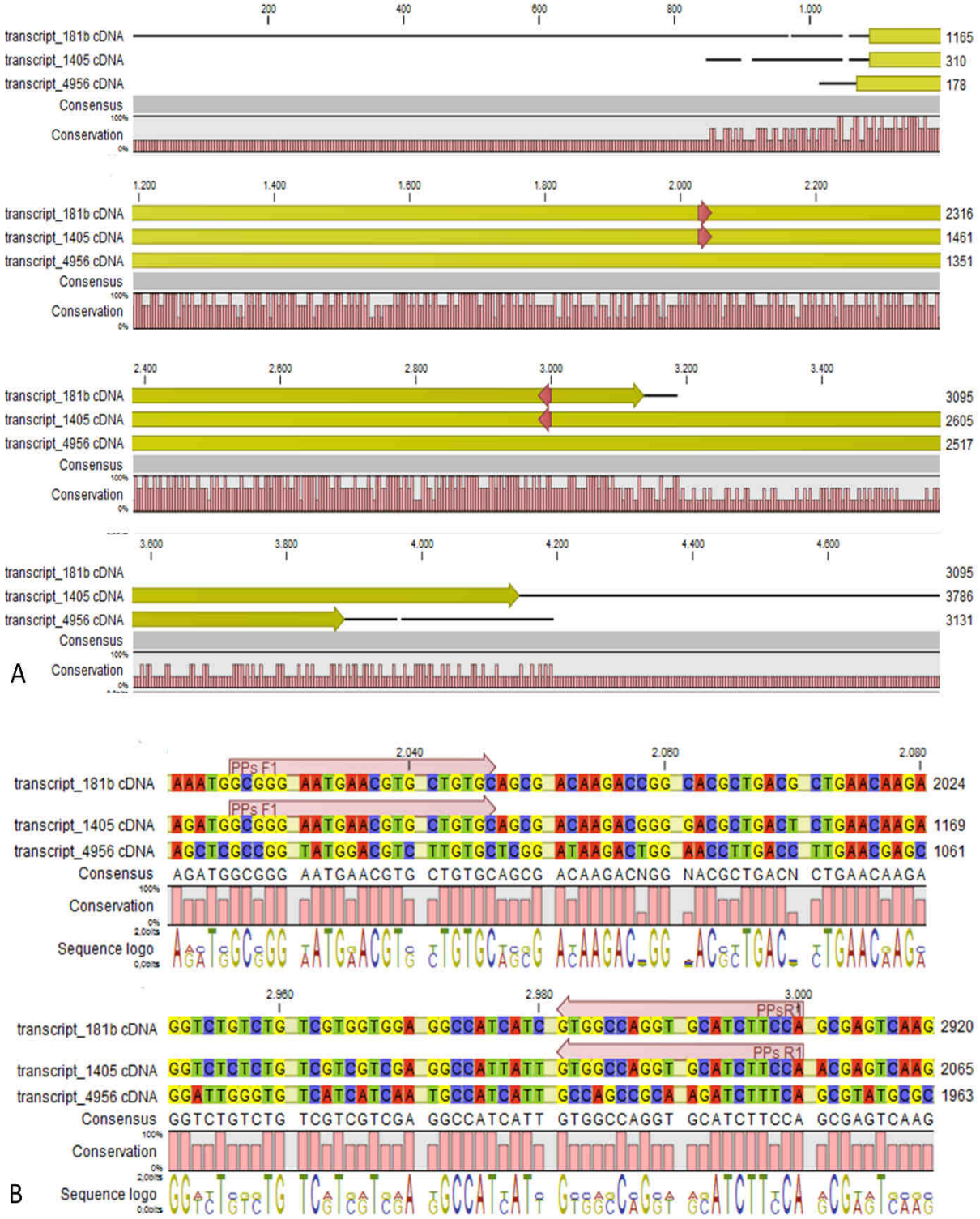

Figure 1. Alignment of the three contigs (transcript_181b, transcript_1405 and transcript_4956) with CLC workbench 7. (A) Alignment of the contigs with the predicted ORF in yellow and red arrows indicating the primers used for amplifying the conserved part. (B) Sequence information in detail regarding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

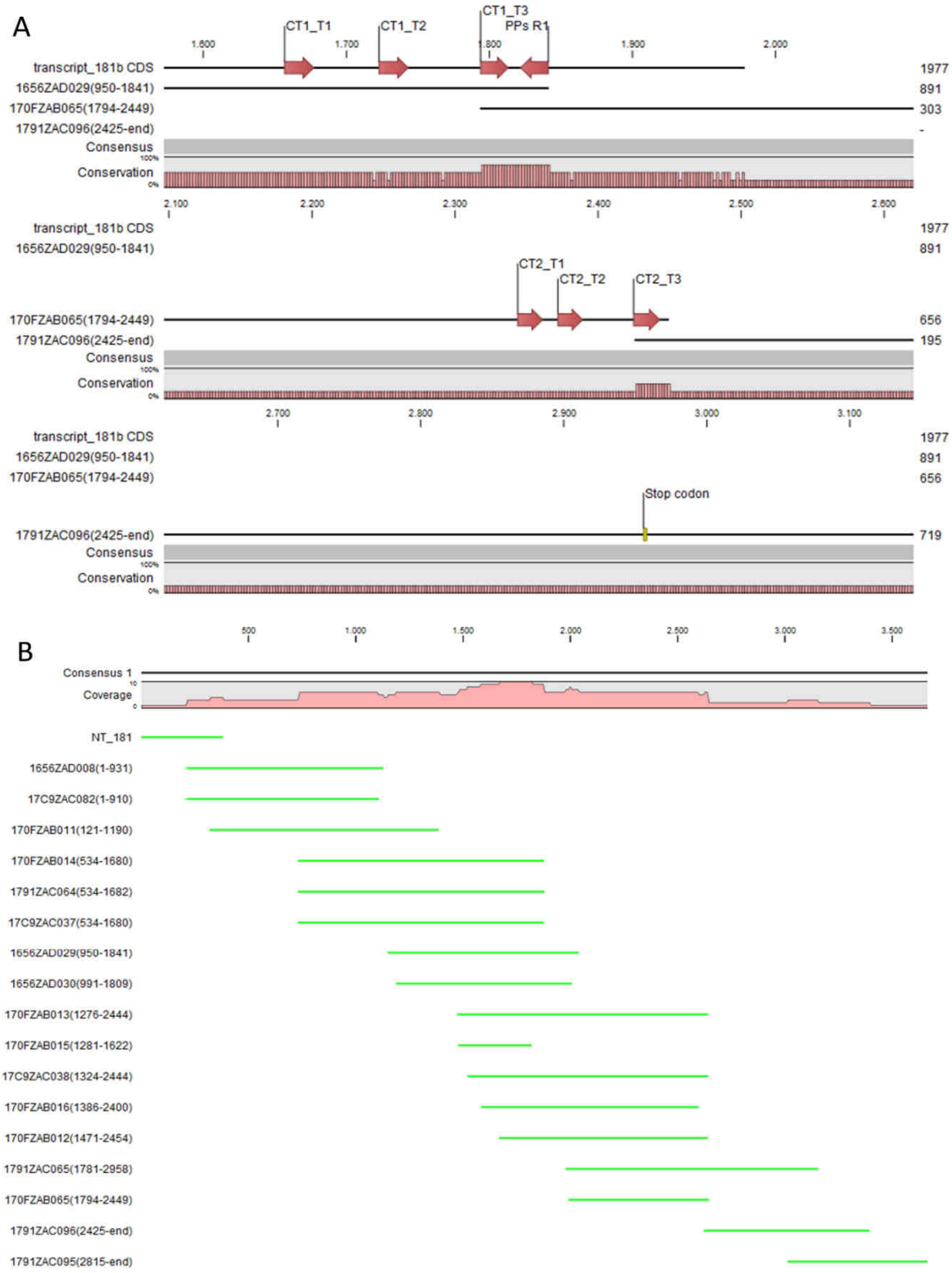

Figure 2. Alignment and assemble of PCR fragments for *CHA1* isolation. (A) Alignment of two step-wise tail PCR fragments for *CHA1* C-terminal extension with the primers indicating in red arrows. (B) Assemble of all PCR and tail PCR results for *CHA1*.

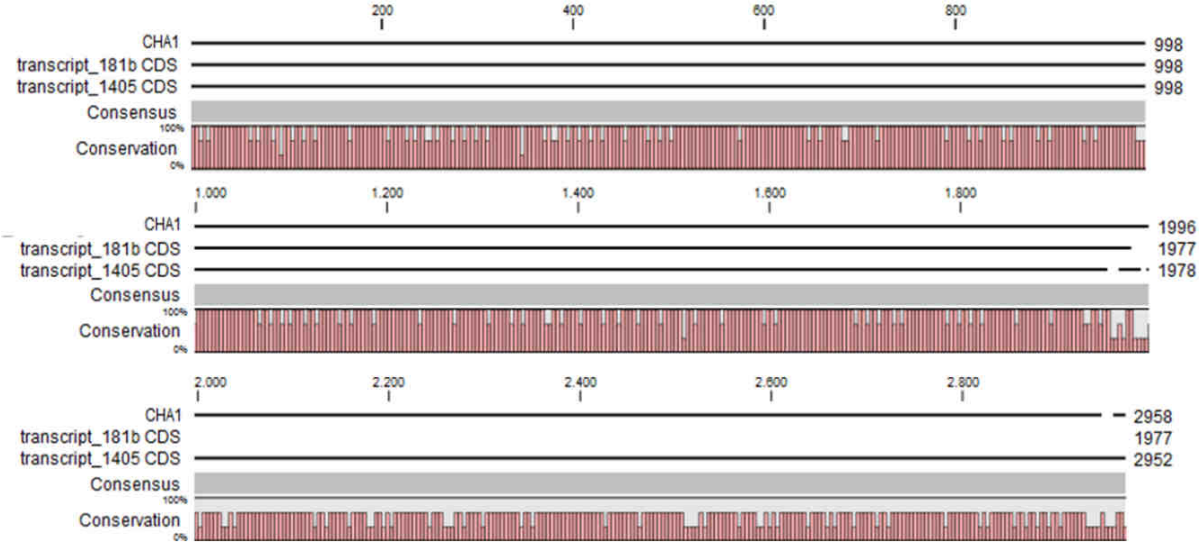

Figure 3. Sequence alignment on CLC workbench 7 of the assembled full *CHA1* (from start to stop codon) with predicted coding sequences of transcript_181b and transcript_1405.

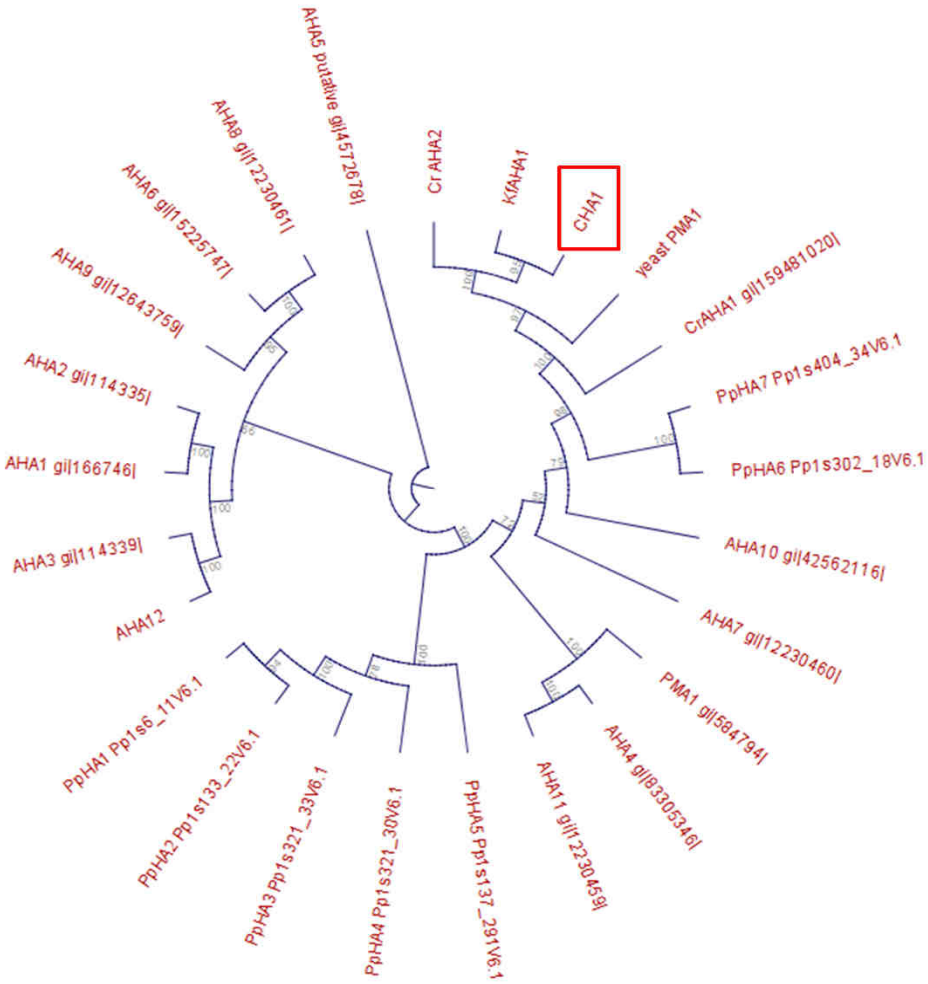

Figure 4. Phylogenetic tree of P3A ATPases, with *CHA1* highlighted in a red box.

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,

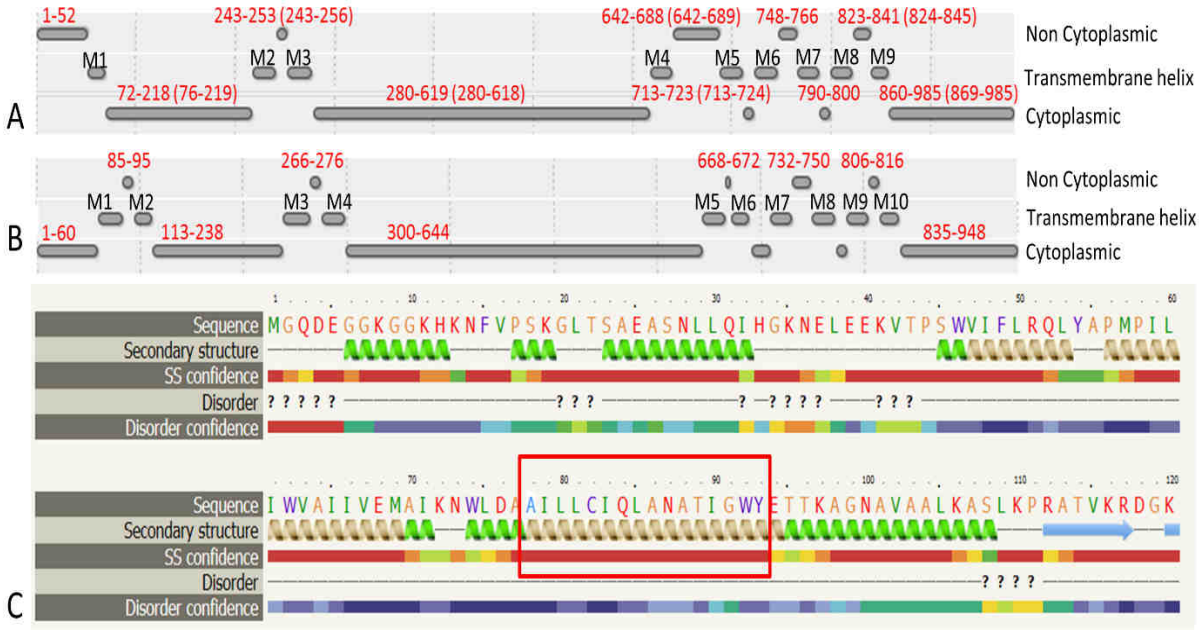

Figure 5. Predicted transmembrane segments of CHA1 from different tools. (A) Result of CHA1 from Interpro, including the prediction from Phobius and TMHMM server v.2.0 (the numbers are in brackets when they are different from Phobius). (B) Result of AtAHA2 from Interpro, including the prediction from 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

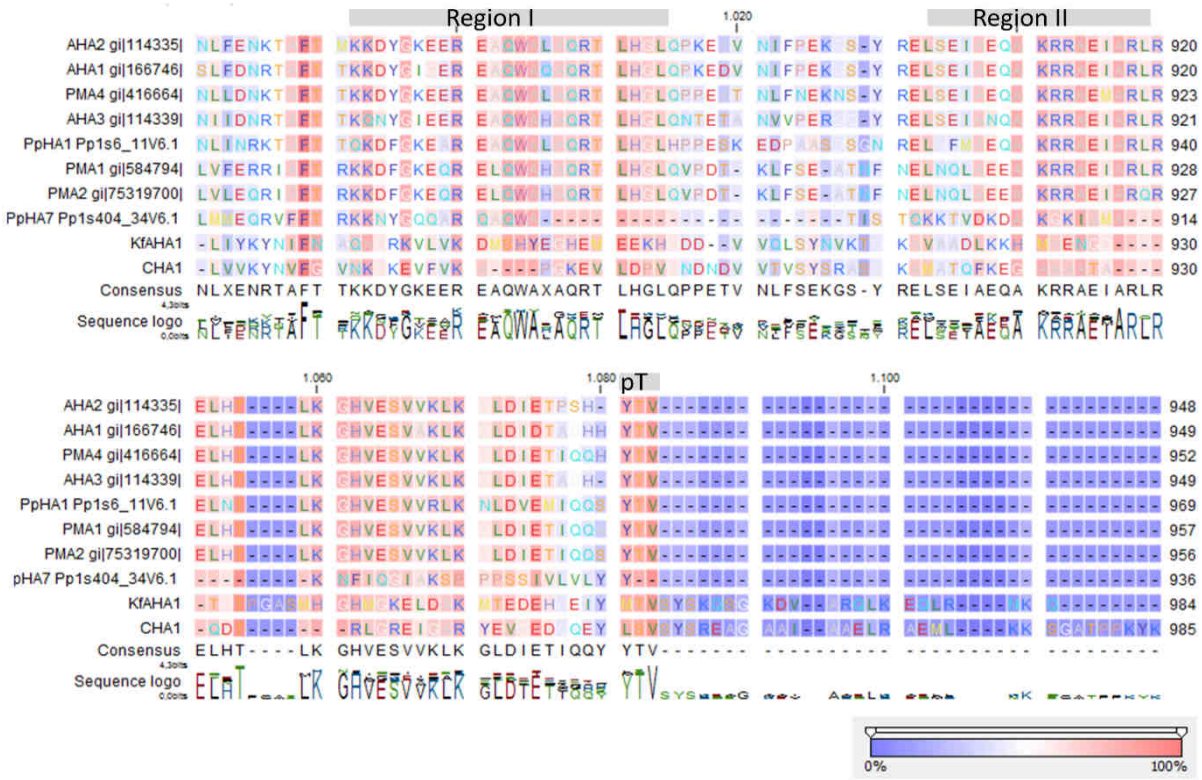

Fig 6. C-terminal alignment of CHA1 with other PM H^+-ATPases. Color bar below indicates the conservation degrees of the alignments. Gray bars above indicate the conserved C-terminal regions in land plants PM H^+-ATPases.

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

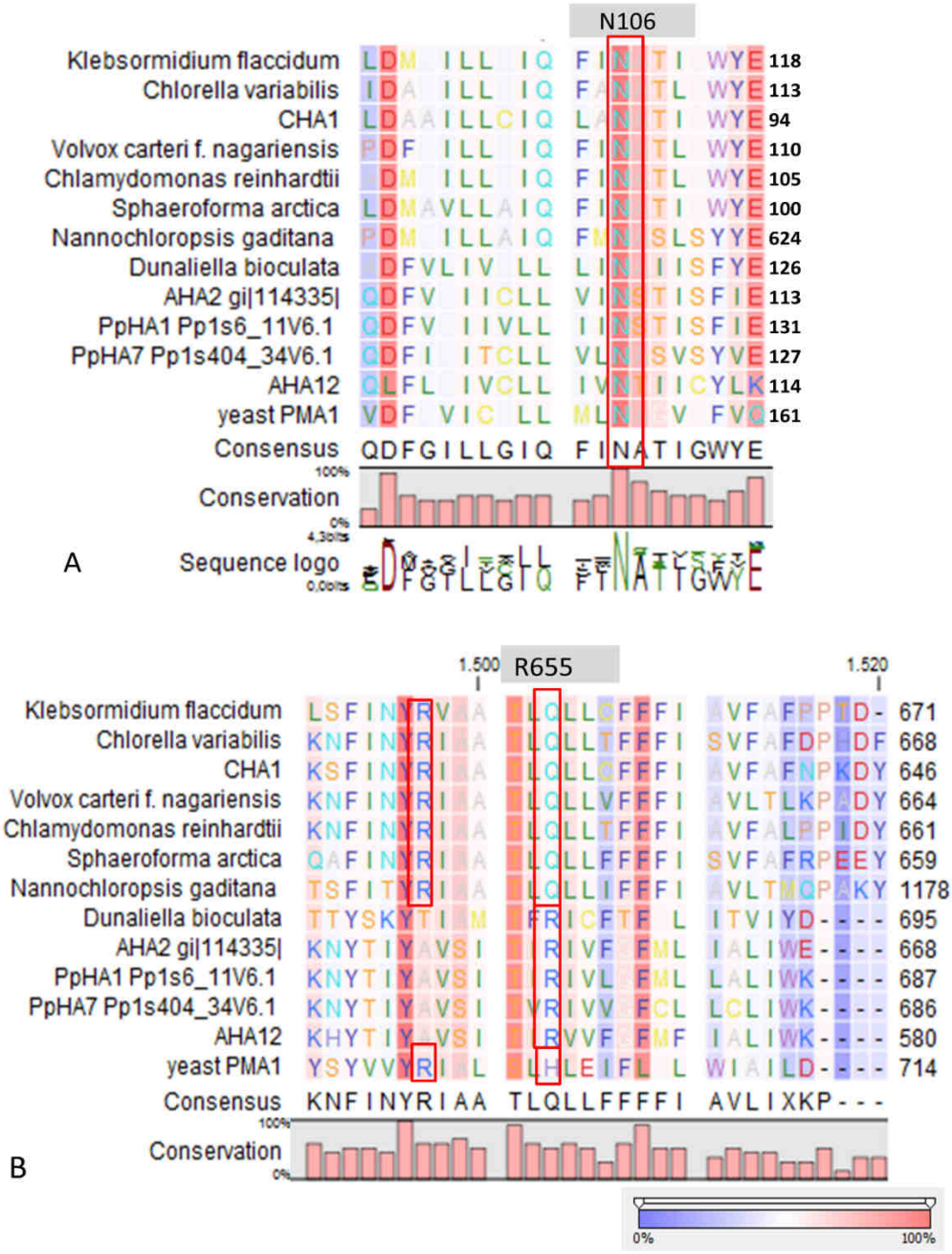

Fig 7. Alignment of transmembrane segments M2 and M5 (based on AtAHA2) of CHA1 and other P3A H^+-ATPases. (A) Alignment of M2. (B) Alignment of M5. Gray boxes indicate the residue and number in AtAHA2. Red boxes indicate certain conserved residues which may evolved with the proton up-hill transport ability.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

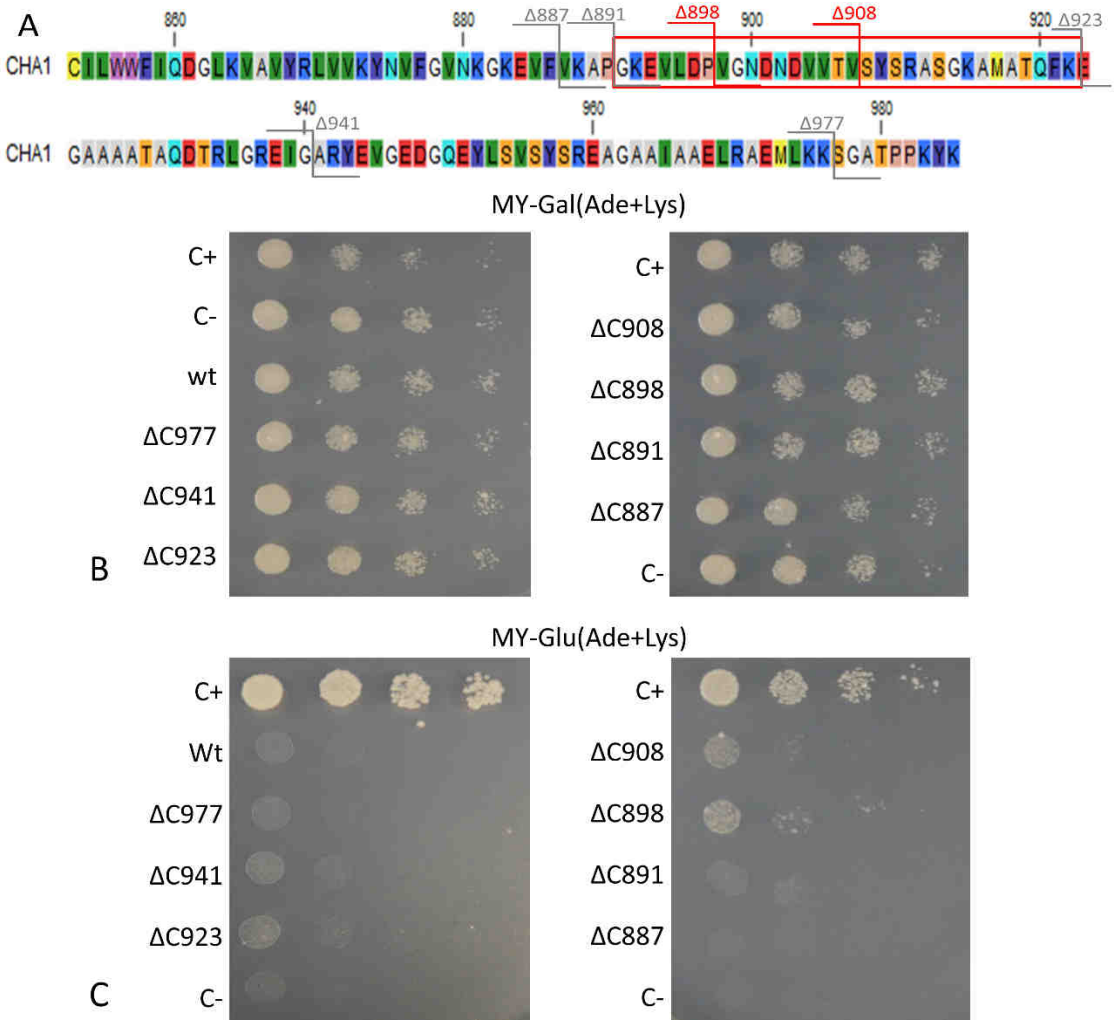

Figure 8. (A) C-terminus of CHA1. Red numbers and cutting lines indicate the positions in CHA1 from where C-terminal deletions where made that rescue the null mutant yeast strain. Gray numbers and cutting lines indicate the truncation sites of CHA1 that failed to rescue the null-mutant yeast strain. 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).

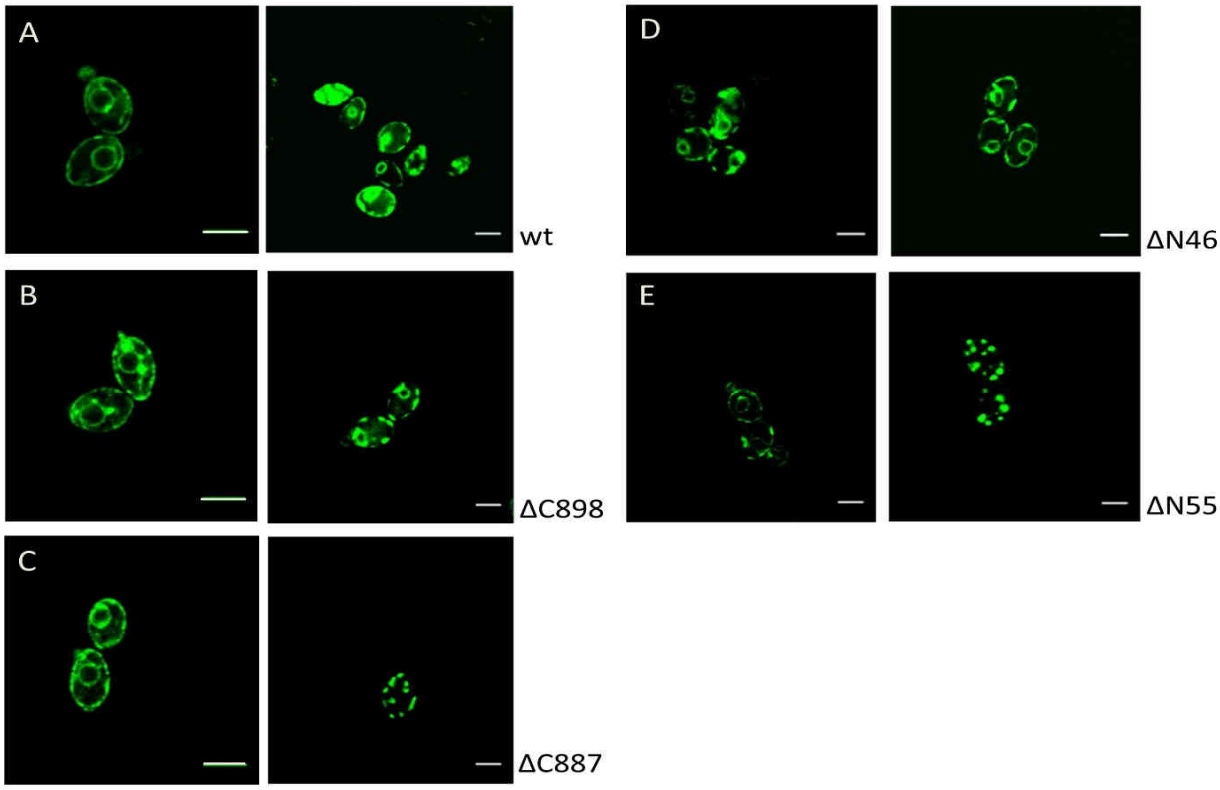

Figure 9. Subcellular localization of N-terminal GFP-CHA1 fusion protein and its N/C-terminal truncated versions in yeast BY4743 strains. Two identical localization samples of each line are presented from 3 independent transformations. (A) Wild-type YFP-CHA1 shows uneven PM and perinuclear localization. (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.

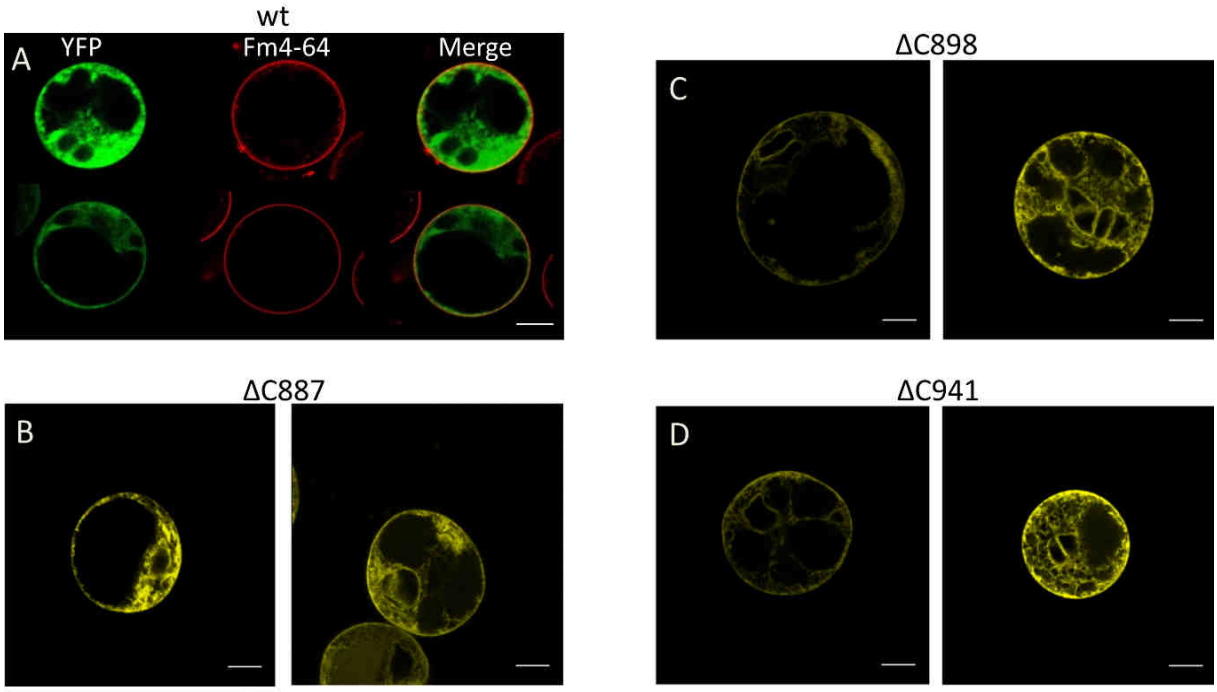

Figure 10. Localization of N-terminal YFP-CHA1 fusion and C-terminally truncated mutant verions in *Arabidopsis thaliana* Col-0 protoplasts. (A) two representative cells expressing N-terminal YFP fusion with wildtype CHA1 in green (artificial color, indicated YFP), Fm4-64 plasma membrane dye in red (indicated 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	

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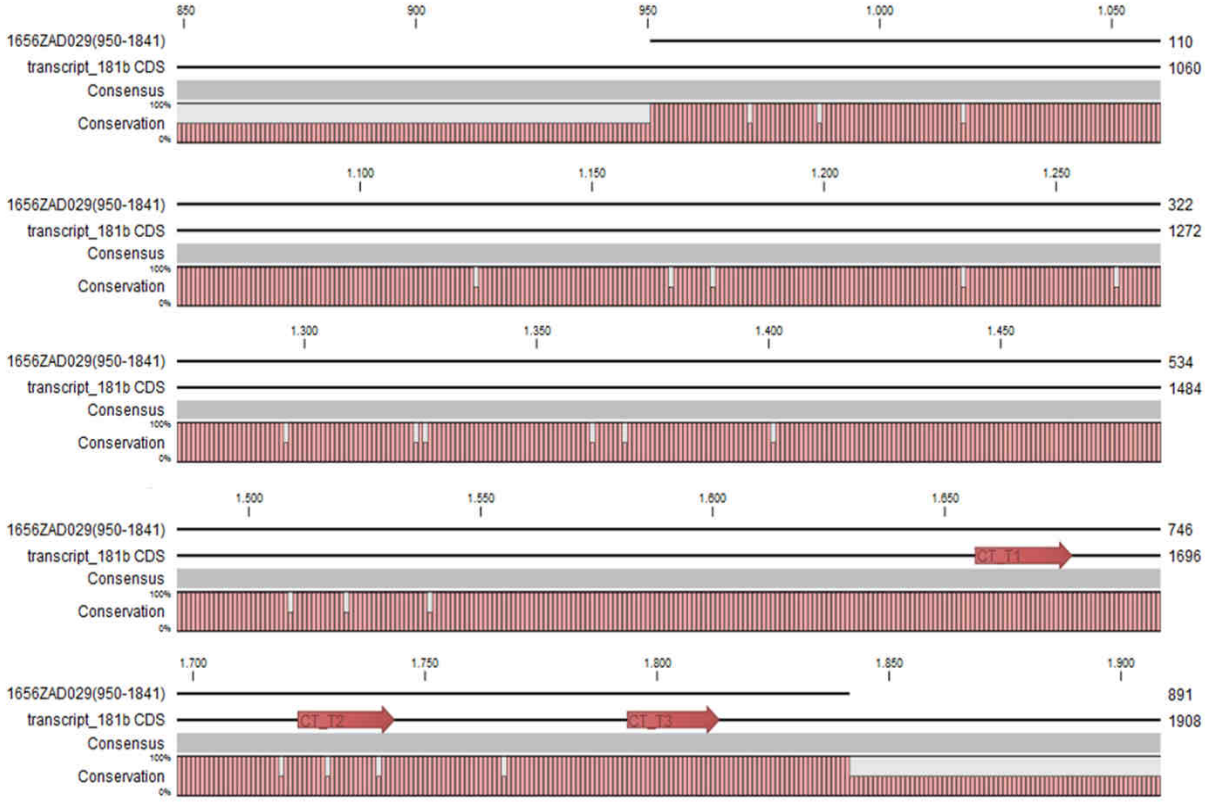
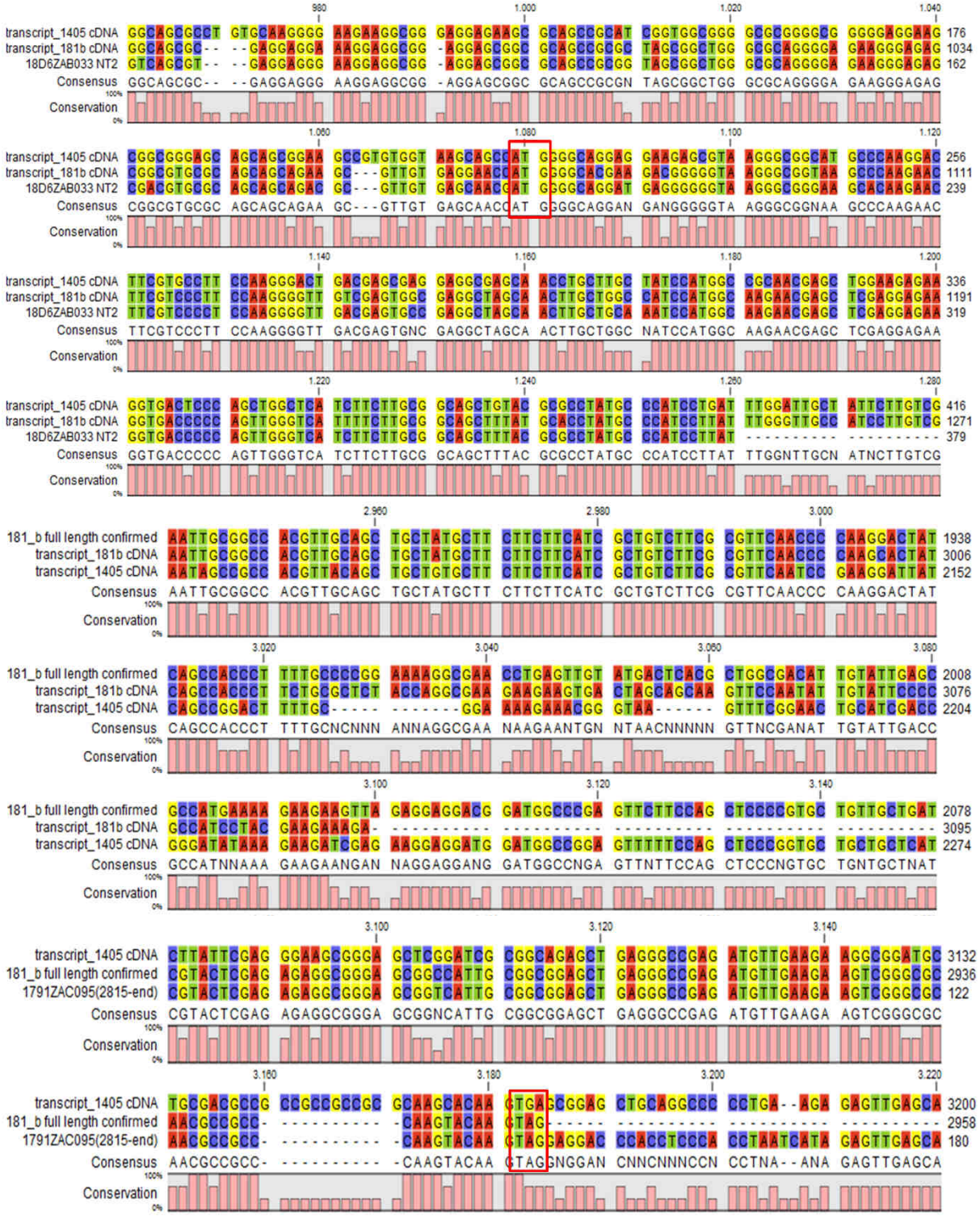
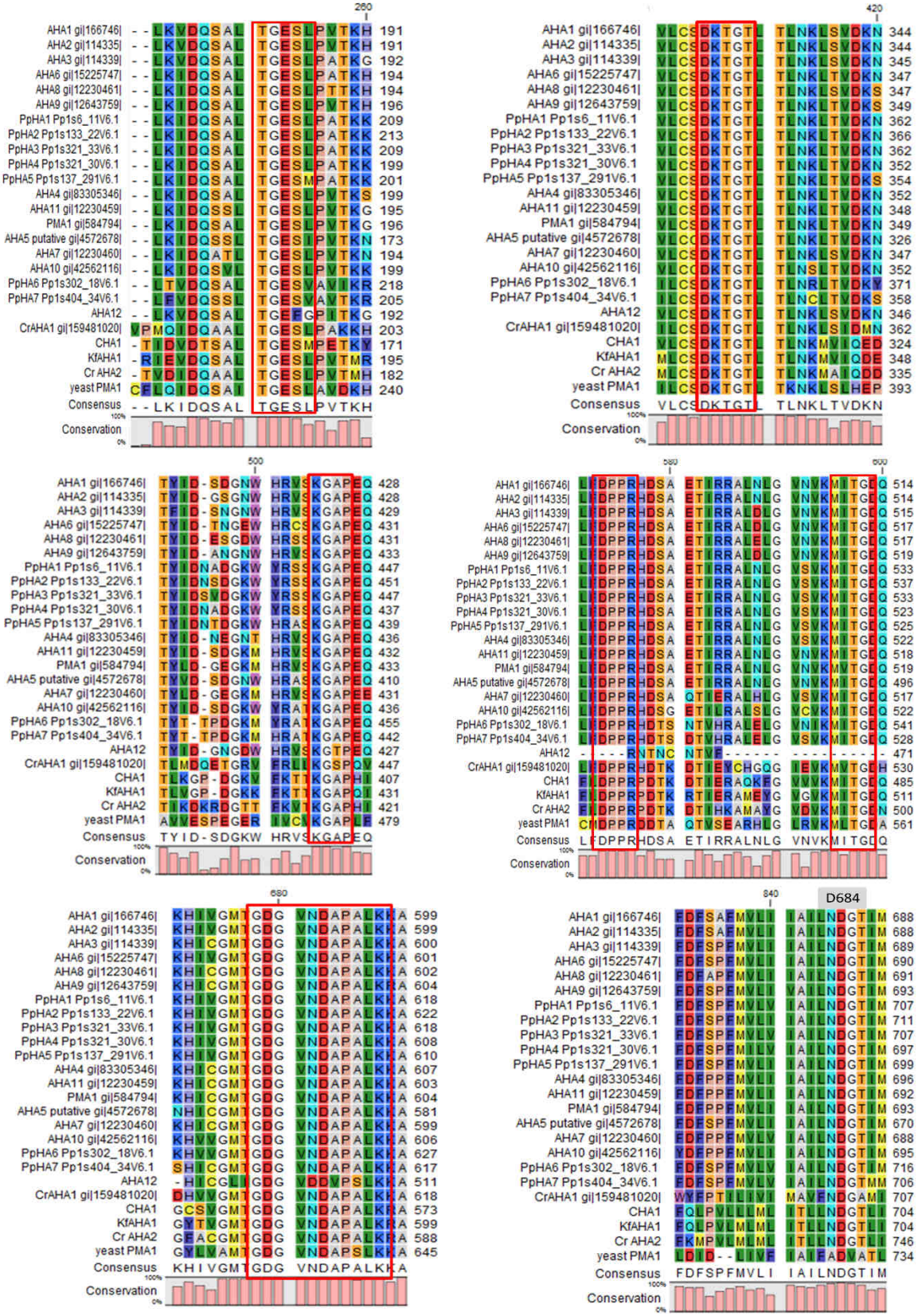


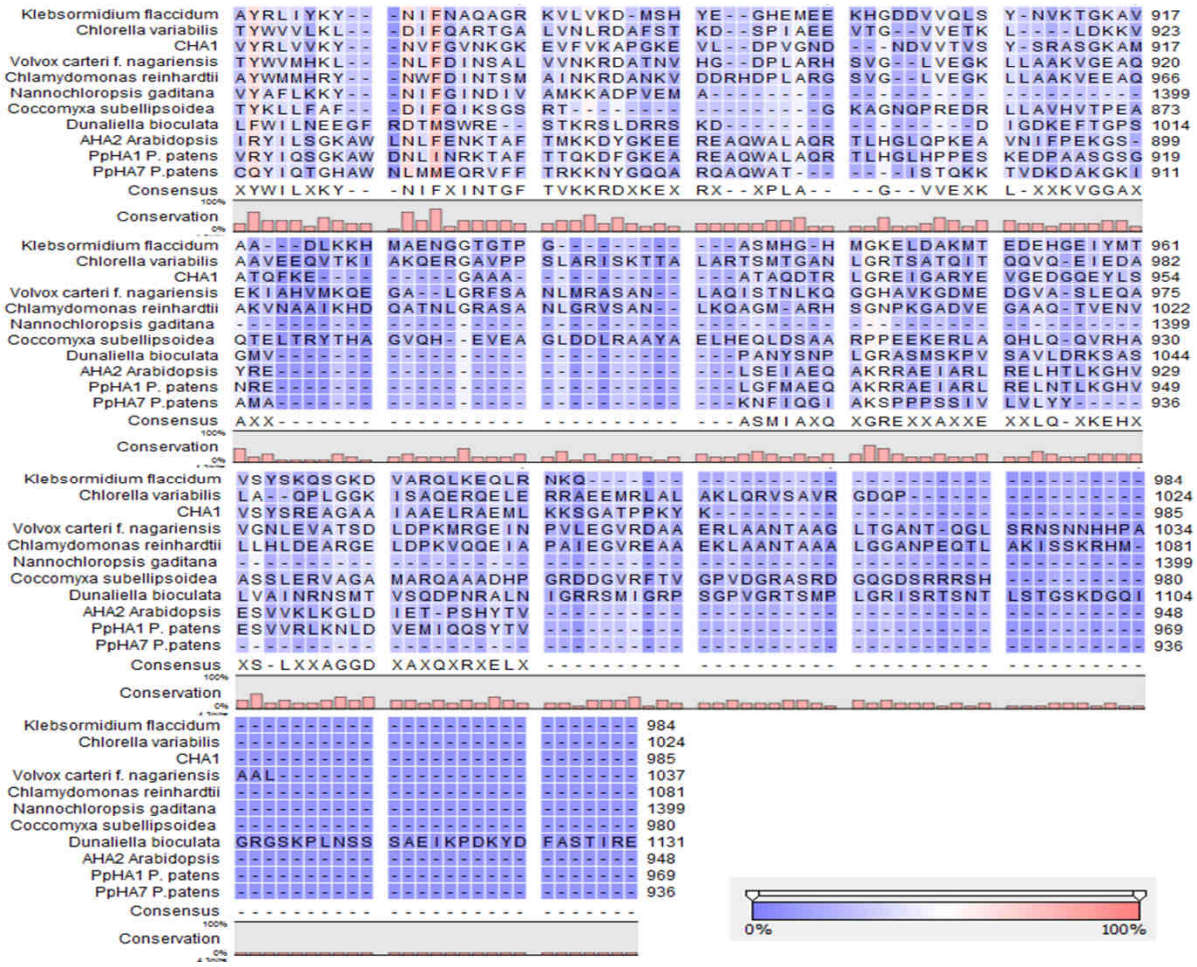
Supplementary figure 1. Alignment of transcript_181b CDS with PCR fragment of the conserved part (with primers PPs F1 and PPs R1). With red arrows indicate the primers designed for C-terminal tail PCR.



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.

Chapter 6

General discussion and conclusion

Charophytes (basal streptophytes) are a group of green algae closely related to the land plants (embryophytes). Evolved from a same single aquatic green algal ancestor, extant charophyte algae share remarkable similarities and differences with the land plants, which makes them efficacious material for studying fundamental plant biology and contribute to solving the evolutionary puzzle behind the profound transition- "terrestrialization". Six taxa are included in the extant Charophytes based on the newest taxonomy study (De Vries & Archibald, 2018), including three early divergent taxa (MCK group) of Mesotigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, and three late divergent taxa (CCZ group) of Charophyceae (Charales), Coleochaetophyceae and Zygnematophyceae.

Charophyceae were first defined as the closest relatives to land plants. This was based on single-gene analysis, and studies of the cellular structure and morphology (Karol, et al., 2001; Delwiche & Cooper, 2015). Recently, a new topology has been proposed, with Zygnematophyceae as the closer sister to land plants, supported by the analyses of large datasets derived from high-throughput transcriptome sequencing (Wickett et al., 2014). Yet this new proposal is still far from solid. Considering the huge diversity of charophyte algae and the low coverage of taxon sampling, the short nature of the assembled transcription sequences and the notion that gene expression is usually time and tissue specific, the supporting data is still relatively weak and debatable on the one hand (Wang et al., 2015). On the other hand, the fossil record for charophyte algae is sparse in general (except for the Charophyceae family), which blocks an alternative way of back-ups for either Charophyceae or Zygnematophyceae as the closer sister lineage of land plants (Delwiche, 2016; Martin-Closas et al., 2017). Nevertheless, following the nuclear genome sequencing project of *Klebsormidium flaccidum*, with the help of fast developing next generation sequencing technology, several genome sequencing projects are underway within the charophyte algae group. This will fill in the missing pages of plant adaptations from water to land and boost our exploration in all different areas including evolution, ecology and basic biology (Hori et al., 2014). In the meantime, combining the known results and current studies in physiology, cell biology of charophyte algae with the increased availability of sequencing data, will be an important and effective way to get a deeper and thorough view in fundamental knowledge of charophyte algae life, in connection with the researches in land plants.

Among these 6 classes, Charophyceae is the largest and the most complex group of Charophytes (Delwiche, 2016). Several species of Charophyceae algae are also happening to be the most extensively studied ones as model systems, such as *Chara*, due to their remarkable similarities to land plants, including cell organizations and the presence of biosynthesis and activity of many plant growth regulators (plant hormones) (Beilby, 2016).

Combining previous studies and new trends in basic research involving Charopyte algae, in this thesis, *Chara spp.* was used for the investigations aiming to support the building and testing of a *Chara* based model system for the study of evolution and basic biological principles. The perspectives that have been addressed and studied in the thesis are discussed below.

Lab culture for *Chara*

The growing interests in using *Chara* as model system for cellular and molecular level research, along with the progress in the *Chara* genome sequencing project, increase the demand for a continuous production of high quality *Chara* material. A standardized, stabilized laboratory culture of *Chara* would be an ideal solution to cope with the seasonal variety of *Chara* thalli in the natural habitat and to avoid the inconsistency in results due to the difficulties in taxonomy of *Chara* species (Schneider et al., 2016). When comparing to other well-established plant model systems, *Chara* as model system for basic research is still quite new and not wide spread. Only a limited number of research groups have been active in this field, mainly located in Australia, Japan, United States and a few countries in Europe. Establishment of a research network among these labs would be appealing and essential for a boost of *Chara* research.

By sorting out our own experience in *Chara* culture, together with the suggestions from other labs (Austria, Germany, etc.), we present in **Chapter 2** information on do's and don'ts for stable culture of *Chara* research material and hope to trigger additional input from other researchers with experience in *Chara* culture from all over the world. From these we may build a solid "protocol" for *Chara* laboratory culture to support progress in the use of *Chara* as a valuable model system and to encourage more laboratories to adopt *Chara* for their research programs.

Cellular auxin transport and signaling in Chara

Auxin as the most studied plant hormone, is well known for its essential role in plant growth, development and defense. Yet, still little is known about the origin and evolutionary pathways of auxin. In recent years, more attention is given to digging into the origin and evolution of plant hormone networks, including the biosynthesis and signaling mechanisms (Hori et al., 2014; Yue et al., 2014; Wang et al., 2015). Multispecies genome-wide analysis revealed that genes required for auxin biosynthesis and signaling pathways originate in charophyte lineages (Bowman et al., 2017). Although Zygnematophyseae are considered as the closest lineages to land plants, their structure resemblance is poor, and it seems that they have gone through a secondary structure reduction from a more complex ancestor. Meanwhile, the Charophyceae lineage has developed in a more complex direction and a strong resemblance in body structure with the land plants evolved. This is especially visible in the group of Characean algae (e.g. *Chara*, *Nitella*), which develop a body structure with leaf-like branches, stem-like internodal cells, and root-like rhizoid. Polar auxin transport (PAT) along the *Chara* internodal cells has been confirmed (Boot et al., 2012), together with the plant-like development strategy (auxin evolved polar growth and apical dominance) (Beilby, 2016). Thus, a better knowledge of the *Chara* (Charophyceae algae) auxin network would be a complement for auxin research within the land plants, which might reveal new auxin functions and working mechanisms as well as reveal evolutionary aspects of plant hormone systems in general. In addition, it might even offer an ideal simplified model for different basic research areas.

To further explore the role and mechanisms of auxin signaling and transport in *Chara* we first proposed a cell-to-cell auxin transport model for *Chara* comparing with the auxin transport model in land plants in **Chapter 3**. This, partly hypothetical, model is based on published observations within Characean algae related to polar rhizoid development, apical dominance and polar auxin transport through the internodal cells. This model supports the search for the key elements involved in auxin transport and functioning in early development and high-lights the targets which are of primary interest for more detailed investigation.

Based on literature study and the model from **Chapter 3** it is clear that (polar) auxin transport in *Chara* internodal cells on the one hand might be strongly affected by pH differences in the surrounding micro-environment along the cell

membrane. On the other hand, auxin itself might have influence on the activity of trans-membrane ion transporters, such as proton pumps, which in turn might affect the auxin trans-membrane transport directly or via (local) pH changes. To contribute to our understanding of this whole puzzle, we studied the exogenous auxin effects on membrane potential and ion transport (in particular, H^+ and K^+ fluxes) across the *Chara* internodal cell plasma membrane in **Chapter 4**. The membrane potential data give an integrate whole cell picture of the electric signals, while the ion-flux measurement with ion-selective electrodes capture the real-time specific ion movement. This combination links the potential changes to the ion fluxes in detail and gives a more thorough picture of how *Chara* cells respond under the stimulation of auxin.

Interestingly, from our experiments it seems that auxin cannot stimulate the plasma membrane proton pump (PM H^+-ATPase) activity of *Chara* internodal cells. This is in contrast with the effect of auxin in higher plants, where auxin is a well-established stimulator of the PM H^+-ATPases (Takahashi et al., 2012). Neither works the fungal toxic fusiccocin in *Chara* cells, which is commonly used to irreversibly activate the proton pumps in land plants. On the other hand, the addition of auxin did obviously stimulate an influx of K^+, while only lowering the pH of the medium had no effects. It confirms that auxin, known as a growth regulator in land plants, could also influence the growth of *Chara*, such as triggering a transmembrane electric signal or accelerate the nutrition absorption (Christian et al., 2006; Osakabe et al., 2013), while there is no direct evidence in contribution to the regulation of *Chara* PM H^+-ATPase. These findings triggered us to perform a more detailed investigation of the PM H^+-ATPase(s) of *Chara*.

The activity and regulation of Plasma membrane (PM) H^+-ATPase of *Chara*

PM H^+-ATPases are known as the primary plasma membrane transporters of plants and fungi, in primary or secondary charge of nutrients uptake, osmotic balance, signaling etc. Besides the coexistence of Na^+/K^+ pumps and PM H^+-ATPases in some chlorophyte green algae, a more ancient sister group of charophyte green algae, it is reasonable to presume that PM H^+-ATPases also act as the primary transporter in most of the charophyte algae (Pedersen et al., 2012). In addition, in *Chara* a unique phenomenon has been noticed decades ago, which is the pH banding phenomenon along the *Chara* internodal branch cells under the light stimulation in which the H^+-ATPases play a prominent role

(Foissner & Wasteneys, 2014). This pH banding mechanism is believed to facilitate carbon source absorption for photosynthesis and reduce photodamage (Schmolzer et al., 2011). Studies on the formation and cellular organization of pH banding have become available over the last decades and developments in this area are still going on. Although the activation of PM H^+-ATPases is known as one the key elements, and PM H^+-ATPases have been well studied in fungal and land plants, barely nothing is known about the PM H^+-ATPases in green algae, leaving it as an undeveloped treasure (Portillo, 2000).

In **Chapter 5**, the isolation, sequencing and identification of a potential *Chara* PM H^+-ATPase gene (*CHA1*) is the first step to understanding of *Chara* PM H^+-ATPases in functioning and evolutionary perspective. Interestingly, but not surprising in the light of our results presented in **Chapter 4**, the C-terminal of CHA1 is quite different from the rather conserved C-terminal from land plants PM H^+-ATPases. CHA1 shows no conserved penultimate threonine or well recognizable 14-3-3 binding domain (Pedersen et al., 2016). This difference also supports an explanation for the results we got in **Chapter 4**, namely that proton flux was not affected by endogenous auxin or FC. Furthermore, alignment of CHA1 with other PM H^+-ATPases amino acid sequences among the green algae species and land plant species, revealed that there might be a different pattern within the conserved ion transport cavity (transmembrane segments M5 and M6) among the green algae, which is different from the land plants (Buch-Pedersen and Palmgren, 2003). We must bear in mind, however, that with the limited information we have at the moment, the above statements only are an indication and points the direction for further exploration. More upcoming genome sequence information from *Chara* and other green algae species (especially within the charophyte group) is necessary.

Concluding remarks

Boosted by the next generation sequencing technology, whole genomic sequencing projects among the green algae species including *Chara*, are underway and promise an acceleration in our knowledge acquisition. It offers a trend and possibilities to use *Chara* as a model system, representing a simplified version of embryophytes (land plants). In this thesis, I have studied *Chara* algae at the traditional plant physiology level and made a start to expand further into the molecular level. From the well-established membrane potential

measurements dated back in the 1960s, 1970s, through real-time selective ion-fluxes measurements developing in 1990s and early 20th century, till the attempts to isolate a single proton pump gene and to study the functionalities and regulation mechanisms, I followed a route to narrow down from the whole cell electrical signal, to specific ion transporters, including channels and pumps, up to the molecular level of a certain proton pump. It contributes to a better understanding of *Chara* algae itself, shows a route for better understanding of auxin transport and signaling evolution and provides tools for a better usage of *Chara* as a model system for basic plant research.

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Summary

Multicellular giant algae *Chara* species have been widely used in physiological studies for decades. Thanks to the large cell size, and the easy accessible and well-controllable cell membrane system, research on these cells specifically targeted ion channels, cytoplasmic streaming, hormone transporting, cellular organization and so on. With the fast progress in bio-informatics and molecular biology, also for *Chara*, the need to complement this model system with more meticulous and thorough studies at the molecular level is apparent.

In our studies we started to link the physiological phenomena associated to the role of the plant hormone auxin to molecular mechanisms, impelling a more advanced and comprehensive usage of *Chara* as a model system.

Chapter 1 reviews some of the remarkable features of *Chara* within the focus on cell biology and electro-physiological studies. A model is proposed for the possible mechanism behind the *Chara* band formation phenomenon. Two key elements, auxin and plasma membrane (PM) H^+-ATPases are highlighted with regard to their known characters from the higher land plant model systems.

To be able to exploit *Chara* as a research model system beyond classical physiological studies a sustainable and standardized *Chara* culture is not only handy but also necessary. In **Chapter 2**, the experiences and lessons gained from the *Chara* laboratory culture experimentation are listed and discussed. Though in general, the culture of *Chara* in a laboratory environment is inexpensive and needs not much special care, the culture as a whole system with reliable production is quite fragile. A minor disturbance or environmental change may cause a severe damage to the whole culture system. To establish a healthy, long-lasting *Chara* laboratory culture, a high starting density turned out to be required. The right combination of soil, light and temperature is crucial for the dominance of *Chara* against the secondary algae/ fungi/ bacteria. In principle, low nutrient, low light intensity and relatively low temperature could sufficiently diminish the overgrowing of unwanted plankton and Cyanobacteria. Other than the fundamental settings, hygiene is always important when handling the culture.

In **Chapter 3**, the role and effects of auxin in the functioning and membrane transport in algae cells was studied and analyzed in comparison with auxin's role and effects in higher plants. Two parallel models based on Arabidopsis and *Chara* were built to highlight the similarities and differences, which indicates

that *Chara* cells have certain properties to provide a good model system for auxin research. The chapter lists out the unknown from the known and points out new research directions.

In **Chapter 4**, the effects and possible role of auxin in *Chara* cells was investigated. Membrane potential and ion fluxes (K^+ and H^+ in particular) of intact *Chara* internodal cells were measured upon different stimuli, e.g. light, salt, auxin and pH. Through the noninvasive ion-selective vibrating probe scanning electrode technique, the pH banding pattern of *Chara* internodal cells can be monitored, as well as the real time K^+ and H^+ ion fluxes responding to the light and ion solution change. Results show the physiological responses of *Chara* cells towards the application of exogenous auxin (mainly IAA) stimulation, such as a hyperpolarization of the membrane potential. Investigation of K^+ and H^+ ion fluxes separately, reveals that compared to K^+ ion fluxes, there is no remarkable influence on H^+ ion fluxes by the addition of IAA. Thus, the combination of data indicates that, different from the land plants, auxin may change the permeability and/or (K^+) channel activities of the *Chara* plasma membrane, but it has no strong effect on *Chara* PM H^+-ATPases.

To understand the differences in relationship between auxin and *Chara* PM H^+-ATPases, as compared to higher plant H^+-ATPases, and the possible differences between *Chara* PM H^+-ATPases and other well studied PM H^+-ATPases from higher plants, in **Chapter 5**, a potential *Chara* PM H^+-ATPase gene (*CHA1*) was isolated, sequenced and analyzed. Bio-informatics analysis showed a new pattern in the evolutionary perspective between algae and land plants. Heterologous expression of CHA1 in yeast and plant protoplast confirmed that CHA1 can reach the plasma membrane and function as a PM H^+-ATPases with an auto-inhibition domain at the C-terminal. Although, the switch mechanism of CHA1 seems different from the known plant PM H^+-ATPases, as they may not need the involvement of a 14-3-3 protein combination.

In conclusion, this study illustrates that even though the pH difference among/ along the *Chara* cell membrane may influence auxin transport and distribution, auxin itself doesn't show direct effects on the activity of *Chara* PM H^+-ATPases. *Chara* plasma membrane transport under different conditions was studied. In specific, PM H^+-ATPase, as a key player in the membrane transport system, was investigated at the molecular level. Bio-informatics and functional analysis of the isolated *Chara* PM H^+-ATPase reveals a new amino acid pattern which could be

essential for structural stabilization ATP hydrolysis and proton transport. This includes a potentially different regulation mechanism from the known plant and yeast PM H^+-ATPases, which would shed light on the PM H^+-ATPase studies within the algae group and from the evolutionary perspective. By building up a standard lab culture and new progress at the molecular level, the *Chara* cells will offer a re-newed promising platform for different fundamental and practical studies.

Samenvatting

De multi-cellulaire zeer grote Chara algensoorten zijn al decennia op grote schaal gebruikt in fysiologische onderzoeken. Dankzij de enorme celgrootte en het gemakkelijk toegankelijke en goed te controleren celmembraansysteem richtte het onderzoek aan deze cellen zich vooral op ionen transport, cytoplasmastroming, hormoontransport, cellulaire organisatie, enzovoort. Met de snelle vooruitgang in bio-informatica en moleculaire biologie, is de noodzaak om ook het Chara modelsysteem aan te vullen met meer gedetailleerde studies op moleculair niveau duidelijk.

In ons onderzoek zijn we begonnen met de fysiologische verschijnselen die zijn geassocieerd met de rol van het planten hormoon auxine te koppelen aan moleculaire mechanismen, waardoor een meer geavanceerd en uitgebreid gebruik van Chara als modelsysteem inzichtelijk wordt.

Hoofdstuk 1 bespreekt enkele van de opmerkelijke kenmerken van Chara binnen de focus van celbiologie en elektrofysiologische studies. Een model wordt voorgesteld voor het mogelijke mechanisme achter het fenomeen Chara pHbandvorming. Twee sleutelelementen, auxine en plasmamembraan (PM) H^+-ATPasen worden benadrukt met betrekking tot hun bekende functies in de hogere landplant-modelsystemen.

Om Chara te kunnen exploiteren als een onderzoeksmodel dat verder gaat dan de klassieke fysiologische studies, is een duurzame en gestandaardiseerde Chara-cultuur niet alleen handig, maar ook noodzakelijk. In **hoofdstuk 2** worden de ervaringen en lessen uit de Chara-laboratoriumkweekexperimenten benoemd en besproken. Hoewel de kweek van Chara in een laboratoriumomgeving over het algemeen goedkoop is en niet veel speciale zorg behoeft, is de cultuur als geheel met betrouwbare productie van voldoende gezond celmateriaal vrij fragiel. Een kleine verstoring of verandering van het milieu kan ernstige schade aan het hele kweeksysteem veroorzaken. Om een gezonde, langdurige Chara-laboratoriumkweek tot stand te brengen, bleek een hoge aanvangsdichtheid vereist. De juiste combinatie van bodem, licht en temperatuur is cruciaal voor de dominantie van Chara tegen de secundaire algen/ schimmels/ bacteriën. In onze experimenten zien we dat een lage nutriënten concentratie, een lage lichtintensiteit en enn relatief lage temperatuur het overgroeien van ongewenste

plankton en cyanobacteriën voldoende kunnen verminderen. Naast de basale groei condities, is hygiëne altijd belangrijk bij het omgaan met de kweek.

In **Hoofdstuk 3** worden de bekende rol en effecten van auxine in het functioneren en membraan transport in algencellen bijeen gebracht en geanalyseerd in vergelijking met de rol en effecten van auxine in hogere planten. Twee parallelle modellen gebaseerd op Arabidopsis en Chara werden gebouwd om de overeenkomsten en verschillen te benadrukken. Hieruit blijkt dat Chara-cellen bepaalde eigenschappen hebben om een goed modelsysteem voor auxineonderzoek te bieden. Het hoofdstuk benoemt de onbekende aspecten die volgen uit debestaande kennisen wijst op nieuwe onderzoeksrichtingen.

In **Hoofdstuk 4** worden de effecten en mogelijke rol van auxine in Chara-cellen beschreven. De membraanpotentiaal en ionenfluxen (in het bijzonder van K^+ en H^+) van intacte Chara-internodale cellen werden gemeten in reactie op verschillende stimuli, b.v. licht, zout, auxine en pH. Door de niet-invasieve ion-selectieve vibratieprobe-scanningelektrodetechniek kan het pH-bandpatroon van Chara-intermodale cellen worden gevolgd, evenals de real time K^+ en H^+ ionenfluxen die reageren op de verandering van licht- en ionensamenstelling van de extracellulaire vloeistof. De resultaten tonen de fysiologische reacties van Chara-cellen op de toediening van extracellulaire auxine (voornamelijk IAA) stimulering, zoals het optreden vaneen hyperpolarisatie van de membraanpotentiaal. Onderzoek van K^+ en H^+ ionenstromen afzonderlijk laat zien dat vergeleken met K^+ fluxen er geen opmerkelijke invloed is op H^+ fluxen door de toevoeging van IAA. De combinatie van gegevens geeft aan dat, in tegenstelling tot wat bekend is van landplanten, auxine de permeabiliteit en/of kanaalactiviteit voor K+ van de Chara-plasmamembraan kan veranderen, maar dat het geen sterk effect heeft op Chara PM H^+-ATPasen.

Hoofstuk 5 is gericht op de verschillen in relatie tussen auxine en de Chara PM H^+-ATPasen in vergelijking met hogere plant H^+-ATPasen. , Om de mogelijke verschillen tussen Chara PM H^+-ATPasen en andere goed bestudeerde PM H^+-ATPasen van hogere planten te onderzoeken werd een mogelijk Chara PM H^+-ATPase-gen (CHA1) werd geïsoleerd, de sequentie ervan bepaald en geanalyseerd. Een bio-informatica analyse toonde een nieuw patroon in het evolutionaire perspectief tussen algen en landplanten. Heterologe expressie van CHA1 in gist en plantenprotoplasten bevestigde dat CHA1 de plasmamembraan

kan bereiken en kan functioneren als een PM H^+-ATPase met een auto-inhiberend domein op de C-terminus. Het regelmechanisme van CHA1 lijkt anders te zijn dan dat van de bekende PM H^+-ATPasen van planten, omdat ze waarschijnlijk niet de betrokkenheid van een 14-3-3-eiwitcombinatie nodig hebben. Het in het proefschrift beschreven onderzoek illustreert dat, hoewel het pH-verschil tussen/ langs het Chara-celmembraan het auxinetransport en de distributie kan beïnvloeden, auxine zelf geen directe effecten op de activiteit van Chara PM H^+-ATPasen vertoont. Het Chara plasmamembraantransport onder verschillende omstandigheden werd bestudeerd, met nadruk op de PM H^+-ATPase, als een belangrijke speler in het membraantransportsysteem. De PM H^+-ATPase wordt ook op moleculair niveau onderzocht. Bio-informatica en functionele analyse van de geïsoleerde Chara PM H^+-ATPase onthullen een nieuw aminozuurpatroon dat essentieel zou kunnen zijn voor structurele stabilisatie, ATP-hydrolyse en protontransport. Daarnaast wordt een mogelijk ander regulatiemechanisme zichtbaar in vergelijking met de bekende plant en gist PM H^+-ATPasen, Dit kan een nieuw lichtwerpen op de PM H^+-ATPase-onderzoeken binnen de algengroep vanuit het evolutionaire perspectief. Door realisatie van een standaardlaboratoriumcultuur en nieuwe vooruitgang op moleculair niveau, zullen de Chara-cellen een vernieuwd veelbelovend platform bieden voor verschillende fundamentele en praktische studies.

Curriculum Vitae

Suyun Zhang was born on the 28th of December 1986 in Jiaxing, Zhejiang Province, China. In 2005, she started her studies at Southwest University in Chonqing and obtained her Bachelor's degree in Biotechnology in 2009. During the bachelor study, she did her internship at the State Key Laboratory of Silkworm Genome Biology for one and half year. In the summer of 2008, she was selected by the university for an exchange research project in the College of Saint Benedict and Saint John's University, the United States. In autumn 2009, Suyun continued her Master study at the China Agricultural University in Beijing. Her study was focused on nutrient uptake of plant roots systems under the stimulation of different sound waves, under the supervision of Prof. Baoming Li. After receiving her Master's Degree in 2011, she continued her PhD project with Prof. Bert van Duijn and Prof. Remko Offringa at the Institute of Biology, Leiden University, with a financial support by the China Scholarship Council and the Foundation for Single Cell Research. Her work about using Chara algae as a model system to study plant physiology and evolution is described in this thesis. Currently, Suyun is employed at a Post-doc position at Fytagoras BV in cooperation with the Leiden University-European Centre for Chinese Medicine and Natural Products (LU-ECCM).

Publications

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