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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 Charophyte 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 Zygnematophyceae 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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