Chapter 6

Diffusion Limitation and CO_2 Concentrating Mechanisms in Bryophytes

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Summary

This chapter explores how the diffusion of CO_2 into photosynthetic tissues is affected by the morphology and biochemistry of bryophytes from the sub-cellular level to that of leaf-like structures, with an emphasis on the most ancient form of a land plant CO_2 concentrating mechanism, the hornwort pyrenoid. Interest in the control of CO_2 diffusion has increased dramatically over the past 5–10 years due to the discovery of CO_2 transporting aquaporins in

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chloroplast membranes and the ever-increasing interest in photosynthetic carbon fixation as a source of food and biologically generated fuels. The diffusion of CO_2 is of critical importance to our understanding of photosynthesis in land plants because it is inextricably linked to water loss. Photosynthetic tissues need to be well hydrated to function properly, but must lose water in order to capture CO_2 since water vapor can diffuse out of photosynthetic tissues through any pore large enough to allow CO_2 in. At the same time, too much water also limits photosynthesis because even thin films of liquid water present significant barriers to CO_2 diffusion. Furthermore, the partial pressure of CO_2 reaching the sites of carboxylation in the chloroplast is what inherently controls the efficiency of photosynthetic carbon assimilation. The amazing variation in bryophyte morphology provides a broad palette for sampling how plants have balanced these structural and biochemical trade-offs. Here we discuss how studying this variability can generate invaluable insight into both the limitations and opportunities for enhancing land plant photosynthesis.

I. Introduction

The transition to land from aquatic environments presented several challenges for photosynthetic organisms to overcome (Chaps. 2, 3 and 4), but getting CO_2 to the surface of photosynthetic tissues was not one of them. On land, access to CO_2 improved relative to that in aquatic environments due to the faster diffusion of CO_2 through air than water. The problem was, and is, that getting the CO_2 from the surface to the chloroplast generally requires features that also increase water loss. This trade-off has led to the evolution of a wide range of canopy, shoot, organ, and cellular structures in bryophytes that have improved CO_2 transport to the chloroplast with some of that diversity still present today. The impacts of canopy- and shoot-level variation on photosynthesis are discussed in Chaps. 5, 9 and 10. Here we focus on the photosynthetic organ and cellular structures that enhance CO_2 uptake in bryophytes.

The morphology of the photosynthetic organ where the trade-off between CO_2 uptake and water-loss occurs has a large and fundamental effect on the diffusive pathways encountered. There are three generalized forms of photosynthetic, leaf-like, organs that are repeatedly encountered in bryophytes, and they provide insight into the biophysical constraints that influenced the evolution of true leaves. The first form is a simple thallus found in some liverworts and all hornworts. This is a relatively uniform, poorly differentiated, flattened plant body that is closely attached to the substrate and a few to several cells thick with all cells photosynthetic. Second, is a complex thallus of some liverworts where cells have differentiated into several types, providing much more structure to the photosynthetic tissue, including epidermal pores opening into internal air spaces. The third is a simple leaf-like structure attached to a stem that may or may not contain photosynthetically active cells and is found in mosses and most liverworts. These leaf-like structures, or phyllids, differ from true leaves because they are generally one cell layer thick over most of the lamina, may or may not have a midrib, and lack well-developed vascular tissue. However, their form and arrangement is quite diverse and can be aggregated along branches or stems into larger functional units, or in the Polytrichaceae, can have complex structure including upward unistratose extensions and in-rolled margins.

Abbreviations: CA – Carbonic anhydrase; CCM – CO_2 concentrating mechanism; $\delta^{13}C$ – Isotopic composition of carbon ^{13}C and ^{12}C; Δ – Isotopic discrimination; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase

In addition to the gross morphology of photosynthetic tissues, cellular properties also influence the diffusion of CO_2. In this chapter, we discuss the pyrenoid, a unique structure within the chloroplast that is responsible for the hornwort CO_2 concentrating mechanism (CCM). We also discuss morphological aspects of complex thalli, leaf-like photosynthetic organs, and the impacts on diffusion of CO_2, with particular attention to features that impact diffusion from the cell wall inwardly to the chloroplast.

II. Tissue Structure and CO_2 Diffusion

A. Simple Thallus

There have been very few studies of photosynthetic organisms with simple thalli (Smith and Griffiths 1996a, b, 2000; Hanson et al. 2002; Griffiths et al. 2004; Meyer et al. 2008), especially within liverworts. Most of this research has focused on the hornworts, a morphologically and phylogenetically isolated, species-poor land plant lineage. Two studies have compared simple thalloid liverwort CO_2 diffusion with that of hornworts lacking a CCM and found similar limitations between groups (Griffiths et al. 2004; Meyer et al. 2008). All simple thalli that lack CCMs appear to be diffusion limited, especially when covered by a film of water. Aside from slow growth in conditions that are unfavorable to other plants, this limitation would provide a strong selective force for one or more of the following: the development of a CCM, improved Rubisco kinetics, and/or structural features that could reduce the resistance between the atmosphere and the chloroplast stroma.

As discussed later in this chapter, a pyrenoid-style CCM did evolve in the hornworts although it is important to realize that like other CCMs, it requires additional electron transport (Hanson et al. 2002; Griffiths et al. 2004; Meyer et al. 2008) making a CCM less favorable in low-light environments often occupied by bryophytes. Rubisco kinetics in bryophytes have been poorly studied, and more investigations are desperately needed. One study found that CCM-lacking hornworts had intermediate properties between CCM containing hornworts and the C_3 liverwort *Marchantia polymorpha,* which has a complex thallus (Hanson et al. 2002). The net result was a lower investment in Rubisco per chlorophyll and a little more CO_2 assimilation per active site. Additional research is greatly needed to determine if this pattern is common to all organisms forming simple thalli or if it is a generalizable response among all CO_2 limited bryophytes. Lastly, it is unknown if liverworts and CCM-lacking hornworts with simple thalli consistently have structural modifications such as chloroplast (Chap. 8) or mitochondrial positioning within cells to maximize CO_2 recycling, functional CO_2-porins (Uehlein et al. 2008), thallus surface properties to regulate water films, or growth habits to maximize capture of CO_2 respired from soil.

The extent that CO_2 diffusion is limiting photosynthesis and the operation of a CCM can be examined by measuring the differential usage of the isotopologues (^{13}C and ^{12}C forms) of CO_2. This approach is predicated on the biochemical preference of Rubisco for the lighter isotopologue of CO_2, and the faster diffusion of the lighter isotopologue into and through photosynthetic tissues. The combined effect of the biochemical and physical fractionations is referred to as a discrimination (Δ) against the heavier forms of CO_2 and assessed through measurements of the isotopic composition of carbon in samples (Farquhar et al. 1982, 1989; Brugnoli and Farquhar 2000). Isotopic composition ($\delta^{13}C$) is reported in the unit-less notation of per mil (‰), that describes the ratio of ^{13}C content to ^{12}C relative to an international standard ($\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 1{,}000$). Online isotopic gas exchange studies measure the isotopic composition and total CO_2 of air entering and exiting a leaf gas exchange chamber along with the water exchange. The total CO_2 uptake and water loss are used to model an expected Δ caused by diffusion in to the intercellular spaces of leaves (or to the surface of photosynthetic cells).

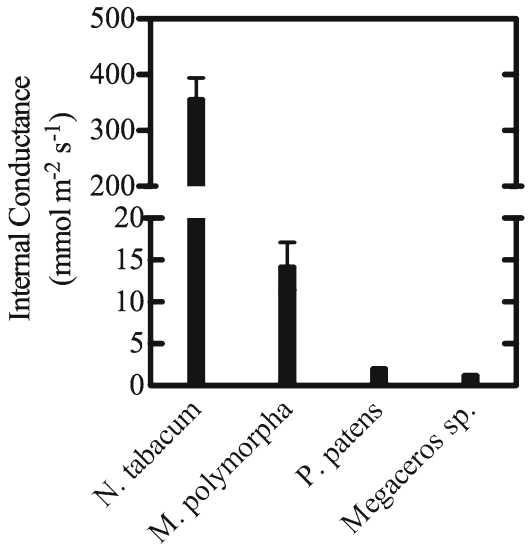

Fig. 6.1. Internal conductance to CO_2 in photosynthetic tissues. Data are presented for the angiosperm leaf *Nicotiana tabacum*, the complex thallus liverwort *Marchantia polymorpha*, the moss *Physcomitrella patens* with unistratose leaf-like tissues, and an unknown species of the hornwort genus *Megaceros* that has a simple thallus and no CCM (Hanson 2004, unpublished). Both *P. patens* and *Megaceros* were measured using tissue-cultured material that had been lightly blotted dry. Internal conductance is often referred to as mesophyll conductance with units of mol m^{-2} s^{-1} in plants with complex photosynthetic tissues that contain mesophyll cells. The more general term, internal conductance, and the smaller units used here are more suited for the anatomical variability and low conductance found in bryophytes.

The modeled Δ is compared with the measured Δ and the difference provides an estimate of the resistance to diffusion from the surface of photosynthetic cells into the chloroplast stroma (Evans et al. 1986).

Measurements of online Δ have shown that organisms with simple thalli have a much smaller Δ than predicted if CO_2 could diffuse through photosynthetic tissues as easily as in angiosperms and gymnosperms with true leaves (Meyer et al. 2008). A simple explanation for this difference is that this "internal" conductance of CO_2 (the inverse of resistance) through simple photosynthetic thalli is two orders of magnitude lower than the conductance through thin leaves with intricate air spaces (Fig. 6.1 Hanson 2004, unpublished). One must also account for changes in "external" conductance because it drops rapidly as surface water film thickness increases (Meyer et al. 2008). Even after accounting for water film effects, and estimating the effect of stomata and intercellular air spaces in true leaves, the difference between leaves and simple thalli for internal conductance is still greater than would be expected based on known structural changes. As discussed in section "Evolutionary trade-off between cell wall structure and CO_2 diffusion", it is possible that the low conductance of simple thalli is due to low cell wall permeability to CO_2. It is also possible, that some of the difference in Δ is not due to internal conductance but instead due to different amounts of cellular respiration and recycling relative to what is found in true leaves. Respired and recycled CO_2 would decrease the apparent rate of photosynthesis and also deplete the isotopic composition of air around a leaf making Δ appear to be less than it is.

B. Complex Thallus

The addition of epidermal pores and internal air spaces to thalloid photosynthetic tissues clearly reduces diffusion limitation for photosynthesis when expressed on either tissue area (Meyer et al. 2008) or chlorophyll content (Hanson et al. 2002). Several lines of evidence, including stable isotopes, have been employed to show that improvements in photosynthesis are achieved without a CCM in *Marchantia* and other liverworts with complex thalli (see Fig. 6.2 Hanson 2004, unpublished) (Smith and Griffiths 1996b, 2000; Hanson et al. 2002; Griffiths et al. 2004; Meyer et al. 2008). There is also no evidence that major changes in Rubisco properties, aside from an increase in content, accompanied the development of more complex photosynthetic thalli (Hanson et al. 2002), although as mentioned above, this is an area where little research has been published. It appears that the roughly two-fold increase in maximal photosynthesis between simple and complex thalli correlates with

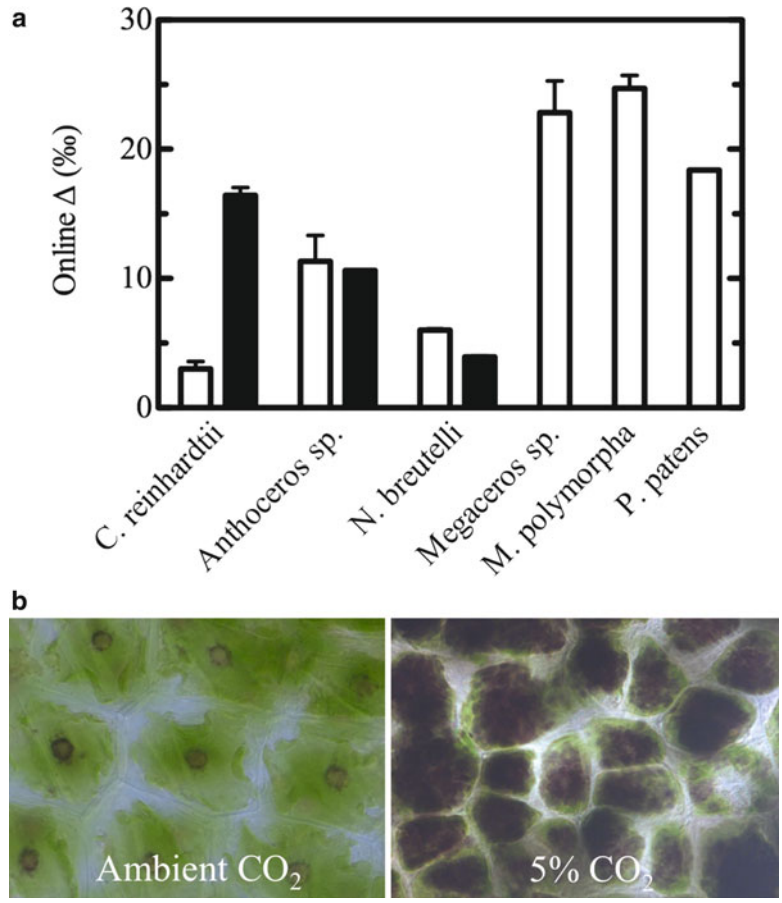

Fig. 6.2. Online photosynthetic ^{13}C discrimination (Δ) measured and the impact of high CO_2 for cultured algae and bryophytes (Hanson 2004, unpublished). *(***a***)* Δ was measured using a combined tunable diode laser/infra-red gas analyzer system (Barbour et al. 2007) for *Chlamydomonas reinhartdtii*, *Anthoceros* sp. (hornwort with pyrenoid), *Notothylas breutelli* (hornwort with pyrenoid), *Megaceros* sp. (hornwort without pyrenoid), *Marchantia polymorpha* (complex thallus liverwort), and *Physcomitrella patens* (moss). *Black columns* represent measurements on organisms growing at 0.7–5% CO_2, which is high enough to down-regulate the CCM and increase *Δ* in *C. reinhardtii*. Hornworts were transferred to ambient CO_2 for the measurement, but all data were collected within 15 min of exposure to the lower concentration. (**b**) Light microscopy showing hornworts grown at ambient and 5% CO_2 that were collected immediately prior to dawn and stained with potassium iodide to show starch grains.

about a five to tenfold increase in internal CO_2 conductance (Fig. 6.1) (Meyer et al. 2008). However, it is interesting to note that plants with true leaves have internal conductances that can be significantly higher; conifers like juniper range from the same magnitude as bryophytes to >100 fold higher (Bickford et al. 2009) and many seed plants like tobacco have an internal conductance >200 fold higher (Fig. 6.1). This higher conductance facilitates 10–20 fold higher photosynthetic rates in plants with true leaves when expressed on an area or mass basis (see Waite and Sack 2010), though the increases are not as evident on a per chlorophyll basis (Martin and Adamson 2001). The combination of this information suggests that internal conductance may be more important in spatial organization of photosynthesis within photosynthetic tissues, impacting how much Rubisco and chlorophyll can be utilized in a given volume more than the inherent relationship between light harvesting and carbon capture.

C. Phyllid

At first glance, the evolutionary progression from multistratose thalli to mostly unistratose leaf-like structures (phyllids) should have greatly reduced the importance of internal conductance as each photosynthetic cell is directly exposed to the atmosphere. However, the fundamental terrestrial problem of water-loss prevents such a simple solution from being a panacea. Unistratose tissues lose water rapidly, and in order to sustain high enough water contents for biological activity they must spend a significant amount of time covered with a film of water. Unistratose tissues also need to tolerate desiccation when water is not readily available. As the classical isotopic gas exchange studies of Rice and Giles (1996) and Williams and Flanagan (1996, 1998) have shown, this water film has a large impact on the diffusion of CO_2 to the surface of photosynthetic cells. The complex three dimensional structures and canopies facilitated by a stem- and leaf-style architecture of phyllid containing species requires balancing water loss with CO_2 diffusion and light penetration rather than optimization for CO_2 diffusion alone (see canopy structure discussion in Chaps. 9 and 10).

The limited extent to which CO_2 diffusion has been maximized in bryophytes is especially evident when examining internal conductance to CO_2. There have only been a few measurements of online Δ for mosses (Fig. 6.2) (Rice and Giles 1996; Williams and Flanagan 1996, 1998). These data show that internal conductance in *P. patens* may be as low as that found in simple thalli of CCM-lacking hornworts (Fig. 6.1), similar between *Pleurozium* and complex thalli, or 50% higher than complex thalli for *Sphagnum* (Williams and Flanagan 1998; Meyer et al. 2008). More data, explicitly separating internal and surface liquid conductance to CO_2 are needed to develop a robust understanding of how bryophytes regulate CO_2 diffusion. However, it appears that the maximum internal conductance in bryophytes is *at least* one order of magnitude lower than maximum internal conductance found in species with true leaves (see Fig. 6.1 for this comparison).

III. Evolutionary Trade-off Between Cell Wall Structure and CO_2 Diffusion

The realization that photosynthetic tissues in bryophytes have a much lower internal CO_2 conductance than has been achieved by angiosperms in particular, leads one to wonder why? What is so different between the mesophyll cells of a tobacco leaf and that of *Marchantia* or the single layer of cells making up most moss phyllids? The relatively recent demonstration of the presence of CO_2 pores in higher plant chloroplast membranes (Uehlein et al. 2008) is one candidate explanation if these are lacking in bryophytes. However, the internal conductance measured for antisense knockouts versus over-expression of CO_2 pores only accounts for a twofold difference at most (Flexas et al. 2006), not 10 or 100 fold. Similarly, chloroplast size and position, carbonic anhydrase activity and location, and cell wall properties all impact CO_2 diffusion. Recently, the potential relative contribution of internal conductance components has been modeled in three dimensions showing that the chloroplast envelope and the cell wall are the largest resistances (Tholen and Zhu 2011). This is of particular interest for bryophytes because cell wall properties of photosynthetic tissues are under very strong selection for properties other than photosynthesis, especially tortuosity associated with desiccation and UV tolerance (see Chaps. 7 and 16). The toughness of bryophyte cell walls has been clearly demonstrated through acid hydrolysis studies (Graham et al. 2004 and see Chap. 2). In some extreme examples, this would be similar to conducting photosynthesis in xylem cells of seed plants. Furthermore, Tholen and Zhu (2011) calculated that the importance of the cell wall resistance in the limitation of photosynthesis by CO_2 diffusion is greatly diminished at elevated CO_2 (60 Pa). Since many bryophytes grow close to the soil, they func-

tion in a high CO_2 environment due to soil respiration. In such an environment, the penalty for developing mechanically tough, diffusion-limiting cell walls in photosynthetic tissues would be small.

The concept that cell wall thickness and/or composition, possibly in combination with the lack of CO_2 pores in chloroplast envelopes are the primary reasons for low bryophyte internal conductance remains to be tested. Historically, the measurement of internal conductance in mosses has been difficult since the online Δ measurements have been time consuming and water content varies rapidly with time unless carefully maintained. However, the recent development of high-frequency, semi-automated, laser-based measurements of isotopic gas exchange for studying internal conductance (Flexas et al. 2006; Barbour et al. 2007; Bickford et al. 2009) and its application for bryophytes (Figs. 6.1 and 6.2) makes this research much more practical. In addition, the necessary instrumentation has become available in over a dozen labs world-wide, which should spur a new era of investigation. Hopefully, the ensuing years will answer the fundamental question of how evolution has influenced CO_2 diffusion limitation of photosynthesis.

IV. The Carbon Concentrating Mechanism (CCM) of Bryophytes

Plants growing under extreme conditions often have CCMs, enabling them to accumulate CO_2 and thereby enhance their ability to fix energy and grow (Badger et al. 1998; Giordano et al. 2005; Raven et al. 2008). For example, many C_4 plants concentrate CO_2 in specialized cells within an elaborate leaf organization known as Kranz anatomy. The C_4 photosynthetic pathway occurs in many grasses and is being explored as an efficient strategy to improve carbon fixation in crops with C_3 photosynthesis (e.g. rice) (Hibberd et al. 2008; Burnell 2011; Whitney et al. 2011; von Caemmerer et al. 2012). In recent years, some non-Kranz C_4 plants have been discovered where single cells maintain two separate populations of chloroplasts (Voznesenskaya et al. 2001; Edwards and Voznesenskaya 2011). Some algae are also thought to have a single-cell C_4 metabolism facilitated by the co-location of Rubisco and phosphoenolpyruvate carboxykinase in the chloroplast pyrenoid (McGinn and Morel 2008). There have also been suggestions of an inducible C_4-like CCM in the moss *Fissidens* (see Chap. 12), a supposition that requires additional research, so as of yet no single-cell C_4 plants have been identified in the bryophytes. The lack of single-cell C_4 in bryophytes could be due to the difficulty of maintaining the large cells with intricately sub-divided cytoplasm (found in single-cell C_4 flowering plants) through dehydration and re-hydration cycles that bryophytes commonly experience. The proposed diatom C_4 system could conceivably work in hornworts as both have pyrenoids, but to date there is no evidence for C_4 metabolism in hornworts and no other land plants have pyrenoids. Even without C_4 metabolism there is substantial diversity in pyrenoid and chloroplast morphology among bryophytes with consequences for photosynthesis that may have impacted their adaptation to life on land.

A. Chloroplast Structure and CO_2 Diffusion

The transition to land from aquatic algal ancestors involved a suite of adaptations to terrestrial life. One important transformation was in the structure and physiology of chloroplasts. Most green algae have few, large chloroplasts with one to several pyrenoids, 90 % of which are composed of the CO_2 – fixing enzyme Rubisco (Vaughn et al. 1990; Badger et al. 1998; Borkhsenious et al. 1998). The pyrenoid functions as an essential component of the CCM as a location where CO_2 can be elevated around Rubisco (McKay and Gibbs 1991; Badger and Price 1992). In pyrenoid containing species, CO_2 and HCO_3^- are actively taken-up into the cell and into the chloroplast where they can diffuse into the pyrenoid. Any CO_2 reaching the pyrenoid

can be directly utilized by Rubisco, however, the high pH of stroma in the light makes it likely that much of the CO_2 will be converted to HCO_3^-. When HCO_3^- arrives at the pyrenoid, it is thought to be transported into the low pH lumen, which would favor the conversion to CO_2 for Rubisco. Although a thylakoid-localized HCO_3^- transporter has not been identified, a lumen-localized carbonic anhydrase has been characterized and shown to be essential for CCM function (Spalding et al. 1983; Karlsson et al. 1998; Hanson et al. 2003). It is also not clear how leakage of CO_2 not captured by Rubisco is limited as leakage would lead to a futile cycle of CO_2 pumping and loss. Pyrenoid and/or chloroplast morphology appears to be important for reducing leakage as species with more compact pyrenoids leak less (Hanson et al. 2002). In algae, the pyrenoid provides an efficient CCM that increases CO_2 levels up to 180 times that in the rest of the cell, enhancing photosynthesis in aquatic environments where CO_2 diffusion is limited (Badger et al. 1998; Giordano et al. 2005). Hornworts are the only land plants that possess a pyrenoid and exhibit this type of CCM (Smith and Griffiths 1996a, b, 2000; Hanson et al. 2002), although the mechanics of its operation are primarily drawn by analogy to algal structures.

The pyrenoid-style CCM in algae is generally down-regulated within a few hours of exposure to high CO_2 ranging from 0.5 to 5% (Badger et al. 1980; Beardall and Raven 1981; Fukuzawa et al. 2001; Vance and Spalding 2005). In general, the regulation of CCM function has been shown via physiological adjustments in photosynthesis and gene expression, but changes in CCM activity based on the differential usage of the isotopologues of CO_2 and/or HCO_3^- were also hypothesized and a simple screen showing these changes was developed in the early 1980s (Beardall et al. 1982; Sharkey and Berry 1985). When a CCM is active at ambient and sub-ambient levels of inorganic carbon (CO_2 and HCO_3^-), the heavier inorganic carbon cannot readily escape fixation by Rubisco and a relatively high proportion is incorporated, resulting in a low Δ. The effect can be seen by comparing Δ calculated from online ^{13}C gas exchange for hornworts containing and lacking pyrenoid-style CCMs (Fig. 6.2 Hanson 2004, unpublished) (Meyer et al. 2008). It is clear that pyrenoid containing species have low Δ values (consistent with CCM activity) relative to pyrenoid lacking species of *Nothoceros, Megaceros* and mosses (Smith and Griffiths 1996b).

Suppression of algal CCMs under conditions of high inorganic carbon availability increases the leakiness of cells to CO_2 (Badger et al. 1980; Beardall and Raven 1981; Raven and Beardall 2003). This facilitates the escape of all inorganic carbon including the heavier forms, allowing Rubisco to exert its high Δ (preference for the lighter forms). Using this approach our data (Fig. 6.2a) show that, unlike most algae (Badger et al. 1998), at least some hornworts are unable to down-regulate their CCM in response to high CO_2 as they have a low Δ under both ambient and high CO_2. Interestingly, we also found a hyper-accumulation of starch grains at high CO_2 in pyrenoid-containing hornworts (Fig. 6.2b), and a small suppression of maximum photosynthesis (data not shown). Thus, even at high CO_2 when daily carbon assimilation greatly exceeds the ability of the cell to utilize assimilated carbon, photosynthesis is only mildly down-regulated.

In addition to online ^{13}C gas exchange, the isotopic effect of CCM activity can also be seen via analyses of the isotopic composition of tissues. In contrast to the measurement of instantaneous ^{13}C usage provided by online gas exchange, tissue analyses integrate the relative accumulation and retention of ^{13}C over the lifetime of a tissue, where species with CCMs incorporate relatively more $^{13}CO_2$ than species lacking CCMs. An integrated lifetime Δ can be calculated from the difference between the $\delta^{13}C$ of the atmosphere (source CO_2) and the $\delta^{13}C$ of the tissue, $\Delta_{lifetime} = [(\delta^{13}C_{atmosphere} - \delta^{13}C_{tissue})/(1{,}000+\delta^{13}C_{tissue})] \times 1{,}000$ (Farquhar et al. 1989). Since $\delta^{13}C_{atmosphere}$ is relatively constant and negative in sign, and since Δ is generally

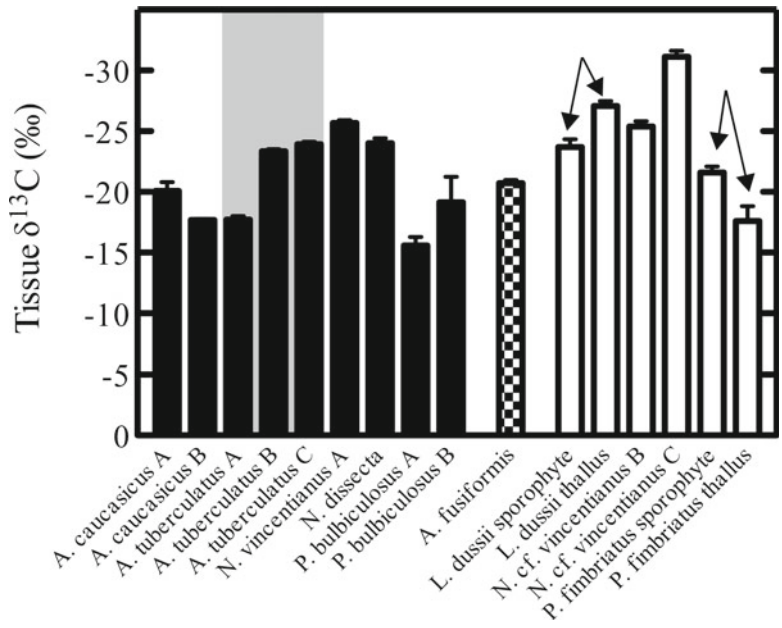

Fig. 6.3. Isotopic composition of ^{13}C of field collected hornwort tissues (Hanson and Villarreal 2008, unpublished). Solid columns are the pyrenoid containing species *Anthoceros caucasicus, Anthoceros tuberculatus, Nothoceros vincentianus, Notothylas dissecta*, and *Phymatoceros bulbiculosus.* Open columns are the pyrenoid-lacking species *Leiosporoceros dussii, Nothoceros c.f. vincentianus*, and *Phaeomegaceros fimbriatus*. The *checkered column is Anthoceros fusiformis*, which has a starch-free area located where a traditional pyrenoid would be expected. The letters A, B, and C following species designations denote that the same species was collected at different locations or times of year. The shaded solid columns highlight that *A. tuberculatus* collections from different locations can differ substantially in their isotopic composition. The paired arrows denote where sporophyte and gametophyte (thallus) tissue were analyzed from the same collected material.

positive in sign, a plant with a small Δ (active CCM) will generate tissues with a less negative $\delta^{13}C$ than a plant with a large Δ (inactive CCM).

We have compiled a figure of unpublished (D. Hanson and J.C. Villarreal 2008) tissue $\delta^{13}C$ measurements to assess the frequency of CCM function among hornworts (Fig. 6.3). Among seed plants, C_3 species tend to have $\delta^{13}C$ values ranging from −22 to −30‰, whereas CCM containing C_4 seed plants range from −10 to −14‰ (Cerling et al. 1997; Osborne 2011). A similar division has been seen within bryophytes, where hornworts with a pyrenoid-based CCM ranged from −13 to −20‰ and C_3 mosses, liverworts, and hornworts ranged from −21 to −35‰ (Griffiths et al. 2004). Overall, our data support the pattern that pyrenoid-containing hornworts have a $\delta^{13}C$ that is −20‰ or less negative, whereas those lacking a pyrenoid are more negative than −20‰. This division is consistent with all existing physiological data that clearly demonstrate an active CCM for hornworts containing pyrenoids and no CCM activity in pyrenoid-lacking species. However, Fig. 6.3 also shows that the pyrenoid-containing species *Anthoceros tuberculatus* can be both above and below the −20‰ threshold depending on collection time and locality. In addition, we found that sporophyte tissue from *Phaeomegaceros fimbriatus* was C_3-like (more negative than −20‰), while the gametophyte thallus $\delta^{13}C$ suggested the presence of a CCM. Since other factors such as water content (Rice and Giles 1996; Williams and Flanagan 1996, 1998; Meyer et al. 2008) and fixation of soil respired CO_2 (which is much more negative than $\delta^{13}C_{atmosphere}$, the normal presumed CO_2

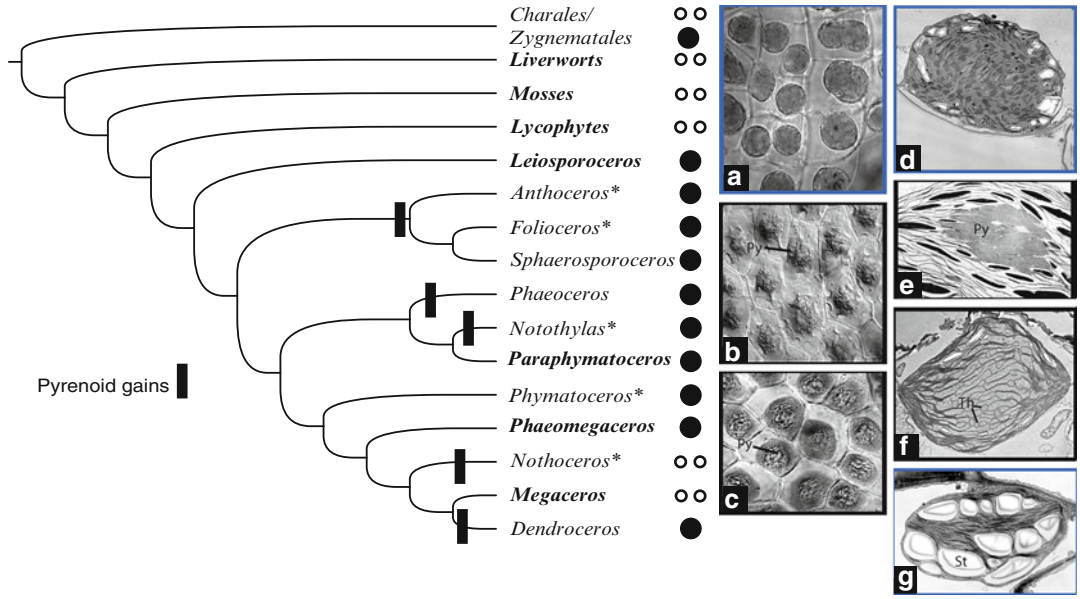

Fig. 6.4. Skeleton phylogeny of hornworts based on three genes (see Duff et al. 2007) with inferences on the evolution of the pyrenoid. Under either scenario of Zygnematales (pyrenoid bearing) or Charales (pyrenoid-less) sister to land plants, hornworts may have re-evolved the pyrenoid after it was lost in liverworts, mosses and tracheophytes. *Leiosporoceros*, the sister taxon to all hornworts, is pyrenoidless. Genera with pyrenoids and without pyrenoids (*bold*) are shown. Pyrenoid-bearing genera with one or more species lacking pyrenoids have an asterisk (*). *Megaceros* and *Phaeomegaceros* are the only genera in which all species lack a pyrenoid, while in *Phymatoceros* (two species) one species has a pyrenoid (*P. bulbiculosus*) and one lacks pyrenoids (*P. phymatodes*). Taxa with multiple plastids per cell are represented by *open circles* and those that are typically uniplastidic are shown with *solid circles*. More sampling would further clarify the evolution of pyrenoids within hornworts. Collage of hornwort chloroplasts: (**a**) *Leiosporoceros dussii* chloroplast without pyrenoids from the *upper* epidermis, (**b**) *Anthoceros* sp. light microscopy of upper epidermis of gametophyte, each cell contains single plastid with pyrenoids (Py), (**c**) *Nothoceros vincentianus* light microscopy of upper epidermal cells of gametophyte, each with modified central pyrenoid (Py) with abundant starch granules, (**d**) *Leiosporoceros dussii* transmission electron micrograph (TEM) of chloroplast without pyrenoid, (**e**) *Phaeoceros carolinianus* TEM of subunits of the multiple pyrenoid (Py) are more electron-opaque than the surrounding stroma, (**f**) *Nothoceros canaliculatus* TEM of an electron translucent pyrenoid area traversed by numerous channel thylakoids (Th), (**g**) *Phaeomegaceros aff. fimbriatus* TEM of a pyrenoidless chloroplast in the *upper cell*s of the gametophyte with abundant central grana and mostly peripheral starch (St).

source) can have large impacts on the $\delta^{13}C$ of tissues, these data are not proof of an exception to the CCM activity of pyrenoids. However, within-species and within-plant variability in $\delta^{13}C$ does open up the possibility that CCM activity is facultative in some species. Definitive resolution of this question will require more physiological and online Δ measurements under controlled conditions.

The presence of pyrenoids is more variable among hornworts than traditionally thought (see Fig. 6.4 and the evolution of pyrenoids section below). Interestingly, species and even cells within a thallus lacking pyrenoids have more and smaller chloroplasts than those containing pyrenoids. Re-examination of chloroplast morphology and their effect on CO_2 diffusion suggests that large chloroplasts hinder diffusion more than small ones (Terashima et al. 2011; Tholen and Zhu 2011), although it is unknown if expression of chloroplast-localized CO_2-transporting aquaporins (Uehlein et al. 2003, 2008; Flexas et al. 2006) increases with chloroplast size to compensate for the slower diffusion. Therefore, there could be a mechanistic linkage between chloroplast size and functioning of a pyrenoid-style CCM.

If CO_2 diffusion is sufficiently limited by chloroplast size, then species with large chloroplasts may require a CCM for rapid growth. However, this slowed diffusion would also reduce the rate of CO_2 leakage from a centrally located pyrenoid where HCO_3^- is presumably converted to CO_2 for Rubisco. This reduced leakage would be a result of the larger distance to escape the chloroplast coupled with increased time to reach equilibrium with HCO_3^- in the high pH stroma of illuminated chloroplasts. So, it is also possible that large chloroplasts are required for terrestrial pyrenoid-based CCMs to function as there is no other known mechanism to reduce the futile cycle caused by CO_2 leakage from the pyrenoid.

B. Evolution of Pyrenoids in Land Plants

Characteristics of hornworts appear intermediate between algal and land plant traits, providing insights into selective pressures that may have faced plants as they colonized the land c. 400 million years ago (Kenrick and Crane 1997; Renzaglia et al. 2000; Qiu et al. 2006). Despite morphological simplicity and homogeneity they have endured through severe and prolonged climatic change. Hornworts exhibit "ancestral" traits, such as poikilohydry (the ability to survive desiccation) (Proctor 2009) and simple, thalloid gametophytes (the gamete-bearing life phase) with 'derived' traits, such as stomata in sporophytes (the spore-producing phase) (Renzaglia et al. 2009) and a pyrenoid (Smith and Griffiths 1996a, b, 2000; Hanson et al. 2002).

Reconstructing the evolution of pyrenoids in hornworts is not a trivial task. First of all, data on chloroplast ultrastructure has been collected in less than 20 % of the taxa and these results indicate great variability across species and within plants. The extensive geological history of this ancient lineage, spanning multiple extinction events, has resulted in ambiguous phylogenetic reconstruction and large genetic distances between hornworts and other land plants. Current phylogenetic hypotheses suggest that the closest relative to land plants is either a member of the Charales (pyrenoidless) or Zygnematales (pyrenoid bearing) (Turmel et al. 2005; Graham et al. 2009). These two outgroups to land plants result in dramatically different reconstructions of ancestral characters in pyrenoids of hornworts. In addition, hornworts are nested among liverworts, mosses and lycophytes, all of which do not have pyrenoids. The appearance of the trait may have been triggered by specific environmental factors when hornworts originated. Pyrenoid presence appears to have evolved at least five times independently within hornworts, although it is lost in several clades (Fig. 6.4) (Villarreal and Renner 2012).

A large drop in atmospheric CO_2 concentration in the Late Devonian is thought to have promoted the evolution of the leaf blade, which allowed multiplication of the number of stomata per mm^2 and increased CO_2 sequestration (Beerling et al. 2001). The evolution of pyrenoids in green algae and carboxysomes in cyanobacteria has also been hypothesized to be a response to this Devonian drop in CO_2 (Raven 1997; Badger et al. 2002). Our recent insight on hornwort pyrenoids suggests that the ancestor of all hornworts about 300 mya lacked pyrenoids (Villarreal and Renner 2012). Therefore, alternative micro-habitat conditions may have influenced the evolution of pyrenoids within some hornwort clades. One study examined Rubisco content in hornworts and found that a species without a pyrenoid had a higher Rubisco content than two species containing pyrenoids, leading to more efficient CO_2 capture in at least one of the pyrenoid containing species (Hanson et al. 2002). Since Rubsico is a major sink for nitrogen in plants, a reduction in Rubisco content without a loss in CO_2 uptake would generate significant savings in the nitrogen budget. Therefore, fluctuations in the available nitrogen could also affect the evolution of pyrenoids. Interestingly, all hornworts are thought to form symbioses with cyanobacteria, though in the basal genus *Leisporoceros* the symbiosis differs morphologically from all others (Villarreal and Renzaglia 2006).

This symbiosis has been shown to provide nitrogen and is under the genetic control of the hornwort in the genus *Anthoceros* (Meeks et al. 1983; Campbell and Meeks 1992; Meeks and Elhai 2002), though the variability and effectiveness of this association is not well quantified in other genera. If environmental conditions reduced the formation of cyanobacterial-hornwort symbioses or their effectiveness, this would increase the benefit of having a pyrenoid CCM irrespective of CO_2 concentrations in the atmosphere.

Ancestral reconstruction of pyrenoid evolution in hornworts would benefit from a solid and time-explicit phylogeny of the group, and, of course, ultrastructural and physiological examination of more taxa. As it stands today, evolution of pyrenoids within hornworts appears to be an excellent example of atavism, involving multiple losses and gains that mirror the situation in green algae (Nozaki et al. 2002; Villarreal and Renner 2012). The sister taxon to all other hornworts, *Leiosporoceros,* lacks pyrenoids. If plant cells containing single chloroplasts without pyrenoids (i.e. the condition in *Leiosporoceros*) are plesiomorphic in hornworts, pyrenoids have been gained independently at least five times (Villarreal and Renner 2012). Additional losses are seen in the five pyrenoid-bearing genera that have one or more species that lack pyrenoids (Fig. 6.4, genera denoted by *). Lack of a pyrenoid typically is associated with reduction in plastid size and increase in plastid number per cell. The pyrenoid was lost in the crown hornwort group that includes *Phaeomegaceros*, *Megaceros*, and most *Nothoceros*, and appears again in *Dendroceros* and two species of *Nothoceros* (Fig. 6.4). Thus, pyrenoidless plastids are ubiquitous in only two genera, *Phaeomegaceros* and *Megaceros*. *Megaceros* has a lower CCM activity than taxa with pyrenoids such as *Anthoceros*, *Notothylas* and *Phaeoceros* (Hanson et al. 2002; Meyer et al. 2008). This rather simple interpretation of pyrenoid evolution in hornworts does not take into consideration that chloroplast microanatomy is highly variable, especially in those plants that do not possess well-defined pyrenoids. Indeed, starch-free or pyrenoid-like zones occupy the central region of chloroplasts in some of these plants and their tissue $\delta^{13}C$ is on the borderline between species with and without CCM activity (Fig. 6.3). If these organisms have functional CCMs, then an even more sophisticated CCM may have evolved. Localizations of *rbc*L and other photosynthetic enzymes within this microanatomy may provide clues to the nature of these evolutionary changes. Moreover, in *Anthoceros,* the largest hornwort genus with ca. 80 species (Villarreal et al. 2010), less than 20 % of species have been examined at the light microscope level, limiting accurate reconstruction of pyrenoid evolution in hornworts.

The advantage of a pyrenoid-style CCM in hornworts is puzzling since pyrenoid containing and lacking species occur in a range of terrestrial environments. Some hornworts are semi-aquatic (e.g. the pyrenoidless *Nothoceros aenigmaticus,* a few *Anthoceros* species and occasionally *Phaeoceros carolinianus*); in these the CCM would provide the same costs and benefits as they do in freshwater microalgae. However, the vast majority of hornwort species are terrestrial, growing on soil banks together with liverworts and mosses. In the case of the epiphytic genera *Dendroceros,* the presence of a pyrenoid may relate to desiccation-tolerance in its habitat (Schuette and Renzaglia 2010). Thus, numerous questions remain in regards to structural and physiological modifications within the unique chloroplast of hornworts. With the diversity and pattern of chloroplast evolution, continued research on pyrenoids in hornworts will yield a wealth of information that will inform the development of crop plants with efficient CCM capabilities.

C. Engineering A Crop Plant Pyrenoid

The increased efficiency of CO_2 utilization and the concomitant savings in nitrogen and water loss achieved by CCM containing organisms suggests that it could be beneficial to engineer a CCM into a C_3 crop. A large consortium of scientists are already working to engineer C_4 metabolism into C_3 crops

(Hibberd et al. 2008; Burnell 2011; von Caemmerer et al. 2012), but some have begun to explore engineering a pyrenoid-style CCM into crops as mechanisms of its assembly are uncovered (Meyer et al. 2012). The pyrenoid CCM is intriguing because it does not require multiple cell or plastid types needed for C_4, so it may be easier to engineer. However, little is known about what is needed to make a pyrenoid-style CCM.

The green alga *Chlamydomonas* is currently the primary candidate donor to implement a single cell CCM in higher plants because of the availability of nuclear genome data, ongoing research on its CCM, and ease of mutagenesis (Spreitzer et al. 1985; Merchant et al. 2007; Genkov et al. 2010; Meyer 2010; Meyer et al. 2012, http://cambridgecapp.wordpress.com/). The enzyme Rubisco is partitioned into a large subunit (*rbc*L, in the chloroplast) and a small subunit (*rbc*S, in the nucleus). A major recent development is the discovery that a specific portion of *rbc*S is responsible for the presence of pyrenoids in *Chlamydomonas* where an engineered *Chlamydomonas* mutant that combined an angiosperm *rbc*S with *Chlamydomonas rbc*L lacked a pyrenoid (Genkov et al. 2010; Meyer et al. 2012). Further essential components of the CCM in *Chlamydomonas* are carbonic anhydrases (CA), inorganic carbon transporters and regulatory factors, though there are a range of other potential genes that are expressed when CCMs are stimulated but that have not been fully characterized (Fujiwara et al. 1990; Spalding 2008; Yamano et al. 2008; Ohnishi et al. 2010; Ma et al. 2011; Brueggeman et al. 2012). Carbonic anyhdrases are part of a gene family and their activity is central to the concentrating mechanism because they maintain equilibrium levels of CO_2 to support assimilation by Rubisco and to facilitate diffusion of CO_2 across membranes (Spalding et al. 1983; Badger and Price 1992; Borkhsenious et al. 1998; Hanson et al. 2003; Giordano et al. 2005; Spalding 2008).

Another potentially crucial hornwort feature, absent from most green algae, is a stacked arrangement of thylakoid membranes (grana) involved in light capture (Mullineaux 2005). Grana result in the spatial separation of photosystems and increase the efficiency of light capturing systems in terrestrial environments (Mullineaux 2005; Cardon et al. 2008). In hornworts, grana consist of stacks of short thylakoids and lack end membranes. Therefore, unlike in other land plants, hornwort grana are devoid of the membrane 'sacs' that fully enclose intra-thylakoid spaces. Presumably the unique channel thylakoid system in hornwort chloroplasts assumes the role of isolating biochemical processes necessary for photosynthesis. Because hornworts are the only group to combine pyrenoids, like green algae, with channel thylakoids and grana that resemble those of other land plants but that lack end membranes, the internal architecture of chloroplasts in this group is unparalleled (Vaughn et al. 1992). Multilocus molecular studies place hornworts sister to vascular plants, thus they are separated from algae by two pyrenoid-less lineages (liverworts and mosses).

Arguably hornworts are better donors than algae for genetic engineering of pyrenoids in crop plants because they are phylogenetically closer, and shared features of chloroplast organization with flowering plants may result in a more efficient way to implement a fully functional CCM in crops. Hornworts offer a promising opportunity to study chloroplast evolution, and in particular, the acquisition and maintenance of an alternative way to concentrate carbon in terrestrial environments. However, it is critical to ascertain if hornwort pyrenoids form in a homologous fashion to those in *Chlamydomonas* and whether features not seen in seed plants such as large chloroplasts and channel thylakoids are important to the operation of terrestrial pyrenoid-based CCMs. A hornwort nuclear genome project is reportedly underway, and a chloroplast and mitochondrial genome from a pyrenoid containing species are already available (Kugita et al. 2003; Li et al. 2009) making it more possible to perform the transcriptomic and mutagenic work that will be essential for unveiling the components of the hornwort CCM. Finally, there is some

suggestion that environmental conditions will control expression based on variability of pyrenoid expression between populations of the same species and between tissues in a single plant. A thorough study of chloroplast diversity in different tissues and structures within a carefully selected taxon would reveal the answer to this question.

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