

Forum

Hornworts: An Overlooked Window into Carbon-Concentrating Mechanisms

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Hornworts are the only land plant lineage harboring a biophysical carbon-concentrating mechanism (CCM). Here, we argue that hornworts are a promising, yet currently overlooked, model system to study the evolution and genetic basis of CCMs. The results of such studies could have translational values toward engineering a CCM in crop plants.

Enhancing photosynthesis has been highlighted as one of the major routes for improving food security. To enable more efficient photosynthesis, certain organisms have evolved unique ways to concentrate and shuttle CO₂ to RuBisCO, the key enzyme in carbon fixation. Such CCMs either involve multiple cells or are organized at the single cell level. The former includes the typical C₄ and CAM photosynthesis in some vascular plants, which separate the biochemical processes spatially and temporally, respectively. By contrast, cyanobacteria, some eukaryotic algae, and an enigmatic land plant lineage, hornworts (Box 1 and Figure 1), have evolved biophysical CCMs within the confinement of a single cell. The pyrenoids (or the analogous carboxysomes in cyanobacteria) are specialized compartments inside chloroplasts that predominately comprise RuBisCO, and are essential for an efficient biophysical CCM. Using inorganic carbon transporters and carbonic anhydrases, up to a

50-fold increase in CO₂ levels can be achieved in pyrenoids, thereby promoting photosynthesis and reducing photorespiration [1,2].

The pyrenoid-bearing unicellular green alga *Chlamydomonas reinhardtii* has been extensively studied with the aim to apply the knowledge to engineer a CCM in crop plants [2]. The translational value of a single cell CCM is that it does not require complex multicellular anatomy, such as those in C₄ photosynthesis, and, hence, potentially confers higher transferability. Key components of a biophysical CCM have been described in *Chlamydomonas*, including several inorganic carbon transporters, carbonic anhydrases that interconvert CO₂ and HCO₃⁻, as well as pyrenoid structural proteins for tethering RuBisCO. Many of these CCM components appear to be unique to green algae, having no homolog in land plants [2]. Introducing a few individual components into *Arabidopsis* was unable to increase photosynthetic efficiency [2], suggesting that a suite of modules is required simultaneously.

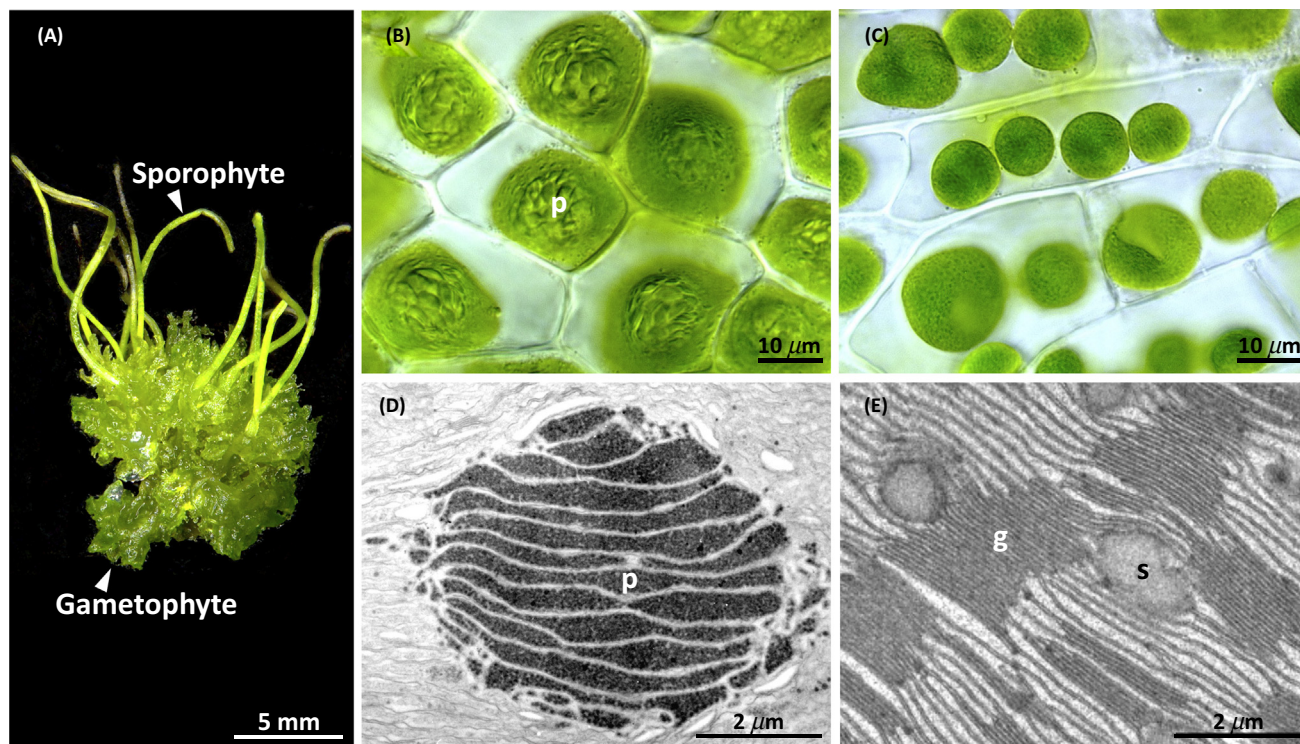
Here, we argue that focusing on a CCM system that is closer to crop species, not only evolutionarily, but also structurally, can provide novel insights into CCM engineering. Hornworts are the only land plant lineage with pyrenoids and capable of a biophysical CCM [3]. One crucial chloroplast feature that differentiates hornworts from algae is the presence of grana [4], a stacked arrangement of thylakoids involved in light capturing. Likely absent

in algae but present in land plants, grana result in more efficient light-capturing systems and the spatial separation of photosystems (which avoids energy spill between them). Given that hornworts uniquely combine pyrenoids, similar to green algae, and grana, similar to land plants, the internal architecture of chloroplasts in this group is unparalleled in photosynthetic organisms.

In most hornworts, the pyrenoid comprises multiple subunits traversed by thylakoids (Figure 1D) and typically lacking an outer starch sheath [4]. Plastid and pyrenoid microanatomy shows considerable variability across hornworts, to the extent that this feature alone is diagnostic of some taxonomical groups. Most notable are the pyrenoid-less plastids with extensive grana in *Leiosporoceros* and some *Nothoceros* (Figure 1), and the star-shaped plastids of the epiphytic *Dendroceros*, with spherical inclusions within pyrenoids [4]; pyrenoid-present species also tend to be uniplastidic (Figure 1B). RuBisCO is highly concentrated in pyrenoids, whereas, in the pyrenoid-less species, it is dispersed throughout the stroma, as in other land plants [4]. Stable isotope analyses showed that the pyrenoid-present species have significantly higher δ¹³C than the pyrenoid-less hornworts and other bryophytes, providing unequivocal evidence for CCM in hornwort pyrenoids [3]. Interestingly, a recent phylogenetic study showed that hornwort pyrenoids have been repeatedly lost and gained during the past 50 million years [5], which suggests that the development of

Box 1. Hornworts

Hornworts are the least-speciose lineage (220 spp.) among bryophytes, which also include mosses and liverworts. The phylogenetic position of hornworts is still debated: they have been placed as a sister lineage to vascular plants, to land plants, or to the other two bryophyte groups [12]. The hornwort life cycle, similar to other bryophytes, comprises a dominant haploid phase alternating with a spore-producing diploid phase. Hornworts have several characteristics connecting them with both algal and land plant lineages. For instance, the presence of solitary chloroplasts per cell with a pyrenoid is only found in algae, while the presence of stomata on their sporophytes supports ties with mosses and vascular plants. Hornworts are particularly exceptional in their associations with microbes; every hornwort species is capable of forming symbiosis with nitrogen-fixing cyanobacteria as well as mycorrhizal glomalean and endogonalean fungi. The key evolutionary position of hornworts and their unique life history make them ideal to use to tackle a range of fundamental questions in plant and evolutionary biology.



Trends in Plant Science

Figure 1. The Diversity of Hornwort Chloroplasts. (A) The model hornwort *Anthoceros agrestis*. (B) *Nothoceros vincentianus* has a single chloroplast per cell and a prominent pyrenoid (p). (C) *Nothoceros fuegiensis* has multiple chloroplasts per cell and lacks pyrenoid. (D) The pyrenoid in *Notothylas breutellii* chloroplast, shown as the electron-dense units traversed by thylakoids. (E) The pyrenoid-less chloroplast of *Leiosporoceros dussii*, with grana (g) and starch grain (s) but no pyrenoid. Reproduced, with permission, from J.C. Villarreal (B,C,E) and K. Renzaglia (D).

pyrenoids is controlled by a few master switches. The fact that there are closely related species with pyrenoid present in one, but absent in the other, makes comparative studies possible and potentially powerful.

We believe that the time is ripe to take hornworts as an emerging model to understand the evolution of pyrenoid-based CCMs. Genomic resources are becoming available for hornworts. The One Thousand Plant project (1KP) has generated transcriptomes for eight hornwort species [6], covering both pyrenoid-present (three) and -absent (five) species. Draft genome assemblies are also available for *Anthoceros punctatus* and *Anthoceros agrestis* [7,8]. Recently, the pyrenoid-bearing *A. agrestis* was established as an emerging model system [9]. Laboratory protocols have been developed to maintain and complete the life

cycle of *A. agrestis* within 2–3 months under axenic conditions. The draft genome of *A. agrestis* is being improved with the goal to bring the assembly to the chromosomal scale [8]. To facilitate classical forward genetic screen, axenic cultures for two polymorphic *A. agrestis* strains are available and data on inter-strain polymorphisms have been released [9]. The recent successful use of the CRISPR/Cas9 system in the two other bryophyte lineages (mosses and liverworts [10,11]) further points to the potential to carry out genetic manipulations in *A. agrestis*.

There are three research directions that we think are worth pursuing in the immediate future. First, the fundamental physiology of hornwort pyrenoids is poorly understood. In particular, it is unknown whether pyrenoid structure and function change with varying CO₂ levels, and

whether they show any diurnal regulation, as seen in *Chlamydomonas* [1]. Characterizing these basic physiological responses will be critical for subsequent genetic studies. Second, hornwort pyrenoids represent an independent origin from those in green algae, but it is unclear to what extent this convergence translates to the molecular level. Did hornworts recruit similar CCM genes to those found in green algae? Although many of the *Chlamydomonas* CCM components [often referred to as low-CO₂ inducible (LCI) genes] are thought to be unique to green algae, homologs of LCIB have been identified in hornwort transcriptomes and genomes. Interestingly, besides hornworts, no LCIB homolog has been found in the land plant genomes sequenced to date. In *Chlamydomonas*, LCIB proteins localize around pyrenoids and are hypothesized to prevent CO₂ leakage from the pyrenoids [1]. Whether LCIB

homologs have similar function and localization in hornworts remains to be tested, but we anticipate that further comparative genomic studies will allow us to assess the genetic commonality between algal and hornwort CCMs. Finally, the presence and absence of pyrenoids spreading across the phylogeny also provide a unique comparative framework for candidate gene discovery. Comparing gene expression under different CO₂ levels and across multiple pyrenoid-present and -absent species should provide a list of candidate genes, which can then be validated in *Chlamydomonas* and also in the model hornwort *A. agrestis*.

In conclusion, hornworts are a unique, yet overlooked, window into understanding CCMs at the single cell level. The close evolutionary relationship of hornworts with crop species (compared with green algae) makes them particularly attractive, and the natural diversity of hornwort chloroplasts represents an untapped resource for comparative studies. With

the genomic and genetic tools becoming available for hornworts, future work on elucidating the genetic basis of hornwort CCM should prove fruitful.

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