Hornwort pyrenoids: a terrestrial exception with engineering lessons

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) underpins nearly all primary production yet remains a slow, error-prone enzyme because it catalyzes both carboxylation and oxygenation, the latter initiating photorespiration and reducing net carbon gain. Many organisms mitigate these limitations not by improving Rubisco selectivity for CO_2 directly, but by modifying its local environment using CO_2-concentrating mechanisms (CCMs). In algae, a prominent biophysical CCM strategy is the pyrenoid: a phase-separated, Rubisco-rich condensate coupled to bicarbonate transport, local carbonic anhydrase activity, and diffusion barriers that elevate CO_2 at Rubisco active sites. Although pyrenoids have been most intensively studied in algal models, a pyrenoid-based CCM has evolved independently in a single land-plant lineage—the hornworts—providing a powerful comparative system for understanding how chloroplast organization can be tuned to terrestrial CO_2-delivery constraints. Here we synthesize a century of hornwort pyrenoid research in ecological, phylogenetic, and mechanistic context. We summarize bryophyte anatomical and microhabitat features that impose strong CO_2 diffusion limitation, and compare hornwort and algal pyrenoids in ultrastructure, molecular parts lists, and regulation. We highlight emerging models for hornwort pyrenoid formation, inorganic-carbon delivery, CO_2 generation and recapture, and recent biochemical/structural work revealing distinctive hornwort Rubisco properties and biogenesis. Finally, we discuss how hornwort pyrenoids complement efforts to engineer algal pyrenoid components into C_3 crops, and propose modular, hybrid engineering strategies that leverage hornwort compatibility with the embryophyte chloroplast, while selectively importing algal modules. Together, hornwort pyrenoids illustrate both the convergent logic and the lineage-specific solutions of biophysical CO_2 concentration, and they open avenues for mechanistic discovery and photosynthesis engineering.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the primary step of carbon fixation and underpins the production of nearly all organic carbon on Earth. Despite this central role, Rubisco is an unusually poor catalyst: its catalytic turnover is slow, its affinity for CO_2 is modest, and its active site often fails to discriminate between CO_2 and O_2. When O_2 is incorporated instead of CO_2, Rubisco initiates photorespiration. While photorespiration is a key plant process linked to a variety of key metabolic processes (for review see: (Busch, 2020)), excessive photorespiration is metabolically costly and reduces net carbon gain (for reviews see: (Hagemann and Bauwe, 2016; Walker et al., 2016)). Across evolutionary history, organisms have adapted to the limitations of this essential and

imperfect enzyme in several ways. Many compensate through sheer abundance: in C_3 leaves, Rubisco can account for up to half of all soluble protein (for reviews see: (Whitney et al., 2011; Prywes et al., 2023)). Others have achieved modest kinetic improvements through sequence and structural variation (Bouvier et al. 2024). However, perhaps the most transformative solutions have not come from altering Rubisco itself, but from modifying the environment in which Rubisco operates.

These environmental strategies are collectively known as CO_2-concentrating mechanisms (CCMs). CCMs elevate CO_2 levels around Rubisco, reduce oxygenation events, and greatly enhance the efficiency of carbon fixation (for reviews see: (Wunder et al. 2019; Liu 2022; Mattinson and Kelly 2025; Monson et al. 2025; Winter and Smith 2022). They occur in multiple forms depending on lineage and ecology. Biochemical CCMs, such as C_4 and CAM in vascular plants, rely on metabolic partitioning and specialized leaf anatomy to increase CO_2 supply. Biophysical CCMs, found in algae and cyanobacteria, instead use microcompartments, pyrenoids or carboxysomes, that compartmentalize and co-localize Rubisco with carbonic anhydrases to generate CO_2 directly at the active site.

Pyrenoids are phase-separated, protein-rich condensates inside the chloroplast stroma, typically forming a dense Rubisco matrix associated with one or more intrinsically disordered "linker" proteins (Barrett et al., 2026). Over the past decade, a surge of work, driven largely by algal (including diatom) model systems, has transformed pyrenoids from enigmatic Rubisco-containing "blobs" into tractable molecular assemblies, revealing principles of condensate organization and motivating ambitious efforts to reconstitute pyrenoid-like structures in vascular plant chloroplasts (Hennacy and Jonikas, 2020; Long et al., 2025; Barrett et al., 2026).

However, pyrenoids are not only an algal innovation. A pyrenoid-based CCM (pCCM) has evolved in a single lineage of land plants: the hornworts (Vaughn et al., 1990; Li et al., 2017; Robison et al., 2025; Robison et al., 2026). Hornworts are a lineage of bryophytes, which are non-vascular, seed-free terrestrial plants (Fig. 1A-D). Bryophytes and vascular plants diverged from each other approximately 500 million years ago, based on molecular clock estimates (Morris et al., 2018). This deep evolutionary separation makes hornwort pyrenoids a powerful comparative system: they likely represent an independent Rubisco condensation solution, and they have achieved this in a terrestrial context (Li et al., 2017). Further, bryophytes have evolved under unique ecological and anatomical photosynthetic constraints that limit chloroplast CO_2 availability (Proctor 2000). Thus, the repeated evolution and retention of a Rubisco condensate is not just a curiosity; it demonstrates how chloroplast organization can be tuned to terrestrial microenvironments.

Hornwort pyrenoids have historically received far less attention than their algal counterparts. In this review, we provide a historical perspective of hornwort pyrenoid research, discuss constraints on bryophyte photosynthesis, and summarize hornwort evolution and phylogeny in the context of pyrenoids. We also compare and contrast algal and hornwort pyrenoid molecular composition and ultrastructure, and review the assembly and functional divergence exhibited by hornwort Rubiscos. Finally, we discuss how hornwort pyrenoids inform current efforts to engineer biophysical CCMs in vascular plants, and present outstanding questions and future research opportunities.

Hornwort pyrenoid research: a historical perspective

Hornworts are one of the three major bryophyte lineages (together with liverworts and mosses) (Nishiyama et al., 2004; Wickett et al., 2014; One Thousand Plant Transcriptomes Initiative, 2019; Li et al., 2020) and are the least species rich (Söderström et al., 2016). Perhaps because of their deep divergence, hornworts have a perplexing combination of traits, including being the only land plant to have pyrenoids (Renzaglia, 1978; Villarreal Aguilar and Renzaglia, 2015; Li et al., 2017; Li et al., 2020; Frangedakis et al., 2021).

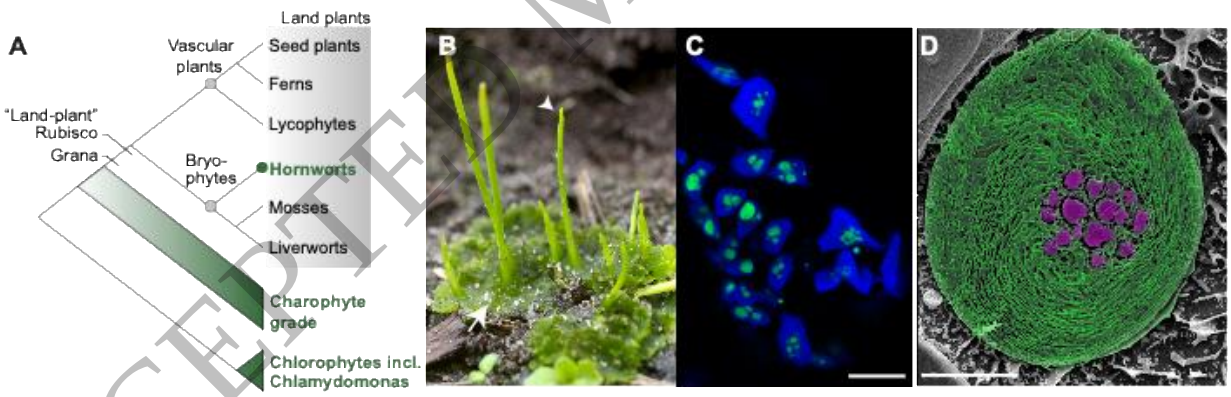

Figure 1. **Hornworts are the only land plants with pyrenoids.** (A) Phylogeny of major plant groups. Lineages that have pyrenoids are colored green. (B) A hornwort plant with sporophytes (2n; arrowhead) and gametophytes (n; arrow) indicated. (C) The model hornwort *Anthoceros agrestis* expressing GFP-tagged Rubisco activase (RCA; green), which localizes to pyrenoids and therefore serves as a fluorescent marker for pyrenoid position within chloroplasts (blue). Scale bar, 10 µm. (D) Pseudocolored scanning electron microscope image of a hornwort chloroplast (green), showing multiple pyrenoids (purple). Scale bar, 5 µm.

The first detailed investigations of hornwort pyrenoids came from McAllister in 1914, where he described the *Phaeoceros laevis* pyrenoid as a "multiple pyrenoid" consisting of dozens to hundreds of small bodies, drawing a clear contrast to the single, spherical

bodies found in algae (McAllister, 1914; Lacoste-Royal and Gibbs, 1987; McKay and Gibbs, 1989). Subsequent light microscopic studies would largely confirm these observations (Scherrer, 1914; Kaja, 1954), although Kaja described the pyrenoid of *Notothylas orbicularis* as a single body. Despite the structural differences, McAllister hypothesizes (on the basis of cytological dyes like Millon's Reagent) that the hornwort pyrenoids, like in algae, are protein rich and are very often surrounded by starch granules. In a later study that included *N. orbicularis*, McAllister observed that pyrenoids appear to be more clearly defined in plastids closer to the dorsal surface of the plant, noting a possible association of pyrenoids with photosynthesis (McAllister, 1927).

With the advent of electron microscopy (EM) came new, more detailed investigations of hornwort pyrenoids. Contrasting with previous reports, these electron micrographs revealed several pyrenoid units in a chloroplast (Menke, 1961; Manton, 1962; Sun, 1962; Wilsenach, 1963; Wujek, 1966), rather than dozens or hundreds. This discrepancy might be explained by artifacts of the staining preparation employed by McAllister (1914) and Scherrer (1914). Electron microscopy also made clear that individual pyrenoids were often divided only by tightly appressed thylakoids, but no serial sections had been performed to determine if pyrenoids are in fact separate or just a highly dissected structure.

These EM investigations, however, focused on *Notothylas, Phaeoceros* and *Anthoceros*, while other genera had been largely ignored and no work had been done on pyrenoid-lacking hornworts. A more complete survey of hornwort diversity as well as an attempt to integrate ultrastructural differences in a phylogenetic context was conducted by Burr (1970). Based on ultrastructural features, Burr arranged species from "primitive" (algae-like) to "advanced" (vascular plant-like) in a ladder-like fashion (Burr, 1970). Despite this flawed approach, Burr's detailed ultrastructural investigations revealed the presence of channel thylakoids, which were previously thought to be artifacts and provided a more detailed view of hornwort chloroplast ultrastructure. Channel thylakoids are unique to hornworts: tubular structures running perpendicular to the grana, enriched in photosystem I, which likely originated from infolding of thylakoid membranes (Burr 1970, Vaughn et al. 1992). Their developmental origin and function in hornwort photosynthetic machinery remains to be elucidated.

At the same time, biochemical fractionation and isolation of pyrenoids from the green alga *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) showed strong enrichment of Rubisco (Holdsworth, 1971; Lacoste-Royal and Gibbs, 1987), helping to establish the pyrenoid as a central feature of algal carbon fixation and laying the

groundwork for its subsequent placement within the CCM framework (Badger and Price, 1992). Building on these insights, Valentine et al. (1986) suggested that hornwort pyrenoids might likewise contribute to a CCM. Later immunogold labeling revealed that, like observed in algae, hornwort pyrenoids were indeed the site of Rubisco accumulation (Vaughn et al., 1990). Vaughn et al. also noted that in the pyrenoid-lacking hornwort species, Rubisco was dispersed throughout the stroma (Vaughn et al., 1990; Vaughn et al., 1992), suggesting a lack of a CCM and providing an early comparative framework for testing CCM-related hypotheses across hornwort lineages.

Physiological comparison of the pyrenoid-containing hornwort *Anthoceros punctatus (A. crispulus* in the study) to the pyrenoid-lacking liverwort *Pellia endivifolia* demonstrated that *A. punctatus* has lower carbon isotope discrimination, a lower CO_2 compensation point, and Ci pool accumulation, which are clear hallmarks of a CCM (Smith and Griffiths, 1996). Smith and Griffiths also later established that carbonic anhydrase (CA) inhibitors suppress photosynthesis far more strongly in hornworts than in other bryophytes, consistent with a CA-dependent CCM that is functionally analogous to biophysical algal CCMs (Smith and Griffiths, 2000).

Comparison of gas exchange characteristics of hornworts with and without pyrenoids (e.g. *Megaceros*) further revealed that even pyrenoid-lacking hornworts display weak CCM characteristics, suggesting that the presence of a pyrenoid might strengthen a CCM, but it is not a prerequisite (Smith and Griffiths, 1996; Hanson et al., 2002). Hanson et al (2002) also notes that hornworts have additional electron transport requirements compared to liverworts, especially at low CO_2, possibly because of additional energy requirements for the CCM (Burlacot et al., 2022).

Over the past century, the somewhat sporadic research into hornwort pyrenoids clearly established the presence of a biophysical CCM. However, the fundamental question remains: why did a terrestrial plant lineage evolve a CCM that is otherwise characteristic of aquatic algae?

Terrestrialization and the constraints of bryophyte photosynthesis

Some 500 million years ago the colonization of land by plants began (Morris et al., 2018). This course of events radically transformed the composition of the atmosphere, global nutrient cycles, and the trajectory of life on earth (de Vries and Archibald, 2018). Today, the near ubiquity of plants on our planet belies the physiological, morphological, and molecular adaptations required for terrestrialization. Extant land plants can be divided into two lineages which diverged approximately 450 million years ago: the vascular plants and the bryophytes (mosses, liverworts, and hornworts) (One Thousand Plant Transcriptomes

Initiative, 2019; Li et al., 2020). This deep divergence is reflected in the vast differences in life cycle, ecological niche, and adaptive traits that these groups evolved to cope with life ashore. While seed plant adaptations are plainly evident in the sequoias towering over the forest floor, in the cactus enduring the harsh desert, or the grasses that cover the prairies, seedless plant adaptations—and bryophytes in particular—are less apparent, leading to the common misconception that they are "primitive", "simple", or "ancient" (Rousk and Villarreal A, 2025).

As plants transitioned to life on land, CO_2—which diffuses some 10,000 times slower in water than air—availability to photosynthetic tissue was greatly enhanced, but so too was the risk of desiccation (Graham et al., 2014). Even today, this tradeoff between conductance for CO_2 and transpirational water loss is a fundamental constraint of plant physiology that has guided many of the striking biochemical, structural, and physical adaptations seen in land plants (Meyer et al., 2008; Clark et al., 2022). In vascular plants, stomata can respond to environmental cues to balance CO_2 uptake with transpirational water loss. However, bryophyte gametophytes—the dominant life phase—lack stomata, precluding such dynamic transpirational regulation (Renzaglia et al. 2017; Pressel et al. 2018). While Fortin and Friedman (2024) proposed that hornwort gametophytic pores could be homologous to stomata, such pores function as the entry points for symbiotic cyanobacteria and not for gas exchange (Renzaglia et al., 2009).

Bryophytes have largely adapted to minimize transpirational losses at the expense of their internal conductance for CO_2, consequently lowering their maximum rate of photosynthesis (Meyer et al., 2008; Hanson et al., 2014). Even accounting for confounding factors such as stomata in vascular plants and water films present on the surface of many bryophytes, conductance for CO_2 is at least an order of magnitude lower in moss leaves (phyllids) and up to two orders of magnitude lower in thalloid liverworts and hornworts compared to vascular plants (Meyer et al., 2008). This difference in conductance can be explained to some extent by the fact that some bryophytes have notably thicker cell walls than vascular plants (Flexas et al., 2021; Roig-Oliver et al., 2021).

In addition to the structural limitations to CO_2 conductance in bryophytes, it is also worth considering the environments they are best adapted to, and the additional challenge this presents. Bryophytes often inhabit damp microhabitats where plants are frequently covered by thin water films following rain, dew, or high humidity, which is crucial for successful reproduction. Even micron-scale water layers may impose severe diffusional resistance to CO_2, slowing gas exchange by orders of magnitude. As a result, bryophytes periodically experience CO_2 limitation similar to that of aquatic phototrophs. Taken together, the adaptive and ecological constraints placed on bryophyte carbon fixation are

considerable, and these constraints worsen when we view them in the biochemical context of carbon fixation.

Hornworts are uniquely "CCM-ready" among land plants

Why, then, do only hornworts—and no other bryophytes—possess a biophysical CCM? We speculate that this could be explained by a combination of hornworts' thallus structure, monoplastidic nature, and carbon-handling machinery that may have made pyrenoid evolution comparatively accessible.

While mosses and liverworts can certainly experience severe diffusion limitation when wet, they often mitigate CO_2 delivery constraints (via non-pyrenoid routes), potentially reducing the selective payoff for a biophysical CCM. For instance, many complex thalloid liverworts possess air pores and internal air chambers that enhance internal diffusion ("ventilation" anatomy). Furthermore, mosses and liverworts contain numerous chloroplasts that are distributed along the cell periphery, maximizing exposure to incoming CO_2 when it is available. In contrast, many hornworts possess a single large chloroplast (MacLeod et al., 2022), which has a comparatively low surface area to volume ratio and may therefore restrict CO_2 delivery. Under these conditions, concentrating CO_2 around Rubisco may be an adaptation to overcome the limitations of relying on diffusion alone. Consistent with this idea, hornwort species with multiple epidermal chloroplasts (2–8 per cell), which would be expected to provide greater chloroplast surface area for gas exchange, invariably lack pyrenoids (Villarreal and Renner, 2012). This explanation is, however, not entirely satisfactory as even these multiplastidic hornworts could have a CCM of some kind, and there are many monoplastidic hornworts that lack pyrenoids (Hanson et al. 2002; Hanson et al. 2014; Meyer Moritz et al. 2008).

However, selection pressure alone is not enough: a functional pyrenoid requires the existence, and appropriate expression and localization, of the CCM machinery. Just as C_4 photosynthesis evolved repeatedly by repurposing existing plant genes (Hibberd and Covshoff, 2010; Emms et al., 2016; Sage, 2016), pyrenoid evolution may be relatively readily achievable when lineages already possess much of the necessary carbon-handling toolkit and need only rewire localization and expression. From this perspective, hornworts may have been effectively 'CCM-ready'. As we discuss below, hornworts, including pyrenoid-less species, are the only land plants to have retained orthologs to both of the chloroplastic carbonic anhydrases (CAH3 and LCIB) that are essential for the Chlamydomonas CCM, although many mosses and liverworts also have a CAH3 ortholog. In this context, pyrenoids may be comparatively evolvable in hornworts because their core innovation is organizational rather than enzymatic: a phase-separated Rubisco matrix can

emerge from multivalent binding interactions, and many unrelated proteins could, in principle, evolve the required linker-like properties.

Diversity and evolution of hornwort pyrenoids

The evolution of pyrenoids in hornworts is notably complex and dynamic. Previous phylogenetic analyses of one third of all hornwort species and all genera estimated a Carboniferous crown age for hornworts (and hence potentially for pyrenoids), between 318 and 348 million years ago, coinciding with a precipitous drop in atmospheric CO_2 near the end of the Devonian period (Peñaloza-Bojacá et al., 2025). As the only plant group inferred to have diversified under such low atmospheric CO_2, hornworts may have experienced selection for successful photosynthesis under these conditions, potentially favoring pyrenoid evolution. Additional studies have suggested that hornwort pyrenoids have likely evolved independently multiple times within hornworts in the last 35-50 million years, coinciding with fluctuating oscillations in atmospheric CO_2, and that most extant species diversity is the result of radiation from this period (Villarreal and Renner, 2012). While pyrenoid-lacking genera *Phaeomegaceros* and *Megaceros* appear to have also diversified during this period, clades with pyrenoids such as *Anthoceros* and *Dendroceros* tend to be more species rich.

Consistent with recurrent origins, as in C_4 plants, pyrenoid structure and morphology varies substantially across hornwort lineages. However, the lack of 3D reconstructions of hornwort chloroplasts limits a full assessment of hornwort pyrenoid architecture: most studies rely on single-plane EM micrographs (surface views or transverse sections) from selected tissues, often the gametophyte epidermis. Moreover, only a handful of taxa have been examined in detail. Based on the available literature (Vaughn et al., 1992), hornwort pyrenoids can be divided into three main morphological types. *Anthoceros*-type pyrenoids (*Anthoceros*, including the subgenus *Folioceros*, and most *Phaeoceros* species) are darkly stained and traversed by multiple thylakoids or even grana, often giving the appearance of multiple pyrenoid units (Fig 2A). *Notothylas*-type pyrenoids are typically compact and darkly stained, with a single thylakoid traversing the matrix. *Dendroceros*-type pyrenoids comprise irregularly shaped subunits and contain numerous dark inclusions with thylakoids, including short grana, interrupting the pyrenoid. Channel thylakoids are rarely observed within the pyrenoid matrix, except in lightly-stained pyrenoids reported for *Nothoceros* and *Phymatoceros* species. Starch grains can accumulate around the pyrenoid in most species, except *Dendroceros*, especially in mature plastids but they do not form a solid *Chlamydomonas*-like shell around the pyrenoid.

Structure and organization of hornwort pCCMs

Across eukaryotes, pCCMs share three core functional components: (i) Rubisco, (ii) compartmentalization of Rubisco into pyrenoids, and (iii) delivery of inorganic carbon (typically as HCO_3^-) to the vicinity of the pyrenoid, coupled to local conversion of HCO_3^- to CO_2 near Rubisco active sites to create a high-CO_2 microenvironment.

The machine: hornwort Rubisco

Rubisco is the central piece of the pCCM. Recently, hornwort Rubisco was found to have unconventional assembly factor dependencies and kinetic behaviors that depart from canonical plant Rubisco (Oh et al., 2024). A typical green-type Rubisco is composed of 8 large subunits (RbcL, encoded by the plastid genome) and 8 small subunits (RbcS, encoded by the nucleus genome). In the green lineage, Rubisco is produced through a multi-step biogenesis pathway in which general plant chaperonins (Cpn60/Cpn20) and Rubisco-specific assembly factors (Raf1, Raf2, BSD2, RbcX) shepherd RbcL folding, L_8 core formation, and RbcS incorporation into the L_8S_8 holoenzyme (Aigner et al., 2017). In angiosperms, this network is strikingly species-selective, such that "mix-and-match" assembly across distant lineages often fails, creating a practical bottleneck for both basic comparative enzymology and Rubisco engineering (Loh and Gunn, 2025).

Our recent biochemical and structural work revealed that hornwort Rubisco biogenesis can markedly diverge from that of other land plants (Oh et al., 2024). Unlike angiosperm Rubisco, *Anthoceros agrestis* Rubisco assembles efficiently without Raf2 or RbcX, depending instead only on hornwort-specific combinations of Cpn60α/β, Cpn20, Raf1, and BSD2. Removing one of the assembly factors, RbcX, essential for assembly of angiosperm Rubiscos not only failed to disrupt hornwort Rubisco assembly, but increased catalytic turnover by roughly 50%, implying that hornwort RbcX acts as a structural modulator rather than an obligate chaperone.

Hornwort Rubiscos, including those expressed in the absence of RbcX, exhibit a range of catalytic turnover rates (~3–10 s^{-1}), yet show comparatively similar CO_2 affinity (~30 μM) and CO_2/O_2 specificity (~70). This kinetic pattern is notable for two reasons. Firstly, CO_2 affinity and specificity appear somewhat arbitrarily fixed, regardless of presence/absence of a CCM. Typically, organisms with CCMs tend to produce Rubiscos with high catalytic turnover rates, but with reduced specificity consistent with a loosened selection pressure for high specificity in the high CO_2 CCM environment (Sage, 2002). Secondly, the Rubisco literature has historically emphasized a tight inverse coupling between turnover and specificity (particularly within land plants; (Tcherkez and Farquhar,

2021), even if larger modern datasets suggest these correlations may be weaker than previously assumed (Bouvier et al., 2021). Hornwort Rubiscos thus appear to decouple the canonical trade-off between catalytic speed and specificity, placing hornwort Rubiscos in a previously unexplored kinetic space (Oh et al., 2024). Structurally, hornwort Rubisco resembles other land plant Rubisco enzymes. However, when assembled in the absence of RbcX, subtle structural differences at the large subunit interfaces loosens the active-site environment, which could explain the enhanced CO_2-fixation rates.

It should be emphasized that the *A. agrestis* Rubisco synthesized and characterized by Oh et al (2024) included only one type of RbcS (*Aa*RbcS1). As we discuss below, *A. agrestis* harbors another unconventional RbcS isoform that serves as an 'innate' Rubisco linker.

Rubisco condensation

Across phylogenetically distant algal lineages, pyrenoid matrices are nucleated by multivalent "linker" proteins that physically crosslink Rubisco into a dense condensate (Barrett et al., 2026). Although algal pyrenoid linkers generally lack obvious sequence similarity (consistent with convergent evolution), these linkers typically follow a sticker-spacer design: short, repeated "sticker" motifs bind RbcL and/or RbcS, and are separated by intrinsically disordered spacer regions (Fig. 2B). Because Rubisco presents many repeated binding sites, a single linker can engage multiple holoenzymes at once, "crosslinking" Rubiscos into an interaction network. As these weak, multivalent interactions accumulate, the network demixes from the surrounding stroma to form a dense condensate. Candidate linkers can often be identified by repetitive motif content, predicted alternation between locally ordered and disordered segments, and compositionally biased low-complexity sequences (e.g., regions enriched in glycine/serine/glutamine/proline and/or charged residues) (Barrett et al., 2024).

These linker-Rubisco interactions have now been mechanistically resolved for several systems. In the diatom *Phaeodactylum tricornutum*, the tandem-repeat/prion-like protein PYCO1 binds Rubisco via sticker motifs that insert a conserved tryptophan residue into a phenylalanine-rich hydrophobic cleft on RbcL, stabilized by a small hydrogen-bond network (Oh et al., 2023). PYCO1 stickers also bind the RbcS ring that lines the central solvent channel of the Rubisco holoenzyme (at/near the channel entrance), with a key tryptophan-containing motif contributing to binding. In *Chlamydomonas*, the intrinsically disordered repeat protein EPYC1 uses multiple short helical stickers to bind RbcS helix αA and αB via salt bridges and a hydrophobic interface (Mackinder et al., 2016; He et al., 2020). *Chlorella sorokiniana*, from a separate green algal lineage, encodes CsLinker, which likewise engages Rubisco through electrostatic and hydrophobic contacts. However, unlike

EPYC1, CsLinker binds a conserved surface on the RbcL N-terminus rather than RbcS (Barrett et al., 2024). A candidate linker, PPAP28, has also been reported from the chlorarachniophyte *Amorphochlora amoebiformis* (Moromizato et al., 2024). PPAP28 is a largely disordered, repeat-rich pyrenoid-localized protein identified by proteomics, and its direct role in Rubisco condensation remains to be fully validated.

The validated hornwort linker from *A. agrestis* departs sharply from the canonical algal sticker-spacer arrangement. Here, the linker is a special RbcS bearing an ~100 amino acid C-terminal extension, termed SequesTration Associated Region (STAR; (Robison et al., 2026)).

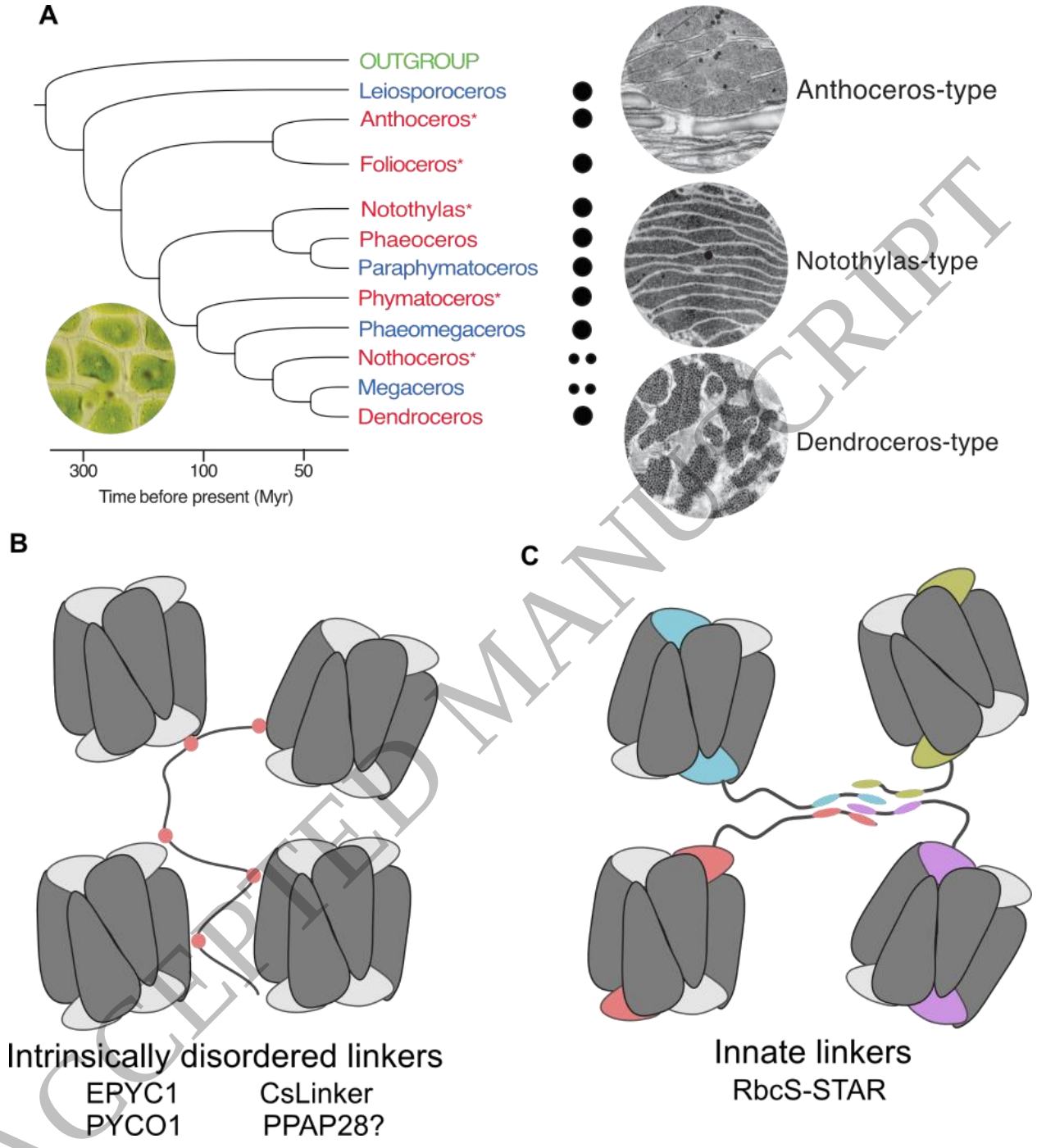

Figure 2. **Pyrenoid diversity. (**A) Simplified hornwort chronogram with mapped plastid (uni or multiplastidic) and pyrenoid evolution. Pyrenoid-containing lineages and pyrenoid-lacking lineages are colored red and blue, respectively. An asterisk (*) indicates that some species in the genus lack pyrenoids. (B) Depiction of intrinsically disordered linkers, which have motifs (red circles) that bind to either the Rubisco large or small subunit. (C) Depiction of RbcS-STAR, an innate linker which is integrated into Rubisco and binds to other linkers, rather than Rubisco. Rubisco large subunits, canonical RbcS, and RbcS-STAR are shown in dark gray, light gray, and different colors, respectively.

STAR contains three repetitive 23 amino acid motifs (m1, m2, and m3). Compared with m2 and m3, the m1 sequence shows lower conservation and is predicted to lack defined secondary structure. Contrastingly, m2 and m3 form coiled coils, displaying canonical heptad repeats, which are required to produce their distinctive amphipathic α-helices. RbcS-STAR is incorporated into the Rubisco holoenzyme in place of a canonical RbcS, and pyrenoid assembly is driven by coiled-coil interactions between STAR extensions protruding from neighboring Rubisco, potentially engaging up to four STAR domains. Thus, unlike algal systems in which condensation is driven primarily by heterotypic linker-Rubisco interactions, hornwort pyrenoid formation is (thus far) unique in being driven by self-association of an innate Rubisco linker (Fig. 2C). Notably, while EPYC1 can form bundled, self-associated assemblies, it does not appear to phase-separate via homotypic interactions alone; instead, condensation arises primarily through heterotypic EPYC1-Rubisco crosslinking (Küffner et al., 2024).

Possible repeated evolution of RbcS-STAR

Curiously, innate linkers, such as RbcS-STAR, might not be restricted to *A. agrestis*. Similar RbcS isoforms with a putative C-terminal STAR are also found in *Phaeoceros* species (Schafran et al., 2025; Robison et al., 2026), which evolved pyrenoids independently (Villarreal and Renner, 2012). In *Phaeoceros*, these putative innate linkers arose from a distinct RbcS lineage, and its STAR-like extension shares no detectable sequence homology with that of *A. agrestis*. Despite this lack of sequence conservation, the *Phaeoceros* STAR is likewise predicted to form coiled-coil structures, a feature required for Rubisco condensation in *A. agrestis* (Robison et al., 2026). These observations suggest that recurrent convergent origins of innate linkers may have underpinned pyrenoid evolution in hornworts, although functional validation of *Phaeoceros* RbcS-STAR remains necessary. Conversely, the absence of RbcS-STAR analogs in some pyrenoid-bearing hornworts indicates that additional distinct mechanisms for Rubisco condensation must exist in hornworts.

Hornwort pyrenoid properties

Pyrenoids differ strikingly in their lifetime and regulatory dynamics. In *Chlamydomonas*, the pyrenoid matrix behaves as a liquid-like condensate that dissolves and re-condenses during cell division, and the formation is responsive to environmental cues (Freeman Rosenzweig et al., 2017; Neofotis et al., 2021; He et al., 2023). This dynamic regulation is

achieved through modulating the EPYC1-Rubisco interaction by phosphorylating EPYC1 via the kinase KEY1 (He et al., 2025). In other algae, classical ultrastructural studies also report pyrenoid regression or disappearance before division followed by re-formation in daughter cells (e.g., *Tetracystis excentrica*, *Euglena gracilis*), consistent with cell-cycle–coupled disassembly/rebuilding in multiple lineages (Brown and Arnott, 1970; Pellegrini, 1980; Meyer et al., 2017).

In contrast, live imaging of *A. agrestis* pyrenoids suggest they are constitutively present and persist through cell division, with multiple pyrenoids often observed within a single chloroplast (Lafferty et al., 2024; Robison et al., 2025). Moreover, the hornwort pCCM has thus far been shown to be constitutive and unresponsive to external CO_2 concentrations (Hanson et al. 2002; Nötzold et al. 2025). This apparent lack of regulatory flexibility may stem from the innate nature of the *A. agrestis* linker. Because RbcS-STAR is an integral component of *A. agrestis* Rubisco, pyrenoid disassembly would require either turnover of the existing Rubisco pool or a mechanism to weaken, compete with, or actively remodel the coiled-coil interactions between STAR motifs. Reversible condensates driven in part by coiled-coil interactions have been demonstrated in both engineered and natural systems (Ramšak et al. 2023; Yeh et al. 2024; Jiang et al. 2021), indicating that coiled-coil-mediated matrix assembly is, in principle, compatible with regulated disassembly, although no such mechanism has yet been identified in hornworts.

Fluorescence recovery after photobleaching (FRAP) experiments indicate different levels of liquidity depending on which pyrenoid component was targeted (Robison et al., 2026). Rubisco activase (RCA) recovered quickly after photobleaching, while RbcS-STAR was relatively immobile. The conventional RbcS isoform, RbcS1, exhibited a range of mobility, from rapid (like RCA) to slow (like RbcS-STAR). One possible explanation is that RbcL:RbcS-STAR stoichiometry plays a key role in determining pyrenoid properties. Fluorescently-tagged RbcS-STAR expression was driven by a strong promoter, and hence might have artificially increased the ratio of RbcS-STAR to other RbcS isoforms in Rubisco thereby making the matrix denser. Rubisco stoichiometry heterogeneity could also explain variability in RbcS1 mobility; because RbcS-STAR occupies the canonical small-subunit position within the Rubisco holoenzyme, it is competing for that position with RbcS1. Changing the relative expression of the small subunits, then, may also change the stoichiometry of RbcL:RbcS-STAR, thus altering the density of the matrix. Since factors like insert copy number and genomic position can't be controlled in biolistic transformation, the degree of RbcS1 expression–and therefore mobility–might vary significantly between transformation events.

HCO3⁻ delivery systems

A "Rubisco house" (i.e., a pyrenoid) can, in principle, provide a basal CO_2-fixation advantage via condensation-driven partitioning and mass-action effects (Küffner et al. 2024; Long et al. 2021), but robust and efficient CO_2 concentration typically requires additional "plumbing" to accumulate inorganic carbon and generate CO_2 near Rubisco. Bicarbonate is transported to the pyrenoid with the help of a series of bicarbonate channels. Outside of *Chlamydomonas*, *A. agrestis*, and a handful of additional models, bicarbonate delivery to pyrenoids is often supported by physiological and comparative evidence rather than molecular pathways. For example, there is evidence of bicarbonate acquisition and pumping across chloroplast membranes in haptophytes, but thylakoid transport and pyrenoid-proximal CA localization is generally unknown (Nimer and Merrett, 1996; Herfort et al., 2002). Similarly, physiological work supports the presence of CCM activity in symbiotic dinoflagellates (Leggat et al., 1999; Raven et al., 2020) and in diverse algal groups including red algae and cryptophytes (Meyer and Griffiths, 2013), but precise localization and molecular details are not yet resolved. *C. sorokiniana* and *A. amoebiformis* currently offer either partial localization evidence or proteomic candidate lists rather than a fully resolved pathway (Villarejo et al., 1998; Moromizato et al., 2024).

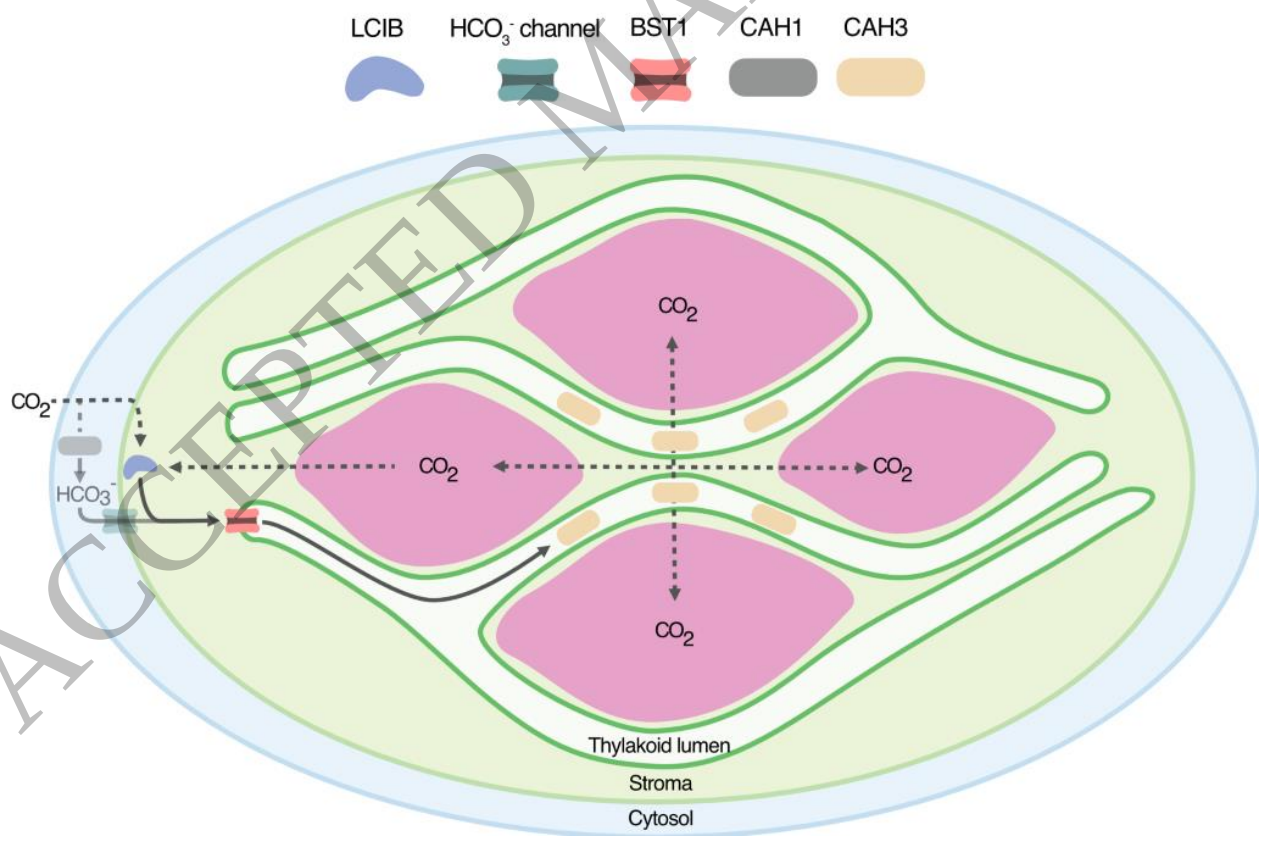

Figure 3. **A spatial model of the pCCM in hornworts.** This schematic summarizes a working model for Ci fluxes and CO_2 elevation around condensed Rubisco in *A. agrestis*. Ci is predicted to enter the cytosol and chloroplast primarily as CO_2. BST1 at the thylakoid membrane is hypothesized to mediate HCO_3^- entry into the thylakoid

lumen. LCIB at the chloroplast envelope is proposed to retain stromal Ci by trapping incoming CO_2 and recapturing CO_2 that leaks from the pyrenoid region. CAH3 is enriched in specialized, centrally positioned thylakoids associated with the pyrenoid and is hypothesized to generate CO_2 near the matrix. The matrix is a phase-separated, Rubisco enriched compartment (depicted in magenta), and the surrounding thylakoids are proposed to act as a diffusional barrier. Possible bicarbonate import from the cytosol and into the chloroplast is depicted with lower opacity vectors to indicate a lack of evidence. Dashed arrows indicate CO_2 movement; solid arrows indicate HCO_3^- movement. Ci, inorganic carbon; LCIB, Low Carbon Inducible protein B; CAH3, Carbonic Anhydrase 3; BST1, Bestrophin-like channel 1.

There is strong evidence in diatoms, including *P. tricornutum*, for a pyrenoid-associated thylakoid-lumen CO_2-generation step but a less unified spatial map (Nakajima et al., 2013; Kikutani et al., 2016; Raven and Giordano, 2017; Nam et al., 2024). Nonetheless, many lineages appear to converge on a design in which HCO_3^- is accumulated and then dehydrated to CO_2 by a pyrenoid-associated, often lumenal, carbonic anhydrase positioned in specialized membranes that traverse or closely contact the pyrenoid (Sinetova et al., 2012; Kikutani et al., 2016; Young and Hopkinson, 2017; Wang and Jonikas, 2020; Fei et al., 2022; He et al., 2023).

A compartment-resolved inorganic carbon delivery route is best established in *Chlamydomonas*, and emerging in hornworts (Robison et al., 2025). In *Chlamydomonas*, bicarbonate channels are located on the plasma (HLA3/LCI1), chloroplast (LCIA) and thylakoid (BST1) membranes. There is currently limited evidence supporting extracellular bicarbonate utilization by hornworts. In pH drift experiments, *A. agrestis* reached equilibrium at slightly higher pH than other bryophytes, but still much lower than aquatic plants and algae known to utilize bicarbonate, suggesting possible, but weak bicarbonate utilization in hornworts (Bain and Proctor 1980). CO_2, therefore, is likely the dominant inorganic carbon species entering the cytosol (and potentially the chloroplast), rather than HCO_3^-. However, support for intercellular bicarbonate utilization by the hornwort CCM is more clear. Hornworts accumulate light induced inorganic carbon pools even when the Calvin-Benson-Bassham Cycle is inhibited and their photosynthesis is greatly reduced when treated with CA inhibitors (Smith and Griffiths 1996; Smith and Griffiths 2000; Hanson et al. 2002). Whether hornworts actively import HCO_3^- into the chloroplast is uncertain as hornworts lack a clear LCIA ortholog. Two non-mutually-exclusive entry scenarios are plausible: (i) CO_2 diffuses into the cytosol, is converted to HCO_3^- by a cytosolic carbonic anhydrase, and HCO_3^- is then imported across the chloroplast envelope by as-yet-unidentified transporters; and/or (ii) CO_2 diffuses across the chloroplast envelope and is converted to HCO_3^- in the stroma by the hornwort LCIB ortholog (Fig. 3).

Once bicarbonate has been delivered to the pyrenoid, it is converted to CO_2 near Rubisco by the carbonic anhydrase CAH3. In *Chlamydomonas*, CAH3 is positioned within the thylakoid-derived "pyrenoid tubules" that traverse the Rubisco matrix, providing a membrane-associated site for HCO_3^- to CO_2 conversion adjacent to the condensate (Mackinder et al., 2017; Hennacy and Jonikas, 2020). However, traversing membranes are not universal across pyrenoids: while many algal pyrenoids are penetrated by thylakoid tubules or sheets, others show a homogeneous matrix that is not traversed by thylakoids, indicating that different lineages can achieve pyrenoid function with distinct internal membrane architectures (Kowallik, 1969; Stoyneva et al., 2009; Gärtner et al., 2012; Herburger et al., 2016).

In *A. agrestis*, the architecture is explicitly non-traversing and CAH3 is not concentrated in matrix-penetrating tubules. Instead, it is enriched in thylakoids in the central chloroplast space, around which multiple pyrenoids are organized (Fig. 3). CAH3 signals form puncta that are concentrated adjacent to the interior-facing side of pyrenoids (Robison et al., 2025; Ruaud et al., 2025). The thylakoid HCO_3^- channel BST1 is distributed across the thylakoid network but shows elevated signal on thylakoids immediately adjacent to pyrenoids (Robison et al., 2025), consistent with localized delivery of HCO_3^- to the lumen near pyrenoids. Thus, pyrenoids can couple lumenal CO_2 generation to Rubisco using either (i) intrapyrenoid tubules within a more continuous matrix (e.g., *Chlamydomonas*) or (ii) distributed thylakoid contacts around multiple pyrenoid units (e.g., *A. agrestis*). Hornwort pyrenoid diversity includes more *Chlamydomonas*-like organizational endpoints (e.g., *Notothylas*), suggesting a continuum of solutions for positioning CCM transport modules relative to the condensate.

CO_2 recapture

Pyrenoids are also accompanied by a CO_2 recapture system to minimize loss of CO_2 that leaks from the Rubisco-rich matrix. In *Chlamydomonas*, the CA-like protein LCIB accumulates at the pyrenoid periphery under very low CO_2 (i.e., sub-ambient CO_2 levels; Toyokawa et al., 2020), where it is positioned to rehydrate leaked CO_2 back to HCO_3^- (often described as localizing around gaps in the starch sheath), effectively acting as a "peripheral CO_2 trap." In *A. agrestis*, by contrast, fluorescently tagged LCIB does not concentrate around individual pyrenoids and instead localizes to the chloroplast envelope, co-localizing with an inner chloroplast envelope marker (Fig. 3) (Robison et al., 2025; Ruaud et al., 2025). This envelope localization is functionally logical because hornwort CAH3 is enriched outside the pyrenoid matrix (in the inter-pyrenoid thylakoid network), placing LCIB around each pyrenoid could "steal" CO_2 immediately after it is generated,

short-circuiting the CCM. Consistent with reaction-diffusion modelling for thylakoid-encased pyrenoids (Fei et al., 2022), LCIB positioned at the envelope is predicted to be optimal: far enough away to avoid intercepting CO_2 near Rubisco, but still able to serve as a "last line of defense" for CO_2 leakage. Finally, envelope-localized LCIB could also rapidly convert any CO_2 that diffuses into the chloroplast into HCO_3^- before it diffuses back out, potentially supporting a partially "passive" mode of CO_2 chloroplast entry in *A. agrestis*.

No starch sheath, no problem?

In many green algae, the pyrenoid matrix is surrounded by a starch sheath that is induced or enhanced under CCM conditions and is thought to reduce CO_2 leakage from the Rubisco-rich condensate, although there is no direct evidence for this role (Itakura et al., 2019; Toyokawa et al., 2020; Fei et al., 2022). While, the starch sheath is important for CCM efficiency in *Chlamydomonas* (Adler et al., 2025), it is difficult to disentangle the diffusion barrier hypothesis from its organizational role in the CCM. Beyond acting as a diffusion barrier, the sheath also helps organize the peri-pyrenoid CO_2-recapture system: in *Chlamydomonas*, starch-sheath mutants mis-localize LCIB and show impaired CCM function, consistent with the sheath providing spatial cues/scaffolding for recapture around the matrix (Toyokawa et al., 2020).

While many green algal pyrenoids are surrounded by starch plates, starch sheaths are not universal: diatom and hornwort pyrenoids lack a starch sheath, but may employ alternative boundary layers. For example, a proteinaceous casing in diatoms (Shimakawa et al., 2024), non-starch polysaccharide deposits in *Micromonas pusilla* (van Baren et al. 2016), or multiple stacked thylakoid layers that encase the pyrenoid matrix in hornworts. "Naked" pyrenoids that lack a discernable boundary layer have also been observed in *Porphyridium purpureum* (*Porphyridium cruentum* in the study; (Brody and Vatter, 1959)). Together, these examples highlight that different lineages can evolve distinct diffusion-barrier architectures to support the same core CCM objective: retaining elevated CO_2 in the vicinity of Rubisco.

Engineering opportunities

Multiple modeling studies suggest that elevating CO_2 around Rubisco via a biophysical CCM could substantially increase photosynthetic performance in C_3 crops (Long et al., 2025). For example, modelling suggests that introducing a cyanobacterial-style CCM into C_3 leaves could increase CO_2 uptake by ~60%, and, by analogy to yield responses observed

under elevated-CO_2 field conditions, this magnitude of improvement could plausibly translate to ~36–60% yield increases under appropriate constraints (McGrath and Long, 2014; Atkinson et al., 2016). More recently, detailed reaction-diffusion and energetic pCCM modeling has provided an explicit engineering roadmap for pyrenoid-based CCMs, and predicted that appropriately configured architecture could markedly boost chloroplast CO_2 fixation (Fei et al., 2022).

Proto-pyrenoids, which we define as condensates comprising Rubisco and a linker protein, have been successfully assembled in C3 plant chloroplasts. Such condensates have been induced using EPYC1 in *Arabidopsis thaliana* (Atkinson et al., 2020), and by expressing RbcS-STAR in both *Anthoceros fusiformis* (a pyrenoid-absent hornwort species) and *Arabidopsis* (Robison et al., 2026). However, an efficient and functional pyrenoid requires "plumbing" to/from Rubisco within the pyrenoid matrix. This involves thylakoid-associated bicarbonate transport, CO_2 generation near the pyrenoid matrix, and (likely) diffusion-barrier architecture. Mechanistic work in *Chlamydomonas* has identified proteins that organize key architectural features of the native pyrenoid, including matrix-starch sheath organization and specialized pyrenoid-associated membrane structure formation (Itakura et al., 2019; Hennacy et al., 2024). In a plant-engineering context, expressing additional algal components has begun to push proto-pyrenoids toward more pyrenoid-like architecture and functionality. For example, SAGA1 and SAGA2 are two pyrenoid-associated proteins with motifs that bind both Rubisco and starch, effectively connecting the Rubisco matrix to the starch sheath. Introducing SAGA1/SAGA2 into *Arabidopsis* drives the formation of starch plate-like structures around engineered proto-pyrenoids (Atkinson et al., 2024).

In parallel to algal-to-crop transplantation efforts, hornworts provide an appealing second blueprint because they are the only embryophytes with pyrenoids, meaning their condensates operate inside a land-plant cellular and plastid environment (Villarreal and Renner, 2012). This matters because a pCCM is not just a Rubisco droplet: performance depends on the spatial organization and choreography of membranes, lumenal pH gradients, carbonic anhydrase localization, and diffusion barriers relative to the Rubisco-rich matrix. Hornwort pyrenoids exhibit diverse ultrastructures, and many species show strong integration of the Rubisco matrix with land-plant-like thylakoid architecture (including grana/lamellae), highlighting that a "land-plant chassis" can support pyrenoid operation. Our recent *A. agrestis* spatial model sharpens this engineering appeal: rather than relying on a *Chlamydomonas*-like traversing pyrenoid tubule system, a hornwort "land-plant pyrenoid chassis" may reduce some of the architectural mismatch that could arise when transferring algal systems wholesale into crops.

A particularly attractive hornwort feature is the innate Rubisco linker. Where EPYC1-, PYCO1-, and CsLinker-driven condensation depends on specific Rubisco subunit interaction surfaces (and thus typically requires installing algal Rubisco sequence-structure), RbcS-STAR self-associates. As mentioned earlier, expressing RbcS-STAR in *Arabidopsis* is sufficient to induce Rubisco condensates. Appending just the STAR domain from RbcS-STAR to *Arabidopsis* RbcS was also sufficient for proto-pyrenoid formation in *Arabidopsis* chloroplasts (Robison et al., 2026). Thus, RbcS-STAR represents a truly Rubisco-agnostic linker system that requires no modification of the host Rubisco structure-function.

A pragmatic path forward may be a modular, and explicitly hybrid, engineering strategy: leveraging hornwort-derived components for embryophyte chloroplast environment compatibility, while importing algal parts where they provide uniquely well-resolved functions. In practice, this could mean driving condensation with the *A. agrestis* STAR linker, which can drive proto-pyrenoid formation without requiring modification of native crop Rubisco, and then layering in *Chlamydomonas* bicarbonate delivery modules as needed. For example, thylakoid-coupled bicarbonate delivery and lumenal CO_2-generation concepts that have strong mechanistic and modeling support (Fei et al., 2022), along with additional algal components that fine-tune boundary conditions (e.g., starch-associated organization factors), where beneficial. Framed this way, hornwort pyrenoids are not a replacement for *Chlamydomonas*-inspired engineering, but a more directly translatable foundation for crops that can be strengthened by selectively importing algal parts with clear, modular functionality.

Conclusions

As the only land plants that possess a pCCM, hornworts provide not only critical insights into the evolution of pyrenoids but also key components for engineering pCCMs in crop plants. Recent studies leveraging the emerging model species *A. agrestis* have revealed a CO_2-concentrating chassis broadly similar to that of *Chlamydomonas*. However, *A. agrestis* employs a drastically different strategy to condense Rubisco, relying on the innate, self-associating linker RbcS-STAR. Together, these features position hornworts as a uniquely tractable bridge between algal and terrestrial pCCMs.

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Author contributions

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Conflict of Interest statement

The authors have no conflicts to declare.

Box 1. Recent advances in hornwort pyrenoid biology

- The emergence of *Anthoceros agrestis* as a tractable land-plant pyrenoid model system has enabled routine live imaging, localization studies, and genetic manipulation of pCCM components.
- Hornwort pCCMs use a spatial architecture distinct from *Chlamydomonas*, with different organization and placement of membranes and CO_2-delivery systems.
- Hornwort pyrenoid condensation can be driven by an 'innate' Rubisco linker (RbcS-STAR), in which Rubisco-incorporated STAR extensions self-associate.
- Independent pyrenoid origins in hornworts are accompanied by distinct linker solutions, highlighting convergent routes to Rubisco condensation.
- Hornwort Rubisco has unconventional assembly-factor dependencies and kinetic behaviors compared with canonical land-plant Rubisco, expanding the known landscape of Rubisco biogenesis and structure-function relationships.
- Proto-pyrenoids have been assembled in C_3 plant chloroplasts using algal and hornwort linkers.
- Hornwort pyrenoids provide a land-plant chassis for hybrid pCCM engineering.

Box 2. Outstanding Questions

- How does inorganic carbon enter hornwort chloroplasts, and what are the chloroplast-envelope Ci transporters that replace the role of LCIA in lineages lacking a clear LCIA ortholog?
- Where do core pCCM components (e.g., CAH3, BST1, and LCIB) localize in pyrenoid-absent hornwort species?
- What diversity of linker mechanisms exist in hornwort species?
- What is the functional role of channel thylakoids in hornwort photosynthesis?
- Are hornwort pCCMs truly constitutive, or are they environmentally tunable across CO_2, hydration state, light, and/or development?
- What are the energetic costs of operating hornwort pyrenoids, and how are these balanced?
- What is the minimal module set required to achieve a functional pyrenoid CCM in crop chloroplasts?

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Figure Legends

Figure 1. **Hornworts are the only land plants with pyrenoids.** (A) Phylogeny of major plant groups. Lineages that have pyrenoids are colored green. (B) A hornwort plant with sporophytes (2n; arrowhead) and gametophytes (n; arrow) indicated. (C) The model hornwort *Anthoceros agrestis* expressing GFP-tagged Rubisco activase (RCA; green), which localizes to pyrenoids and therefore serves as a fluorescent marker for pyrenoid position within chloroplasts (blue). Scale bar, 10 μm. (D) Pseudocolored scanning electron microscope image of a hornwort chloroplast (green), showing multiple pyrenoids (purple). Scale bar, 5 μm.

Figure 2. **Pyrenoid diversity. (**A) Simplified hornwort chronogram with mapped plastid (uni or multiplastidic) and pyrenoid evolution. Pyrenoid-containing lineages and pyrenoid-lacking lineages are colored red and blue, respectively. An asterisk (*) indicates that some species in the genus lack pyrenoids. (B) Depiction of intrinsically disordered linkers, which have motifs (red circles) that bind to either the Rubisco large or small subunit. (C) Depiction of RbcS-STAR, an innate linker which is integrated into Rubisco and binds to other linkers, rather than Rubisco. Rubisco large subunits, canonical RbcS, and RbcS-STAR are shown in dark gray, light gray, and different colors, respectively.

Figure 3. **A spatial model of the pCCM in hornworts.** This schematic summarizes a working model for Ci fluxes and CO_2 elevation around condensed Rubisco in *A. agrestis*. Ci is predicted to enter the cytosol and chloroplast primarily as CO_2. BST1 at the thylakoid membrane is hypothesized to mediate HCO_3^- entry into the thylakoid lumen. LCIB at the chloroplast envelope is proposed to retain stromal Ci by trapping incoming CO_2 and recapturing CO_2 that leaks from the pyrenoid region. CAH3 is enriched in specialized, centrally positioned thylakoids associated with the pyrenoid and is hypothesized to generate CO_2 near the matrix. The matrix is a phase-separated, Rubisco enriched compartment (depicted in magenta), and the surrounding thylakoids are proposed to act as a diffusional barrier. Possible bicarbonate import from the cytosol and into the chloroplast is depicted with lower opacity vectors to indicate a lack of evidence. Dashed arrows indicate CO_2 movement; solid arrows indicate HCO_3^- movement. Ci, inorganic carbon; LCIB, Low Carbon Inducible protein B; CAH3, Carbonic Anhydrase 3; BST1, Bestrophin-like channel 1.