1 **For: *CO₂ concentrating mechanisms in photosynthetic organisms: evolution, efficiency and***
2 ***application for crop yield improvement. Current Opinion in Plant Biology***

4 **Passive CO₂ concentration in higher plants**

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8 Photorespiratory limitations on C₃ photosynthesis are substantial in warm, low CO₂ conditions.
9 To compensate, certain plants evolved mechanisms to actively concentrate CO₂ around Rubisco
10 using ATP-supported CO₂ pumps such as C₄ photosynthesis. Plants can also passively
11 accumulate CO₂ without additional ATP expenditure by localizing the release of photorespired
12 and respired CO₂ around Rubisco that is diffusively isolated from peripheral air spaces. Passive
13 accumulation of photorespired CO₂ occurs when glycine decarboxylase is localized to vascular
14 sheath cells in what is termed C₂ photosynthesis, and through forming sheaths of chloroplasts
15 around the periphery of mesophyll cells. The peripheral sheaths require photorespired CO₂ to re-
16 enter chloroplasts where it can be refixed. Passive accumulation of respiratory CO₂ is common in
17 organs such as stems, fruits and flowers, due to abundant heterotrophic tissues and high diffusive
18 resistance along the organ periphery. Chloroplasts within these organs are able to exploit this
19 high CO₂ to reduce photorespiration. CO₂ concentration can also be enhanced passively by
20 channelling respired CO₂ from roots and rhizomes into photosynthetic cells of stems and leaves
21 via lacunae, aerenchyma and the xylem stream. Through passive CO₂ concentration, C₃ species
22 likely improved their carbon economy and maintained fitness during episodes of low
23 atmospheric CO₂.

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Introduction

The past 30 million years have been a time of relatively low CO₂ availability, when atmospheric concentrations fell below 400 ppm, reaching a low near 180 ppm during the last ice age [1, 2]. During these low CO₂ episodes, photosynthetic carbon gain in C₃ plants of warm climates would have been limited by reduced availability of CO₂ as a substrate and the inhibitory reactions of photorespiration [2]. To minimize these limitations, many plant and algal species evolved ATP-dependent mechanisms to concentrate CO₂ around Rubisco in what can be termed active CO₂ concentrating mechanisms (CCM's). C₄ and CAM photosynthesis are two well-known active CCM's in the terrestrial flora, and each has independently evolved dozens of times over the past 30 million years [3, 4[•], 5[•]]. In algae, ATPase-dependent bicarbonate pumps boost CO₂ levels around Rubisco over ten-fold, typically within pyrenoids of eukaryotic algae and carboxysomes of cyanobacteria [6]. Active bicarbonate-pumps also evolved independently in algae lineages during low CO₂ episodes in Earth's history [6, 7].

While there has been much focus on active CCM's, there is also the potential for concentration of CO₂ around Rubisco without additional expenditure of ATP, in what we term "passive CCM's" (*pCCM*). Here, the term passive is not used in the strict thermodynamic sense but rather a broad sense that encompasses many biological transport processes that do not involve direct energy expenditure although they are ultimately dependent on a distal energy source. A *pCCM* would arise through the strategic release of respiratory or photorespiratory CO₂ within a tissue or cellular region that is diffusively isolated from intercellular air spaces near stomatal channels. Through such compartmentalization of (photo)respiratory CO₂ release, a fraction of the Rubisco in a plant can experience elevated CO₂ concentrations, such that photorespiration is suppressed, carbon gain is improved, and the fitness of the plant increased. In

this review, we discuss the various *p*CCM's that may exist in land plants, and close by noting their potential significance for maintaining the high diversity of the world's C₃ flora during times of low atmospheric CO₂.

General requirements of a passive CCM

People often view the release of photorespired and respired CO₂ as a major cost for C₃ plants; however, this pool of CO₂ can become a valuable resource if its production is manipulated in a manner that increases Rubisco efficiency and offsets photorespiratory costs. We identify four criteria that can delineate a *p*CCM. To begin, there should be no additional expenditure of ATP or reducing power beyond that already used in respiratory or photorespiratory metabolism. Second, the *p*CCM should arise via specific adaptations that enable the enhancement of CO₂ above concentrations that would otherwise occur via inward diffusion of atmospheric CO₂. Third, CO₂ enrichment occurs through either the localized release of metabolic CO₂ within a tissue or cellular region where chloroplasts occur, or from structural adaptations that channel photorespired or respired CO₂ towards a subpopulation of chloroplasts within the plant. Finally, there should be a diffusive barrier between the high-CO₂ compartment and the intercellular air spaces near the stomata in order to restrict the efflux of photorespired and respired CO₂ and thus facilitate its accumulation.

Categories of passive carbon concentrating mechanisms

Table I outlines the numerous active and passive CCM's that have been identified in plants or may potentially meet the criteria of a *p*CCM. The *p*CCM's are subdivided into two types, those concentrating photorespiratory CO₂ and those involving respiratory CO₂

enhancement. Strong evidence supports the existence of the photorespiratory *pCCM*'s; however, the respiratory *pCCM*'s are less well supported and it can be debated whether they meet the criteria listed above. To promote discussion and test of such possibilities, we discuss some of the evidence for considering certain patterns of respiratory CO₂ accumulation as potential *pCCM*'s.

Photorespiratory glycine shuttling (C₂ photosynthesis)

The most effective *pCCM* in higher plants is the shuttling of photorespiratory glycine from the mesophyll (M) tissues where it is generated, to bundle sheath (BS) tissues where it is decarboxylated by the mitochondrial enzyme glycine decarboxylase (GDC) (Figure 1A). The shuttling of glycine between M and BS cells is established by a reduction of GDC expression in the M tissue, leaving mitochondria in the BS cells as the major site of glycine decarboxylation [8, 9, 10^{••}]. As mesophyll GDC activity declines, high Rubisco oxygenase activity leads to a build-up of glycine in the M cells, thereby creating a diffusion gradient between M and BS tissue that allows glycine to passively diffuse into the BS tissue [9]. The BS-specific decarboxylation of glycine produces a pool of CO₂ that can accumulate around Rubisco in nearby BS chloroplasts, thus suppressing photorespiration (Figure 1A). The reduction in mesophyll GDC activity can be partial as appears to occur in early-stage forms, or complete as occurs in the well-developed forms [10^{••}, 11[•]]. In species with complete reduction of M GDC, all of the glycine produced in M tissues passively diffuses into BS cells for decarboxylation, and the resulting reduction in the CO₂ compensation point of photosynthesis (Γ) can be 20 to 40 ppm below C₃ values at 30°C (Figure 2) [11[•], 12-14]. By restricting glycine decarboxylation to the BS cells, the CO₂ concentration around BS Rubisco is elevated two- to three- fold over levels in the mesophyll [15-16], thus satisfying criteria 2 and 3 for a *pCCM*.

In all species that shuttle glycine into the BS for decarboxylation, there is a marked enhancement in mitochondria and chloroplast numbers in the BS tissue relative to C₃ sister species [11[•], 12, 17[•], 18-19]. In most versions of this metabolism, the vast majority of mitochondria are localized along the inner periphery of BS cells, typically against the vascular tissue (Figure 3A). Numerous chloroplasts form a layer over the mitochondria, or are intermixed with the mitochondria [11[•], 12, 19]. This close association of chloroplasts and mitochondria in the BS interior enables rapid refixation of the photorespired CO₂ before it can escape the inner BS [9]. The positioning of the mitochondria and chloroplasts along the inner BS periphery also meets criterion #4, because a large BS vacuole and the outer BS wall act as diffusive barriers to slow escape of photorespired CO₂ from the BS interior [15].

Passive CCM's based on shuttling glycine into vascular sheath cells have been identified in approximately 50 species of higher plants from close to 20 evolutionary lineages [19]. This number is likely a low estimate, because most of the Earth's flora has not been screened for this type of metabolism [19]. Many of the species using the glycine shuttle are closely related to C₄ evolutionary lineages, and it is now understood that this *p*CCM is an important intermediate phase in the evolution of the C₄ pathway [8, 9, 20^{••}, 21[•]]. It is imprecise, however, to equate the glycine shuttling *p*CCM to C₃-C₄ intermediacy, as was the common practice over the past 30 years. C₃-C₄ intermediacy involves more traits than just the glycine shuttling *p*CCM and equating the two fails to recognize that glycine shuttling is a bonified CCM that enhances fitness in its own right, including in many species with no close relationship to C₄ lineages [20[•]]. An alternative name recommended for the glycine shuttling *p*CCM is "C₂ photosynthesis" [18, 20^{••}]. The logic behind C₂ photosynthesis is that it follows the example of C₄ photosynthesis by

naming the glycine shuttling metabolism after the number of carbon atoms in the molecule transporting CO₂ into the vascular sheath cells.

In a few genera, notably *Flaveria*, there is a gradation in the degree of C₂ photosynthesis from incipient forms operating within the context of C₃ photosynthesis, to full C₂ photosynthesis operating in tandem with a C₄ metabolic pump [9, 10^{••}, 11[•], 12]. While these phases are interpreted as stages in C₄ evolution, within the context of C₂ metabolism, they demonstrate the variable nature of the C₂-type of *pCCM*. In the incipient forms, numerous mitochondria and chloroplasts co-occur in the inner BS, while many chloroplasts are also present in the outer BS adjacent to intercellular air spaces. The M tissue is completely C₃ in function, with much GDC expression [11[•]]. In these BS cells, a single-celled *pCCM* appears to function, where glycine produced in the outer region of the BS diffuses to mitochondria in the inner BS for decarboxylation and refixation of the released CO₂ by adjacent chloroplasts [11[•], 19]. This can lead to slight yet significant improvements in carbon gain in warm, low CO₂ conditions [11[•], 22]. At the other end of the spectrum, in well-developed C₂ species, it is common to observe the co-expression of a modest C₄ metabolic cycle [9]. This upregulation of C₄ metabolism is proposed to deliver carbon skeletons and reducing power to the bundle sheath cells to facilitate reassimilation of ammonia, which is also released by GDC [23]. Strengthening of this C₄ metabolism leads to the eventual replacement of the C₂-type of *pCCM* with the active C₄ CCM in the late stages of C₄ evolution [9, 21[•]].

Trapping of Photorespired CO₂ in C₃ plants

In C₃ plants that are not nutrient-limited, there is a near continuous layer of chloroplasts around the outer periphery of M cells facing the intercellular air spaces. Coverage of the cell periphery facing the intercellular air spaces is commonly over 80%, and in species such as wheat

and rice, it can exceed 90% [24^{••}, 25]. Often, the chloroplasts produce extrusions termed stromules that fill the gaps between chloroplasts (Fig. 4) [24^{••}, 26[•]]. Mitochondria are typically localized to the inner region of the cell, often along the interior side of the chloroplasts [26[•], 27]. The pattern is most pronounced in rice, where chloroplasts form a thick sheath around the edge of the mesophyll cells (Figure 3B) [26[•]]. Rubisco-containing stromules formed by the rice chloroplasts often loop around the mitochondria [26[•]]. These patterns led Sage and Sage [26[•]] to hypothesize that rice leaves are specifically adapted to trap photorespired CO₂ by forming a sheath of chloroplasts and stromules that prevents rapid CO₂ diffusion out of the cell through the gaps between chloroplasts (Figure 1B). Instead, the CO₂ has to leave the cell via the chloroplast stroma, giving Rubisco a chance to refix the CO₂. Under warm conditions when photorespiration is large, the resulting surge of photorespired CO₂ could be substantial enough to elevate the stromal CO₂ content and thus act as a *pCCM* [26[•]]. Consistent with this hypothesis, estimates of photorespiratory CO₂ refixation in a range of C₃ species indicate it can be substantial, from 40% to 80% ([27-29]).

To directly test this hypothesis, Busch et al. [24^{••}] used carbon isotope tracers to estimate stromal CO₂ concentrations and intrafoliar reassimilation of photorespired and respired CO₂ in wheat and rice leaves at 30°C. At 350 ppm CO₂, chloroplast CO₂ levels were estimated to have increased by 14 ppm, leading to an increased net CO₂ assimilation rate (A_{net}) of 15% to 16%. In rice leaves with 95% chloroplast coverage of the mesophyll cell periphery, Γ was reduced by over 10 ppm compared to what would have been the case in the absence of refixation (Figure 2). At an ambient CO₂ level of 200 ppm, which predominated in the late-Pleistocene around 20,000 years ago [2], the chloroplast CO₂ concentration was increased by 16 ppm, leading to a 33% enhancement of A_{net} [24^{••}]. While these CO₂ enhancements are modest, the proportional

increases in A_{net} due to photorespiratory CO_2 trapping at past levels of low atmospheric CO_2 (e.g. 200 ppm) are equivalent to relative increases in A_{net} observed in C_3 plants following a doubling of atmospheric CO_2 above late-20th century values [30-31].

Does photorespiratory trapping constitute a *pCCM*? The evidence that chloroplast CO_2 is enriched 10% to 20% and improves A_{net} , particularly in low CO_2 conditions, meets a key criterion. The use of stromules to seal gaps between chloroplasts, the inward positioning of mitochondria with respect to chloroplasts, and the wrapping of chloroplasts and stromules around mitochondria indicate clear adaptations to trap (photo)respired CO_2 for Rubisco refixation, fulfilling another criteria. One criterion that is not clearly met is whether there is an enhanced resistance within chloroplasts on the outward diffusive pathway. Thylakoid membranes could act as such a resistance. It would also be interesting to know if Rubisco is heterogeneously distributed in the stroma, for example, if more is present along the chloroplast envelope adjacent to mitochondria. Such a pattern would be consistent with the criteria for a *pCCM*.

Enrichment of Respiratory CO_2

Refixation of respiratory CO_2 as a means of improving plant carbon economy has been discussed for decades, and although it can enhance CO_2 and reduce photorespiration, it has not been generally recognized as a specific CCM [32, 33, 34[•]]. If natural selection has strategically co-localized respiratory CO_2 release around interior chloroplasts, or positioned chloroplasts along channels where respiratory CO_2 is abundant, then the possibility that the plant has evolved a *pCCM* based on respiratory CO_2 should be considered. Two general types of respiratory *pCCM*'s are envisioned. In the first, high resistance around the periphery of thick heterotrophic tissues could slow the efflux of respiratory CO_2 , thereby enabling CO_2 enrichment within the

tissue (Figure 1C). This is common in many non-foliar tissues such as stems, strobili, floral tissues and fruits, which commonly have few if any stomata yet form distinct zones where chloroplasts are abundant [33, 35-36]. In woody stems such as those from aspen and birch, the secondary phloem and pith region contains chloroplasts that are diffusively isolated from the atmosphere by the periderm in older stems, or a layer of schlerenchyma in young stems, as shown in Figure 3C [37, 38[•]]. In green fruits, stomata are often infrequent, enabling substantial accumulation of respiratory CO₂ arising from the dense, rapidly growing tissue subtending an outer region of chloroplasts [33]. CO₂ levels within such tissues can accumulate well above 1000 ppm, and the chloroplasts inside effectively refix a large fraction (50% to 100%) of the respired CO₂ in illuminated conditions [33, 34[•], 35]. For example, in stems of birch, photosynthetic cells in the stem can refix up to 97% of respired CO₂ in the stem, with chloroplasts experiencing an average daytime CO₂ concentration of 618 ppm, which is more than double the typical CO₂ concentration in leaf chloroplasts [38[•]]. In stems of western white pine, mean CO₂ concentration during the day was 869 ppm, which is high enough to suppress photorespiration by over 50%, consistent with the criteria of a *pCCM* [39]. How significant this is to overall carbon gain remains uncertain. The contribution of stem photosynthesis to carbon balance is variable, ranging from 10-15% in trees with large leaf mass, to over 80% in desert shrubs with low leaf mass (34[•]). While much of this fixed carbon enters via the stomata, during periods of stress when stomata close, or during low atmospheric CO₂ when leaf photosynthesis is reduced, the contribution of CO₂ refixation in stems could be important for maintaining a plant's carbon balance.

The second type of respiratory *pCCM* generally involves long distant channels from belowground heterotrophic tissues to chloroplasts in aerial portions of the plants (Figure 1D).

212 These channels can be formed by aerenchymatous tissue, lacunae from roots through stems, or
213 inflated regions of stems and leaves [40-42, 43[•]]. It has also been suggested that xylem conduits
214 can also move substantial amounts of CO₂ dissolved in xylem water from below ground tissues
215 and lower stems to petioles and leaves where it can be efficiently refixed [34[•]]. Marsh plants in
216 particular form such channels as they facilitate O₂ flow to belowground tissues in flooded
217 environments. In *Typha* species (cat-tails), for example, CO₂ concentrations in lacunae of leaves
218 are always 100 ppm greater than in intercellular air spaces, and often well above 1000 ppm [44].
219 The fraction of A_{net} supported by these lacunar CO₂ pools can equal that supported by stomatal
220 CO₂ sources, indicating substantial enrichment of the stromal CO₂ pool by respiratory sources
221 [45]. In a similar vein, chloroplasts can function in mangrove pneumatophores, suggesting they
222 may exploit elevated CO₂ concentrations along lacunae arising from roots [46]. In a synthesis
223 based on patterns observed in Lycopoid fossils of the Carboniferous period (360 to 300 million
224 years ago), Green [43[•]] hypothesizes that many species in the ancient Carboniferous forests
225 evolved a distinct CCM termed the Lycopoid Photosynthetic Pathway (LPP), where chloroplasts
226 lining lacunae experienced enriched CO₂ atmospheres channelled up from belowground tissues
227 and stems. Green [43[•]] (Figure 1D) suggests low CO₂ and elevated O₂ conditions of the
228 Carboniferous may have favoured the evolution of the LPP mechanism. The LPP may still
229 function in modern Lycopoids, as indicated by stems of *Selaginella* species which exhibit
230 chlorenchymatous tissue along interior lacunae (Figure 3D) [43[•]]. Species in the Lycopoid genus
231 *Isoetes* are typically submerged aquatic CAM species that appear to supply respiratory CO₂ via
232 lacunae that support both night-time CO₂ uptake by PEP carboxylase, and direct daytime uptake
233 by Rubisco [41, 43[•], 47]. These species lack stomata when submerged, allowing the epidermal
234 cuticle to provide a resistance against CO₂ loss during the day, while the respiratory CO₂ arising

from the roots provides a significant pool that augments what the CAM cycle releases, and may maintain an elevated CO₂ environment when the CAM pool of CO₂ is depleted [47]. As such, *Isoetes* may represent a *pCCM* supplementing the CAM CO₂ concentrating mechanism.

Experimental evaluation of passive carbon concentrating mechanisms

Demonstrating that (photo)respiratory CO₂ can accumulate and suppress photorespiration around some of the chloroplasts in a tissue is readily feasible [16, 24^{••}, 34[•], 38[•], 39]; however, the challenge for delineating a *pCCM* is identifying whether this occurs because there is a simple abundance of respiratory tissue versus a specific set of adaptive traits that enrich CO₂ around a subpopulation of chloroplasts in the tissue. Distinguishing adaptive traits can be challenging in the absence of selection experiments or comparative analyses of multiple independent lineages where the trait arose [48]. In the case of photorespiratory CCM's, evolutionary comparative approaches are amenable because there are multiple lineages where C₂ photosynthesis and respiratory trapping have independently evolved ([11[•], 19, 24^{••}]. Thus, for example, in *Flaveria* and other lineages where C₂ photosynthesis evolved, enhanced *A*_{net} at low CO₂ repeatedly corresponds with organelle accumulation and GDC enhancement in the BS tissue [12, 18-19]. It is also possible to manipulate chloroplast density, refixation rate and Γ along the cell periphery by age and nitrogen status, thus supporting hypotheses that high chloroplast coverage has adaptive value through the formation of photorespiratory CO₂ traps [24^{••}, 49]. Demonstrating respiratory-based *pCCM*'s could be a greater challenge, however. Simple accumulation of respired CO₂ in heterotrophic tissues would not constitute a *pCCM*, and it would be necessary to show that the traits enabling a *pCCM* have arisen due to selection pressure to improve carbon use efficiency under low CO₂. This may be possible by growing multiple generations of plants in

elevated CO₂ and examining whether the putative CCM traits are lost. Such an approach with single-celled algae has demonstrated loss of the bicarbonate pump after 1000 generations [50]. Alternatively, evaluation under very low CO₂ can enhance the contribution of a putative *p*CCM to fitness. By proposing the potential existence of respiratory based *p*CCM's, it is hoped that such experimental evaluations could be developed, and in doing so, provide a greater appreciation of how plants have dealt with the challenges of low atmospheric CO₂ episodes in Earth's history.

Conclusion

In recent geological time, low atmospheric CO₂ conditions prevailed, creating conditions favouring the evolution of active CCM's. Despite the repeated evolution and ecological success of species with active CCM's, the large majority of plant species use C₃ photosynthesis. Less than 10% of the estimated 250,000 land plant species use an active CCM, and at least 75% of the terrestrial primary productivity is produced by C₃ vegetation [49, 51]. Even in the tropics and sub-tropics, where C₄ photosynthesis is clearly superior in warm habits of depleted atmospheric CO₂, the C₃ flora is well-represented, with many tens of thousands of species [52]. A paradox is thus apparent: how can so many C₃ plant species survive in tropical and subtropical environments during low CO₂ episodes of recent geological time, when atmospheric concentrations repeatedly fell below 200 ppm? Photorespiratory potential would have been high in these settings, and the competition from C₄ biomass intense. As suggested here, it may be that many C₃ species in conditions promoting photorespiration evolved specific mechanisms to passively concentrate respired and photorespired CO₂, and in doing so, compensated for the more extreme effects of low atmospheric CO₂.

Acknowledgements: We thank Professor Tammy Sage of the University of Toronto for helpful comments on this manuscript. The work discussed in this paper was supported by funds from the Natural Science and Engineering Research Council of Canada, Discovery Grant program and the Bill and Melinda Gates Foundation, C₄ Rice Engineering program.

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Table I: CO₂ Concentrating Mechanisms in Higher Plants

I. Active CO₂ concentrating mechanisms

A. C₄ photosynthesis

B. CAM photosynthesis

C. Bicarbonate pumping using membrane-bound ATPases

II. Passive CO₂ Concentrating Mechanisms

A. Photorespiration-based

1. Trapping of photorespiratory CO₂ via glycine shuttling

i) Mesophyll to bundle sheath glycine shuttling (C₂ photosynthesis)

ii) Single-cell glycine shuttling in vascular sheath cells

2. Trapping of photorespiratory CO₂ via chloroplast and stromule sheaths

B. Respiratory-based

1. Concentration within non-foliar organs (stems, fruits, flowers, aerial roots)

2. Transport from respiratory sources via aerenchyma, lacunae or xylem water

Figure Legends:

Figure 1: Schematic diagram of four passive CO₂ concentrating mechanisms.

Abbreviations: c, chloroplast; (CH₂O)_n, carbohydrate; G, glycine decarboxylase containing mitochondria; m, glycine decarboxylase-free mitochondria; n, nucleus; P, peroxisome; PP, phloem parenchyma; R, Rubisco; S, sieve tube member. See Figure 3 for microscopic images of the plants that the schematics are based upon.

[Text Box: In C₂ photosynthesis (**panel A**), the oxygenation of RuBP by Rubisco (R) in the mesophyll cells produce p-glycolate, which is converted to glycolate and then glycine in the first steps of the photorespiratory cycle. The glycine produced in mesophyll cells then diffuses to bundle sheath cells where it is decarboxylated by glycine decarboxylase (GDC) localized to bundle sheath (BS) mitochondria (G). Mesophyll mitochondria (m) lack GDC and cannot metabolize photorespiratory glycine. The CO₂ produced by BS mitochondria can accumulate to approximately 3 times the level in mesophyll tissues and is efficiently refixed to carbohydrate, (CH₂O)_n, by Rubisco (R) in chloroplasts of the inner BS. The serine produced by glycine decarboxylase returns to the mesophyll cells to be metabolized to hydroxy-pyruvate (HP) and then RuBP. In photorespiratory CO₂ trapping (**panel B**), photorespiratory glycine is decarboxylated by GDC in mitochondria localized to the interior of a near-continuous sheath of chloroplasts and stromules that line the mesophyll cell periphery. The photorespired CO₂ must then diffuse out of the cell through the chloroplasts where it can be refixed by Rubisco. Under high rates of photorespiration, CO₂ concentrations in the chloroplast can be enhanced 15%. In respiratory CO₂ trapping (**panel C**), as suggested by the anatomy of birch stems (Figure 3C, 38), a sclerenchyma layer just to the outside of the phloem can act as a resistive barrier that slows the diffusive efflux of respired CO₂ from the heterotrophic tissues of the stem, allowing it to accumulate to twice the concentration that may occur in leaf mesophyll cells. Chloroplasts (green ovals) in the phloem parenchyma (PP) and outer pith parenchyma can refix this CO₂ with high efficiency. In respiratory CO₂ channelling (**panel D**), as suggested by *Seleginella* stem anatomy (Figure 3D; 43), lacunae lining the vascular cylinder can channel respired CO₂ from belowground tissues and the lower stem to illuminated regions of the upper stem, enabling chloroplasts in parenchyma cells along the lacunae to experience CO₂ concentrations that might be twice that in leaf mesophyll cells. A central cortical region that is relatively free of chloroplasts can serve as a diffusive barrier to slow efflux of the channelled CO₂, allowing it to accumulate and be efficiently reassimilated.

Figure 2: The response of net CO₂ assimilation rate to variation in intercellular CO₂ concentration in C₃ plants, C₄ plants, and plants using the passive CO₂ concentrating mechanisms of C₂ photosynthesis or photorespiratory CO₂ trapping. The curves are based on data presented in Vogan and Sage [14] and Busch et al. [24]. Note the reduction in the CO₂ compensation point (the x-intercept of the response curves) in C₂ photosynthesis and photorespiratory CO₂ trapping.

Figure 3: Transmission electron micrographs of leaf (panels A, B) and fluorescent light micrographs of stem (panels C, D) sections from plants exhibiting passive CO₂ concentrating

mechanisms outlined in Figure 1. Panel A) Mesophyll (Me) and bundle sheath (BS) cells from a leaf cross sections of the C₂ grass *Steinchisma hians*, showing the clustering of chloroplasts (**c**) and mitochondria (**m**) in the inner bundle sheath cell. B) A longitudinal section of a rice leaf mesophyll cell, showing a complete sheath of chloroplasts around the cell periphery. C) A cross section from a second year stem of paper birch (*Betula papyrifera*), with chloroplasts indicated by red fluorescence in the outer cortex parenchyma (Cp), the phloem parenchyma (Ph), and pith parenchyma cells (Pi). D) A stem cross section from *Selaginella* cf. *uncinata* (peacock fern) showing red-fluorescent chloroplasts in the inner parenchyma cells of the cortex (Cp), which border the lacunae (L) surrounding the vascular bundles. In panel A, note the tight packing of centripetal BS chloroplasts and mitochondria. In panel B, note the interior location of the mitochondria, where photorespired CO₂ is released. In panel C, note the separation of the phloem parenchyma chloroplasts from the outer cortical chloroplasts by a schlerenchyma (Sc) layer. The schlerenchyma may act as a diffusive resistance that enables respiratory CO₂ accumulation in the phloem and pith. Other abbreviations: Co, collenchyma layer; Ep, epidermis; xy, xylem. TEM image in panel B courtesy of Tammy Sage, University of Toronto.

Figure 4: A transmission electron micrograph of a rice mesophyll cell showing a chloroplast stromule, chloroplasts (c), mitochondria (m), peroxisomes, the mesophyll cell wall (w), cytoplasm (cyt) and vacuole (v). Note the immuno-gold labelled Rubisco shown as black dots within the chloroplasts and stromule. Note how the stromule extend between the mitochondria and the mesophyll cell wall.

A: C₂ photosynthesis

B: Photorespiratory CO₂ trapping

C: Respiratory CO₂ trapping

D: Respiratory CO₂ channeling

Sage and Khoshravesh Figure 4