Single Cell C₄ photosynthesis in aquatic and terrestrial plants: a gas exchange perspective

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Abstract

Another “green revolution” is needed for crop yields to meet the demands for food and this has provided a new focus on photosynthesis research. Many important crops have the C₃ photosynthetic pathway and a range of options for enhancing leaf photosynthesis in C₃ species are being tested. George Bowes’ pioneering research elucidated the inducible single cell C₄ photosynthetic mechanism of aquatic plants such as *Hydrilla verticillata*. Here we review the efficacy of single cell C₄ photosynthesis by comparing the physiology of terrestrial Kranz C₄ photosynthesis with that of terrestrial and aquatic single cell C₄ photosynthesis. The comparison shows that the terrestrial single cell system which involves spatially separated cytoplasmic domains and dimorphic chloroplasts equals Kranz C₄ photosynthesis in efficiency; whereas, the simpler aquatic single cell system employed by *H. verticillata* which operates the C₄ pathway between cytoplasmic space and chloroplasts is less efficient due to diffusive constraints. Nevertheless, understanding this readily inducible C₄ pathway may provide valuable lessons for improving C₃ photosynthesis by enhancing chloroplast CO₂ concentrations.

Keywords: CO₂ concentrating mechanism: CCM, Rubisco oxygenase activity, *Hydrilla*, *Bienertia*, *Suaeda*

1. Introduction

To meet the challenge of increasing crop yield for an increasing world population, it has become apparent that photosynthetic efficiency and capacity must increase per unit leaf area to improve yield potential. Many important crops have the C₃ photosynthetic pathway, which is relatively inefficient in CO₂ uptake and assimilation. This inefficiency stems from ribulose biphosphate carboxylase oxygenase (Rubisco), the primary CO₂ fixing enzyme,

which is a poor catalyst under current atmospheric conditions. Therefore, a number of options for enhancing leaf photosynthesis in C_3 species are being researched (Peterhnsel et al., 2008; von Caemmerer and Evans, 2010; Zhu et al., 2010; Parry et al., 2013). Rubisco's oxygenase activity decreases the capacity of CO_2 assimilation and requires the recycling of phosphoglycolate in the photorespiratory pathway resulting in an energy cost and loss of previously fixed CO_2 . Many photosynthetic organisms including cyanobacteria, algae, aquatic and land plants have developed active CO_2 concentrating mechanisms to overcome Rubisco's inefficiencies (Badger et al., 1998; Badger et al., 2002; Bowes et al., 2002; Edwards et al., 2004; Sage et al., 2011; von Caemmerer et al., 2012; Christin and Osborne, 2013). Among aquatic and land plants this led to the development of C_4 photosynthesis, a biochemical CO_2 concentrating mechanism. In the majority of species the pathway operates across two cell types (Edwards and Voznesenskaya, 2011; Sage et al., 2012). Perhaps surprisingly the C_4 photosynthetic pathway has also been found to operate completely within a single cell, which raises many intriguing questions on how CO_2 diffusion barriers are established and biochemistry is compartmentalised to concentrate CO_2 around Rubisco (Fig. 1). Here we review the occurrence of single cell C_4 photosynthesis and compare the function in aquatic and terrestrial plants to provide insights into the efficacy of introducing a functional single-cell C_4 system into crop species.

2. C_4 photosynthesis in species with Kranz anatomy

During C_4 photosynthesis in species with Kranz type anatomy (two-cell system) CO_2 is converted to HCO_3^- by carbonic anhydrase (CA) in mesophyll (M) cells, which is then fixed by phosphoenolpyruvate carboxylase (PEPC) to produce C_4 acids. These acids diffuse to, and are decarboxylated in, bundle sheath (BS) cells to supply CO_2 for Rubisco. This process

elevates the CO₂ partial pressure ($p\text{CO}_2$) in the BS and inhibits Rubisco oxygenase activity allowing Rubisco to operate close to its maximal rate. However, many crops (e.g. rice and wheat) perform C₃ type photosynthesis and in comparison to C₄ crops (e.g. maize and sorghum) they have lower yields and decreased water and nitrogen use efficiency (Hibberd et al., 2008; Langdale, 2011). Edwards et al. (2001) outlined the minimum requirements for the CO₂ concentration mechanism in C₄ plants: 1) cell specific amplification of enzymes of C₄ photosynthesis (such as PEPC in M cells and decarboxylases and Rubisco in BS cells), 2) novel cell specific organelle metabolite transporters, 3) symplastic connections between M and BS cells and spatially separated sources and sinks of metabolites and 4) barriers to CO₂ diffusion between the site of fixation by PEPC in M cells and site of refixation by Rubisco in the BS.

Despite the complexity of the pathway, C₄ photosynthesis has evolved in a large number of dicot and monocot genera with a diversity of anatomical and biochemical features (Edwards and Voznesenskaya, 2011; Sage et al., 2012). Three main biochemical sub-groups have been identified utilising different C₄-acid decarboxylases in the BS cells (Hatch et al., 1975; Furbank, 2011). The NADP malic enzyme (NADP-ME) types carry out C₄ acid decarboxylation in the chloroplast, while in the NAD malic enzyme (NAD-ME) type decarboxylation occurs in the mitochondria, and in the PEP carboxykinase (PCK) types decarboxylation occurs in both the cytosol and mitochondria (Burnell and Hatch, 1988). However, the classification of species into 3 distinct subtypes is not as straight forward as initially thought as there is evidence for metabolic flexibility in some species (Furbank, 2011).

The efficiency of the C₄ pathway in C₄ species with Kranz anatomy is dependent on the diffusive resistance to CO₂ from BS to M cells and the relative biochemical capacities of the

C₃ cycle in the BS and the C₄ acid cycle which operates across the M-BS interface (von Caemmerer and Furbank, 2003). Since the C₄ cycle consumes energy in the form of ATP during the regeneration of PEP, leakage of CO₂ out of the BS is an energy cost to the leaf and therefore the coordination of the two cycles is important. Also, excessive leakage would decrease the ability to concentrate CO₂ in the BS cells. Physiological evidence for coordination has come from indirect measurements of carbon isotope discrimination or measurements of PSII activity by chlorophyll fluorescence analysis and CO₂ assimilation (Krall and Edwards, 1990; Henderson et al., 1992). Both of these leaf measurements suggest that the ratio of CO₂ leakage out of the BS to the inward rate of supply, defined as leakiness, remains constant with short term environmental variation. It is, however, not known how this coordination is achieved. Figure 2a&b show examples of measurements of CO₂ assimilation rate, and leakiness calculated from carbon isotope discrimination using variants of the equation described by Henderson et al. (1992) as a function of light intensity for three NADP-ME Kranz type C₄ species (the monocots *Zea mays* and *Miscanthus x giganteus*, and the dicot *Flaveria bidentis*). These measurements suggest that leakiness is constant over a wide range of irradiance. Carbon isotope discrimination increases at low light to varying degrees, and using the basic equation (see legend to Figure 2) this would imply that leakiness increases at low light. However, the increase in discrimination may not be caused by changes in leakiness because the signal becomes difficult to interpret under these conditions as respiratory fractionations become more important and the basic model makes a number of simplifying assumptions that may not hold at low light (Cernusak et al., 2013; Ubierna et al., 2013). A large number of C₄ species including dicot and monocot species with representatives from the different decarboxylation types have been compared using carbon isotope discrimination with the surprising result that leakiness is very similar

despite biochemical and anatomical diversity (Henderson et al., 1992; Cousins et al., 2008). This means that there is a common relationship between carbon isotope discrimination and the ratio of intercellular to ambient CO₂, C_i/C_a (Fig. 2c). It is difficult to disrupt this coordination through environmental perturbations, however molecular manipulations of *F. bidentis* by RNAi technologies have shown that reductions of Rubisco content disturbs the balance and leads to increased leakiness whereas reductions in C₄ cycle capacity through reduction in NADP malic enzyme reduces leakiness demonstrating that the balancing of biochemical capacities is important (von Caemmerer et al., 1997; Pengelly et al., 2012). However, it is puzzling that a different balance in the capacity of the carboxylases, i.e. maximum activities, and the ratio of PEPC to Rubisco (Table 1), gives rise to very similar C₄ photosynthetic efficiency hinting at extensive metabolic regulation of the C₃ and C₄ cycle which may differ between species.

Resistance to CO₂ diffusion from BS to M cells is considered to be critical for the efficient function of the CO₂ concentrating mechanism. It is thought that structure of the cell wall, the location of chloroplast and mitochondria contribute to a high resistance to CO₂ diffusion between M and BS cells but our understanding remains incomplete (von Caemmerer and Furbank, 2003). Bundle sheath resistance is difficult to estimate and the best estimates have come from experiments using a PEPC inhibitor which allowed direct measurements of CO₂ resistance from intercellular airspace to the site of BS located Rubisco (Table 2, Jenkins, 1989; Brown and Byrd, 1993; He and Edwards, 1996; Kiirats et al., 2002; Edwards et al., 2007). At the leaf level BS resistance is the ratio of the BS resistance across the BS M interface and the BS area to leaf area ratio, S_b. Estimates of S_b in the literature range from 0.6 to 3.1 m²m⁻² which contrast with the much larger ratios of M surface area (appressed to

intercellular airspace) to leaf area ratio, S_m , between 10 and 30 m²m⁻² which facilitates CO₂ diffusion into the M cells of C₃ species (von Caemmerer et al., 2007). Nevertheless the higher BS resistance compared to CO₂ diffusion resistance in C₃ species from intercellular airspace to chloroplasts is not solely due to lower values of S_b compared to S_m but is also due to high resistance across the BS-M interface compared to the intercellular airspace to chloroplast interface in C₃ species (von Caemmerer and Furbank, 2003; von Caemmerer et al., 2007). With recent technological developments it has become easier to make measurement of carbon isotope discrimination concurrently with gas exchange and this has become a useful tool to study efficiency of C₄ photosynthesis (Fig. 2). Additionally, the lack of oxygen sensitivity of the CO₂ compensation point is another measurement that provides unequivocal evidence of C₄ photosynthesis (Fig. 3, Furbank et al., 2009). For example, measurements of both carbon isotope discrimination and the O₂ sensitivity of the compensation points have been used to show that corn husk photosynthesis is C₄ like (Pengelly et al., 2011). Below we have used these measures to look at terrestrial and aquatic single cell C₄ photosynthesis.

3. Terrestrial single cell C₄ photosynthesis

After the discovery of C₄ photosynthesis in the 1960s (for review see Hatch, 1999) it was believed that Kranz anatomy, which provides the spatial separation for the two carboxylases, was essential for terrestrial C₄ photosynthesis. Thus it was a surprise to discover terrestrial species within the family of Chenopodiaceae where C₄ photosynthesis operates within single cells (Freitag and Stichler, 2000; Voznesenskaya et al., 2001). Two novel means of single-cell C₄ photosynthesis have evolved in the subfamily Suaedoideae. The systems function by spatial separation of two cytoplasmic domains which contain

dimorphic chloroplasts. Detailed reviews describing what is known about these species and discussing the issues associated with single cell C_4 photosynthesis have been provided by Edwards et al. (2004), Edwards and Voznesenskaya (2011) and Sharpe and Offermann (2014). In two *Bienertia* species, *B. cycloptera* and *B. sinuspersici*, the two cytoplasmic domains are located at the cell periphery and in a central cytoplasmic compartment. However, in *Suaeda aralocaspica* the spatial separation of functions are achieved by localization of the dimorphic chloroplasts at opposite ends of elongated cells (Fig.1).

With respect to the minimum requirements of C_4 photosynthesis, point 1) is met in the terrestrial single-cell C_4 plants by a cytoplasmic domain specific amplification of C_4 photosynthetic enzymes. This was demonstrated first through immunolocalisation studies of the major enzymes for both types of single cell C_4 species *S. aralocaspica* and *B. cycloptera* (Voznesenskaya et al., 2002; Voznesenskaya et al., 2003a). The results suggested the chloroplasts in one domain function analogous to that in M cells in Kranz type where they support the carboxylation phase of the C_4 cycle (at the point of entry of CO_2 in the cells, Fig. 1). All the mitochondria in the cell are located in the other domain where decarboxylation of malate occurs via NAD-ME which generates CO_2 for assimilation by the Rubisco containing chloroplasts analogous to BS cells in Kranz type NAD-ME species. More recently, an elegant study developed protocols to isolate peripheral chloroplasts and organelles from the central compartment to study the subcellular compartmentalization of enzymes and transporters in *B. sinuspersici* (Offermann et al., 2011). This study provided functional evidence of NAD-ME type biochemistry in *B. sinuspersici*. Additionally, measurements of photosynthetic oxygen evolution revealed that only chloroplasts in the central compartment have a fully operational C_3 cycle, while both chloroplast types have the

capacity to photoreduce 3-phosphoglycerate. This confirmed that peripheral and central chloroplasts function analogous to M and BS chloroplasts in Kranz type C_4 species, respectively. Also curious was the large investment in chloroplast and chlorophyll to the central compartment not dissimilar to the investment of chlorophyll and nitrogen to the BS compartment seen in terrestrial NAD-ME species (Ghannoum et al., 2005). The study by Offermann et al. (2011) also identified a pyruvate transporter in the peripheral chloroplasts for import of this substrate for PPDK, which supports another requirement for C_4 photosynthesis. The third requirement of symplastic connection between the two cytosolic compartments is probably more easily met; however, how the necessary metabolite gradients are established between the two compartments is intriguing. This is also connected with the curious question (point 4) of how the CO_2 diffusion barriers are established. In *Bienertia*, the peripheral and central compartment domains are connected by a complex of sheet-like cytoplasmic channels traversing the vacuole in the medial longitudinal regions of the cell (position illustrated by the arrows in Fig. 1). These channels are suggested to be the path of exchange of metabolites, and likely the main route of leakage of CO_2 from the central compartment (Voznesenskaya et al., 2005). In *S. aralocaspica* the elongated M cells have a large vacuole which is surrounded by the cytoplasm. The exchange of metabolites between the two domains located at the distal end where external CO_2 enters and the proximal end is thought to occur along the thin layer of cytoplasm in the middle which is devoid of organelles (indicated by the arrows in Fig. 1).

These single cell C_4 species have high rates of net CO_2 assimilation as determined by gas exchange studies of CO_2 uptake and carbon isotope discrimination (Edwards et al., 2007; King et al., 2012). The comparison of gas exchange of single cell C_4 with Kranz C_4 species

(Fig.2) shows them to have similar CO₂ assimilation rates and similar values of leakiness at high light. Hence, the lack of Kranz anatomy does not appear to provide a disadvantage. The larger apparent leakiness values at low light are probably not real; but, rather caused by interference of respiratory fractionation and the application of a simplified model equation (Stutz et al., 2013). However, more detailed studies of how these pathways operate at low light would be insightful since in the quantum yield region, where CO₂ assimilation is limited by the amount of light absorbed, the quantum requirement increases steeply with decreasing diffusive resistance. A review of dry matter isotope values supports the instantaneous isotope measurement and has shown these values to be C₄ like. However, some more negative values have also been observed in young leaves and leaves grown under low light where the C₄ type leaf anatomy may not be fully developed (for review see Edwards and Voznesenskaya, 2011). Biochemically the ratio of PEPC to Rubisco activity in mature leaves of *B. cycloptera* and *S. aralocaspica* are very similar to that of Kranz C₄ species, but young developing leaves of *S. aralocaspica* had a lower ratio (Table 1).

As noted, an intriguing question is how the diffusion barriers are established with these two structural forms of single cell C₄; i.e. what provides diffusive resistance to CO₂ between the site of decarboxylation of malate by mitochondria in one domain, to the intercellular air space adjacent to the other domain. As in NAD-ME type C₄ species, the mitochondria are located either internal to, or surrounded by chloroplasts (Fig. 1) analogous to that in BS cells of NAD-ME species. Thus, the chloroplasts surrounding mitochondria may provide resistance as well as the liquid diffusion path to intercellular air space. There has only been one measurement of diffusive resistance using the PEPC inhibitor in *B. cycloptera* which indicated a substantial diffusive resistance, although significantly less than that recorded for

Kranz C₄ species with the same technique (Table 2). Using the simple calculations of CO₂ diffusion resistance per μm of water ($12.5 \text{ m}^2 \text{ s mol}^{-1} \mu\text{m}^{-1}$) the measured resistance is perhaps less than expected from the cytosolic distance of $\sim 20 \mu\text{m}$ deduced from the anatomy for *Bienertia* (Fig. 1, from the center of the central compartment to the periphery of the cell). However, we do not at present have good estimates of appropriate diffusive surface area (equivalent of S_b) to leaf area ratios (Fig. 1, von Caemmerer and Furbank, 2003). From anatomical considerations one might expect *S. aralocaspica* to have higher resistances because of the larger cytosolic distance between the two cytoplasmic zones ($\sim 45 \mu\text{m}$ in the liquid phase from the distal end of the cell where CO₂ is generated by mitochondrial NAD-ME to the intercellular air space, Fig. 1). It clearly would be interesting to make further anatomical measurements coupled with measurement of diffusive resistances using the PEPC inhibitor.

The lack of O₂ sensitivity of the C₄ compensation point is dependent on PEPC activity and diffusion resistance between M and BS cells or the two cytosolic compartments of the single cell C₄ species (Peisker, 1979; Furbank et al., 2009). Using equation 6 from Furbank et al. (2009) it is easy to show that the measured ratio of PEPC to Rubisco activity and the diffusive resistance in Table 2 are sufficient to predict a lack of O₂ sensitivity as is indeed the case for *B. sinuspersici* (Fig. 3). This provides further evidence that the biochemistry and diffusive environment ensures efficient C₄ photosynthesis in this species.

To model C₄ photosynthesis in these single cell species it would be interesting to know whether PEPC and Rubisco have similar kinetic properties as observed for PEPC and Rubisco in Kranz C₄ species. Rubisco of Kranz C₄ species examined have greater catalytic turnover rates and Michaelis Menten constants for CO₂ compared to Rubisco of C₃ species (Seemann

et al., 1984; Badger and Andrews, 1987; Kubien et al., 2008), which enhances their nitrogen use efficiency (Ghannoum et al., 2005). As of yet, no measurements of Rubisco kinetic properties of these single cell C₄ species have been reported. The PEPC from the single cell C₄ species have some kinetic and regulatory characteristics similar to those of Kranz type C₄ species (Lara et al., 2006). The C₄ form of PEPC tends to have a higher K_m for PEP; evolutionary differences of PEPC in the genus *Flaveria* have been studied in detail with respect to differences in the affinity for PEP (Nakamoto et al., 1983; Bauwe, 1986; Bauwe and Chollet, 1986; Jacobs et al., 2008). However, there are very few studies in which the K_m HCO₃⁻ of PEPC has been measured in C₄ species; thus, we know very little about the diversity of PEPC in this regard between Kranz and single cell C₄, and C₃ species (except see discussion below, (Ascencio and Bowes, 1983; Bauwe, 1986).

4. C₄ photosynthesis in aquatic plants

C₄ photosynthesis probably arose in some aquatic organisms in response to limitations in dissolved CO₂ before the advent of C₄ photosynthesis in terrestrial plants (Bowes et al., 2002; Bowes, 2011). A small number of freshwater submerged monocots, including *Hydrilla verticillata*, *Egeria densa*, *Sagittaria subulata*, and *Orcuttia viscida*, have been identified to perform facultative single cell C₄ photosynthesis (Keeley, 1998; Bowes et al., 2002; Bowes et al., 2003). With research interest in implementing C₄ photosynthesis in terrestrial C₃ species, there is renewed interest in understanding their physiology (Bowes et al., 2003; Bowes et al., 2007; Edwards et al., 2007).

George Bowes' research on the aquatic monocot *H. verticillata* provided the first detailed description and subsequent characterisation of a single cell C₄ system (Holaday and Bowes, 1980; Salvucci and Bowes, 1981, 1983a). *H. verticillata* grows under submerged conditions

and C₄ photosynthesis is induced under warm temperatures, limiting CO₂ and high light in the summer. The change from C₃ to C₄ can occur without the production of new leaves and this has been demonstrated in lake and laboratory environments (Bowes, 2011). *Hydrilla* leaves are composed of two morphological distinct chloroplast-containing cell layers (without epidermal cells) illustrated in Fig. 1, and Rubisco and PEPC have been located in chloroplast and cytosol, respectively, in both cell layers (Reiskind et al., 1989a; Reiskind et al., 1989b; Magnin et al., 1997).

When first described, the aquatic single cell C₄ photosynthesis system proposed for *H. verticillata* was not readily accepted because of the firmly held view that Kranz anatomy was essential for providing the necessary CO₂ diffusion barrier. However, evidence that a form of C₄ photosynthesis takes place in *H. verticillata* is now overwhelming. Bowes et al. (2007) and Bowes (2011) have provided schematics outlining the proposed biochemical pathway and summarized the main differences between the C₃ leaf (high compensation point) and the C₄ leaf (low compensation point). Like many submerged monocots, *Hydrilla* secretes H⁺ in the light into the boundary layer on the abaxial side lowering its pH to 4 and enhancing conversion of HCO₃⁻ to CO₂ and passive diffusion of CO₂ into the leaf (Fig. 4). This occurs in both C₃ and C₄ leaves, and is associated with energy cost in the form of ATP (van Ginkel et al., 2001).

The C₄ cycle operates between the cytosol and chloroplast, and its induction is associated with an up-regulation in expression and activity of C₄ enzymes, with the ratio of PEPC to Rubisco activity similar to what is observed for terrestrial C₄ species (Table 1, Salvucci and Bowes, 1981; Ascencio and Bowes, 1983; Bowes, 2011). Carbonic anhydrase activity is also up regulated, and is presumably cytosolic, although its location has not been confirmed

(Salvucci and Bowes, 1983a; Bowes, 2011). *Hydrilla* is characterised as an NADP-ME C₄ subtype. In the proposed C₄ cycle (Fig. 4), the main site of entry of CO₂ is from the abaxial side of the leaf which would supply carbon for photosynthesis by both layers of cells. As CO₂ enters the leaf, cytosolic carbonic anhydrase converts it to bicarbonate which is then fixed by PEPC producing OAA. OAA may be directly imported into the chloroplast, reduced to malate by NADP-MDH utilizing NADPH, the malate decarboxylated by NADP-ME producing CO₂, pyruvate and NADPH, the pyruvate converted to PEP by PPDK, and the PEP exported to the cytosol providing substrate for PEPC. Alternatively, through cytosolic and chloroplastic aminotransferases, the OAA synthesized by PEPC may be converted to aspartate in the cytosol, the aspartate imported in the chloroplast, converted to OAA providing substrate for NADP-MDH and metabolised as above. These shuttles would maintain redox-balance in the chloroplasts, while a shuttle involving import of malate from the cytosol is considered unfavourable for redox balance as reductant would be shuttled from the cytosol to the chloroplast via the C₄ cycle (Bowes et al., 2002; Bowes et al., 2003). In the aquatic single cell C₄ system there is no evidence of dimorphic chloroplasts that perform the function of M and BS chloroplasts in the conventional Kranz type two cell system.

As noted above, the abaxial side of the leaf is considered the main site of entry of CO₂. The abaxial cells have a labyrinth –like membrane structure next to the outer cell wall, analogous to transfer cells (which have an invaginated plasma membrane), which provides more surface area for the pumping of protons out of the cells and for entry of CO₂. On the adaxial side with pH 10 at the boundary layer, bicarbonate and carbonate will be predominant forms of inorganic carbon (Bowes et al., 2002). The cells on the adaxial side have an osmiophilic layer which appears to permeate the cell wall (on the upper and

adjacent sides of the cell) (Joye and Paul, 1992). Perhaps this provides resistance to diffusion of CO₂, and prevents leakage of CO₂ generated by the C₄ decarboxylase in the adaxial cells. *Hydrilla* cells do not appear to have special localization of chloroplasts within the cell (Bowes et al., 2003) although if this happened in the natural environment it might contribute to a diffusion environment to support C₄ photosynthesis. There is some evidence that chloroplasts can change their positions from dark to light, being dispersed in the dark and forming bands located on anticlinal and inner paradermal walls in the light which may be a light avoidance mechanism, see (Edwards et al., 2007). Also, large chloroplasts could favour capture of CO₂ by Rubisco from decarboxylation of malate (a range of sizes were measured from reports in the literature, Fig. 1, with the largest being ~3 fold bigger than those of terrestrial single cell C₄ species).

While there is clear evidence for function of an NADP-ME C₄ cycle in *Hydrilla*, when grown under conditions where the C₄ system is induced, the amount of NAD-ME also increases about 3 fold and the activity was about 6 fold higher than the activity of NADP-ME (Salvucci and Bowes, 1981, Table IV). Magnin et al. (1997) showed with organelles extracted from *Hydrilla* that NADP-ME is in chloroplasts, and NAD-ME is in the mitochondria. It was considered unlikely that NAD-ME is part of C₄ function, since it could result in CO₂ leakage from the cell or set up a futile cycle if NAD-ME is producing CO₂ from the mitochondria, and PEPC in the cytosol is competing for refixation of that CO₂ with Rubisco in the chloroplast. However, the efficiency could be affected by positioning of mitochondria relative to chloroplasts. If the mitochondria are located internal from the site of entry of CO₂, or surrounded by chloroplasts, as in the single cell terrestrial C₄ species and BS cells of Kranz

type NAD-ME species, then potentially *Hydrilla* is utilizing two C₄ cycles to concentrate CO₂ around Rubisco (at amounts required for C₄ function, Reiskind et al., 1997)

There are many intriguing aspects of the inducible single cell C₄ photosynthetic mechanism in *Hydrilla*; one of the major question concerns the efficiency of this single cell C₄ pathway. Substantial increases in the photosynthetic rate occur with the induction of C₄ photosynthesis with similar amounts of Rubisco, along with large increases in the ratio of PEPC: Rubisco as seen in terrestrial C₄ species (Table 3, Salvucci and Bowes, 1981). The diffusive barrier to CO₂ in the C₄ mode is presumably provided in part by the chloroplast envelope, which during C₃ photosynthesis has to allow easy access of CO₂ to Rubisco. Therefore, it would be interesting to know whether the chloroplast envelope changes its diffusive resistance to CO₂ during the transition to C₄ photosynthesis. As noted earlier, *Hydrilla* is induced to perform C₄ in the summer under warmer temperatures. If comparisons can be drawn from temperature dependent measurements of CO₂ diffusion to chloroplasts in M cells in tobacco (Evans and von Caemmerer, 2013), higher temperatures could cause a decrease in resistance to CO₂ diffusion also in *Hydrilla*. This suggests significant control by biochemical processes, which in *Hydrilla* might for example be dependent on the amount of aquaporins for CO₂ transport into the chloroplast envelope (Uehlein et al., 2008; Kaldenhoff, 2012). With improvement of technologies for the measurements of carbon isotope discrimination concurrently with gas exchange it may be possible to more easily use isotope techniques to investigate *Hydrilla* photosynthesis with techniques previously used for algal cultures (Sharkey and Berry, 1985).

¹⁴C pulse chase labelling experiments with *Hydrilla* functioning in the C₄ mode have shown that about 60% of initial label appears in malate and aspartate, and is rapidly chased into

phosphorylated Calvin cycle intermediates (Holaday and Bowes, 1980; Salvucci and Bowes, 1983b). Studies with carbonic anhydrase inhibitor ethoxycarbonylamine and PEPC inhibitors also show only a fractional reduction (~40%) in photosynthetic rate unlike the almost complete inhibition of photosynthesis seen in terrestrial Kranz C_4 species when a PEPC inhibitor is applied or in the C_4 mutants lacking PEPC, or in transgenic plants with very low carbonic anhydrase activity (Salvucci and Bowes, 1983a; Jenkins, 1989; Kiirats et al., 2002; von Caemmerer et al., 2004). If the C_4 system were fully induced in these studies, it suggests that the aquatic single-cell C_4 system is somewhat leaky and does not have the same efficiency seen in terrestrial Kranz or the terrestrial single cell C_4 species. This is also supported by the oxygen sensitivity of the CO_2 compensation point, which is less in leaves where C_4 photosynthesis has been induced but not completely absent (Figure 3). Some of the reduction in the compensation point even at low O_2 is also due to an increase in photosynthetic capacity over respiratory metabolism. Using equation 6 from Furbank et al. (2009) these compensation points (Figure 3) can be modelled with a lower resistance to CO_2 diffusion from chloroplast to cytosol compared to the equivalent resistances of terrestrial Kranz and single cell C_4 species.

The efficiency of the C_4 pathway is also linked to the kinetic properties of Rubisco and PEPC. Rubisco from *Hydrilla* has kinetic constants that are C_4 like with a $K_m(CO_2) = 26$ and $28 \mu M$; the corresponding $K_i(O_2) = 690$ and $734 \mu M$ for C_3 and C_4 leaves respectively, and CO_2/O_2 specificity is 73 (Bowes and Salvucci, 1984; Bowes, 2011). This is similar to Rubisco kinetics from other aquatic plants (Yeoh et al., 1981). This would seem a disadvantage in the C_3 mode when aquatic CO_2 levels are low; but not in the C_4 mode if chloroplasts levels are as high as estimated ($400 \mu M$, Reiskind et al., 1997; Bowes et al., 2003).

Rao et al. (2006) showed that an isoform of PEPC was up-regulated during induction of C₄ with post-translational phosphorylation in the light and changes in allosteric regulation by glucose-6-P and malate, characteristics of C₄ PEPC. However, the PEPC which is up-regulated during the induction of C₄ in *Hydrilla* differs in some properties from the PEPC involved in C₄ photosynthesis in terrestrial Kranz C₄ species. For example, it lacks the C₄ signature of a Ser at the carboxy-terminus (Rao et al., 2002), which is considered a key characteristic of the C₄ isoforms in terrestrial plants which alters the K_m for PEP (but see exceptions in Rosnow et al., 2014). Additionally, it has low affinity for HCO₃⁻ with a K_m (HCO₃⁻) around 220 μM compared to 27 μM for terrestrial Kranz C₄ species (Ascencio and Bowes, 1983; Bauwe, 1986).

While there has been less research on *Egeria densa*, it is clear that it is a facultative C₄ aquatic species. Under low temperature and low light (LTL) it functions in the C₃ mode, while under high temperature and high light (HTL) C₄ is induced (Salvucci and Bowes, 1981; Casati et al., 2000). For example, exposure to HTL induces an increase in PEPC and NADP-ME, enzymes of the C₄ cycle (Casati et al., 2000). The first evidence for changes in the form and kinetic properties of PEPC on induction of C₄ in an aquatic plant was in *E. densa*. Under conditions, which induce C₄, a low molecular mass of PEPC appears which is phosphorylated and changes the kinetic properties to those characteristic of C₄ PEPC (Casati et al., 2000; Lara et al., 2001)

Among algae, there is also interest in whether some marine planktonic diatoms perform C₄ photosynthesis. They are known to actively accumulate CO₂ around Rubisco which suppresses photorespiration and contributes to their productivity; although, the mechanism(s) are poorly understood (Hopkinson et al., 2011). It had generally been held

that this is a biophysical based mechanism (via active transport of inorganic carbon across membranes); although evidence for a role of C₄ photosynthesis has been shown in the diatom *Thalassiosira weissflogii* (Reinfelder et al., 2004). Subsequently, analyses by the gold-standard method of short-term labeling with ¹⁴CO₂ (Roberts et al., 2007) , indicated *T. weissflogii* functions as a C₃-C₄ intermediate, whereas another species in the same genus, *T. pseudonana*, functions exclusively via C₃ photosynthesis. Recently, (Haimovich-Dayana et al., 2013) concluded that C₄ does not contribute to the CO₂ concentrating mechanism in the marine diatom *Phaeodactylum tricornutum*. Roberts et al. (2007) suggest that diatoms may utilize a combination of complementary CCMs allowing acclimation to changing CO₂ levels and availability of other resources.

5. Single-cell C₄ rice

There is growing interest in installing C₄ traits into rice using a dual cell Kranz type system (von Caemmerer et al., 2012). The efforts have focused on expressing a C₄ system in the existing M cells of rice and in fact some C₄ genes have been successfully incorporated into rice with high expression levels (Matsuoka et al., 2001; Kajala et al., 2011; Covshoff and Hibberd, 2012). In order to test the full potential for a C₄ metabolic system to function in rice (single cell or Kranz), genes required for all of the enzymes of the C₄ cycle and metabolite transporters (and possibly genes which regulate some of the enzymes), will need to be expressed at levels equivalent to those in C₄ plants. For a review on previous research and perspectives for incorporating a single-cell C₄ system, see Burnell (2011). *Hydrilla* has been proposed as a model system for introducing single-cell C₄ photosynthesis into rice (Edwards, 1999; Bowes et al., 2003; Bowes et al., 2007).

Modelling experiments examining the possible benefits of single cell C_4 photosynthesis in a C_3 leaf by adding a C_4 cycle to the existing M cells (without structural modifications or development of dimorphic chloroplasts) have shown that although it may be inefficient it could nevertheless reduce the drop in CO_2 partial pressure from intercellular to the chloroplast at high light and high temperature (von Caemmerer, 2003). This is also beneficial in reducing water loss, as plants would be able to operate at lower stomatal conductance. With the chloroplasts located near the plasma membrane in lobed M cells of rice, low efficiency of C_4 is expected if the decarboxylation phase of the cycle occurs in the chloroplast, due to leakage of CO_2 to the intercellular air space. In rice and wild relatives, on average, 89% of mesophyll cell surface area exposed to intercellular air space is covered by chloroplasts. However, about half of the chloroplasts in the cells are located where cells are in contact with adjacent cells where leakage to the Intercellular airspace may be more restricted (Giuliani et al., 2013). Bowes et al. (2003; 2007) have indicated the need to identify the required transporters for exchange of metabolites of the C_4 cycle with the chloroplast, and to resolve what provides resistance to leakage of CO_2 following decarboxylation of malate in the chloroplast (e.g. permeability of the chloroplast envelope to CO_2). Installing a single cell C_4 system in rice will likely result in a mixed system where part of the atmospheric CO_2 is delivered to the chloroplast by the C_4 cycle, and part is diffusing directly to chloroplasts and fixed by Rubisco. The C_4 cycle might be most beneficial by concentrating CO_2 in chloroplasts which are not located at the intercellular air space. Such a mixed C_3 - C_4 pathway system could provide significant improvements on photosynthesis and yield.

Besides considering C₄ cycles via chloroplast based NADP-ME and PCK decarboxylases (see Burnell, 2011), another strategy is to incorporate a C₄ cycle through mitochondrial NAD-ME (Fig. 5). The M cells of rice have lobes, chloroplasts are located at the periphery of the cell with a high percentage of coverage of the cell wall, while mitochondria and peroxisomes are located internally (Sage and Sage, 2009; Giuliani et al., 2013). This structure enhances refixation of the photorespired CO₂ which is generated by mitochondrial glycine decarboxylase. Busch et al. (2013) estimated that rice can refix up to 30% of the photorespired CO₂ and a mathematical model exploring the advantages of refixing photorespired CO₂ and the effects on compensation points was provided by von Caemmerer (2013). A general analogy can be made between the M cell structure of rice, and the central cytoplasmic compartment of *Bienertia* M cells (Fig. 1). This compartment in *Bienertia* has a high density of chloroplasts (C₃ type, Rubisco containing) with mitochondria and peroxisomes (latter not illustrated) located internally. Since CO₂ is generated via glycine decarboxylase in mitochondria during photorespiration, their positioning in the central compartment favors refixation of photorespired CO₂ by the chloroplasts. In *Bienertia*, these mitochondria are also the site of decarboxylation by NAD-ME in the C₄ cycle. Installing an NAD-ME type C₄ cycle in rice would have the benefit of generating the CO₂ internal to chloroplasts, analogous to the configuration in *Bienertia*. By increasing resistance to CO₂ leakage, this could increase the function of C₄ photosynthesis in rice.

6. Concluding thoughts

Facilitating Rubisco's access to CO₂ is an overarching concern in discussions on how to enhance C₃ photosynthesis (von Caemmerer and Evans, 2010). C₄ cycles via malic enzymes come with an energy cost of 2 ATP per turn of the cycle (Berry and Farquhar, 1978).

Photorespiration comes with an energy cost of 3.5 ATP and the equivalent of 2 NADPH per O_2 reacting with RuBP (Farquhar et al., 1980). Thus, under conditions which favour photorespiration (i.e. increased RuBP oxygenase/carboxylase activity), namely warmer temperature and limited water, C_4 can provide both higher rates of photosynthesis and more efficient use of solar energy. However, where there is limited photorespiration, i.e. under cooler climates, or greater availability of CO_2 (less stomatal limitation or rising atmospheric levels), there would be no advantage, or a disadvantage to be C_4 . This makes the readily inducible system which operates in *Hydrilla* very attractive. This system is activated in the summer as CO_2 becomes limited, as temperatures rise, but the induction mechanism is not resolved. The terrestrial forms of single cell C_4 also provides a model which demonstrates a C_4 cycle can effectively function through a mitochondrial C_4 decarboxylase, with partitioning and chloroplast differentiation analogous to that in Kranz type C_4 species. There is much we do not currently understand about the regulation of C_4 photosynthesis in its various forms, but new molecular and genomic tools have already significantly progressed our understanding (Langdale, 2011; Denton et al., 2013; Heckmann et al., 2013). Perhaps the time is right to take another look at various forms of single cell C_4 photosynthesis with the new molecular tools available.

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Figure legends

Figure 1

Schemes illustrating features of the photosynthetic cells in the single-cell C_4 aquatic and terrestrial species. Cells are not drawn to scale, average dimensions are given. Images of cells and chloroplasts are drawn from analysis of microscopy: *Hydrilla* (cell size from Bowes et al., 2003), chloroplast size from (Reiskind et al., 1989b; Joye and Paul, 1992; Bowes et al., 2003); *Orcuttia viscida* (Keeley, 1998); *B. sinuspersici* (cell and chloroplast size from a collection of images by E. Voznesenskaya and N. Koteyeva); *S. aralocaspica* (cell size from a collection of images by E. Voznesenskaya and N. Koteyeva; chloroplast size, (Voznesenskaya et al., 2003b)). The average dimensions for chloroplast size were for *Hydrilla*: $10\ \mu\text{m} \times 6\ \mu\text{m}$ (a wide range was found from measurements on images in different reports, from $17 \times 7\ \mu\text{m}$ to $3.1 \times 1.3\ \mu\text{m}$ see above references); *O. viscida*: $9.5 \times 3.7\ \mu\text{m}$ (Keeley, 1998); *B. sinuspersici*: central compartment chloroplasts, $4.4 \times 2.5\ \mu\text{m}$; peripheral chloroplasts, $5.1 \times 0.7\ \mu\text{m}$; *S. aralocaspica*, proximal chloroplasts $5.4 \times 1.8\ \mu\text{m}$; distal chloroplasts $5.3 \times 0.9\ \mu\text{m}$. In the terrestrial single cell C_4 , the arrows within the cells indicate the suggested paths of diffusion of metabolites between the two cytoplasmic domains (see text). The sites of entry of CO_2 into the cells are illustrated by the broken arrows. The smaller organelles in the figure for terrestrial plants are mitochondria which are located in the central compartment of *B. sinuspersici* and at the proximal end of the *S. aralocaspica* chlorenchyma cells.

Figure 2

Light response of a) CO_2 assimilation rate, b) leakiness measured at ambient CO_2 and $25\ ^\circ\text{C}$ and c) carbon isotope discrimination, Δ , as a function of intercellular to ambient CO_2 , C_i/C_a

(at high light only) in the two cell Kranz C₄ species *Flaveria bidentis*, *Zea mays* and *Miscanthus x giganteus* (open symbols) and two single cell C₄ species (closed symbols) *Bienertia sinuspersici* and *Suaeda aralocaspica*. Data were taken from (Pengelly et al., 2010; King et al., 2012; Ubierna et al., 2013). Measurements for *F. bidentis* (a) were taken from (Pengelly et al., 2010) and for *F. bidentis* (b) from (Ubierna et al., 2013). The line in panel C is given by the equation $\Delta = 4.4 + (-5.7 + (29 - 1.8) * 0.26 - 4.4) * C_i/C_a$ which is based on the simplified theoretical relationship between carbon isotope discrimination, C_i/C_a and leakiness (here 0.26) developed by Farquhar (1983). The value 4.4‰ denotes fractionation during CO₂ diffusion in air, -5.7‰ is the fractionation during CO₂ hydration and PEPC carboxylation, 29‰ the fractionation during Rubisco carboxylation and 1.8‰ is the fractionation associated with the CO₂ dissolution and diffusion through the liquid phase. This simplified equation ignores a ternary correction, respiratory fractionations and assumes that bundle sheath CO₂ partial pressure greatly exceeds mesophyll CO₂ partial pressure (Cernusak et al., 2013).

Figure 3

CO₂ compensation point as a function of O₂ partial pressure for *Oryza sativa* a C₃ species, *Zea mays*, a Kranz C₄ species, the terrestrial single cell C₄ species *Bienertia sinuspersici* and the aquatic plant, *Hydrilla verticillata* grown under different conditions to induce high, intermediate and low photorespiratory (PR) states (Salvucci and Bowes, 1983a; Pengelly et al., 2010).

Figure 4

Proposed model for function of single-cell C₄ photosynthesis in *Hydrilla*. The scheme illustrates active excretion of protons into the boundary layer on the abaxial side of the leaf (acidifying the boundary layer to pH 4) where CO₂ becomes the predominant form of inorganic carbon (pK for the first dissociation constant, CO₂/bicarbonate, is 6.3) and this is associated with energy cost in the form of ATP. The carboxylation phase of the C₄ cycle occurs in the cytoplasm, import of OAA and/or aspartate into the chloroplast and subsequent decarboxylation via NADP-malic enzyme. The exchange of metabolites by the chloroplast is presumably facilitated by unidentified membrane transporters (illustrated by solid circles). AAT, aspartate amino transferase; Glu = glutamate; 2-OG= 2 oxoglutarate; OAA, oxaloacetate. The CO₂ concentrating mechanism reduces competition by O₂ in Rubisco catalysis, and thereby reduces photorespiration, which for clarity is not illustrated. Adapted from Bowes et al. (2002) and Bowes (2011).

Figure 5

Model for development of a mixed C₃-C₄ pathway of photosynthesis in rice mesophyll cell via an NAD-ME type C₄ cycle, without structural modifications. For clarity the photorespiratory cycle which also releases CO₂ in the mitochondria is not illustrated. PPKK, pyruvate, Pi dikinase; PEPC, phosphoenolpyruvate carboxylase; NAD-ME, NAD-malic enzyme.

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Table 1PEPC and Rubisco activity of Kranz and single cell C₄ species

Species	Notes	PEPC activity	Rubisco activity	Ratio <u>PEPC</u> Rubisco	Reference
Kranz C₄ species		$\mu\text{mol m}^{-2} \text{s}^{-1}$	$\mu\text{mol m}^{-2} \text{s}^{-1}$		
<i>F. bidentis</i>	High light grown	127 ^b ± 17	50.2 ± 3.3	2.5	(Pengelly et al., 2010)
<i>F. bidentis</i>	Low light grown	49.8 ± 4.5	23.9 ± 1.7	2.1	(Pengelly et al., 2010)
<i>Zea mays</i> (B73)		237 ± 25	45 ± 3	5.3	(Pengelly et al., 2011)
<i>Zea mays</i> (Sweet corn)		260 ± 22	40 ± 1	6.5	(Pengelly et al., 2011)
		$\mu\text{mol mg chl}^{-1} \text{min}^{-1}$	$\mu\text{mol mg chl}^{-1} \text{min}^{-1}$		
<i>Salsola laricina</i>		20.5	1.13	18	(Voznesenskaya et al., 2002)
Terrestrial single cell C₄ species					
<i>Bienertia cycloptera</i>		22.8 ± 1.6	4.3 ± 0.6	5.3	(Voznesenskaya et al., 2002)
<i>Suaeda aralocaspica</i>	young	6.0	2.3	2.6	(Voznesenskaya et al., 2003b)
	intermediate	11.2	4.9	2.3	(Voznesenskaya et al., 2003b)
	mature	28.9	6.7	4.3	(Voznesenskaya et al., 2003b)
<i>Suaeda aralocaspica</i>		12.8 ± 0.5	2.17 ± 0.15	5.9	(Voznesenskaya et al., 2001)
Aquatic plants					
<i>Hydrilla verticillata</i>	^b Γ=24 μbar	1.93 ± 0.13	0.56 ± 0.03	3.5	(Salvucci and Bowes, 1981)
<i>Hydrilla verticillata</i>	Γ=12±5μbar	2.0 ± 0.4	0.7 ± 0.1	3	(Ascencio and Bowes, 1983)
<i>Hydrilla verticillata</i>	Γ=76 μbar	0.53 ± 0.01	0.74 ± 0.03	0.7	(Salvucci and Bowes, 1981; Ascencio and Bowes, 1983)
<i>Hydrilla verticillata</i>	Γ=141±5μbar	0.5 ± 0.1	0.8 ± 0.17	0.6	(Ascencio and Bowes, 1983)

^a ± denotes plus or minus the standard error

^b A low CO₂ compensation point, Γ , has been shown to be a sign of induction of C₄ pathway, whereas a high Γ value indicates C₃ photosynthesis.

Table 2

Comparison of estimates of diffusive resistance between atmosphere and site of Rubisco carboxylation using a PEPC inhibitor in Kranz and single cell C₄ species and carbon isotope techniques in C₃ species.

Species	CO ₂ diffusion resistance (leaf area basis, m ² s mol ⁻¹)	Reference
Kranz C₄ species		
<i>Panicum maximum</i>	330	(He and Edwards, 1996) ^a
<i>Panicum miliaceum</i>	129	
<i>Sorghum bicolor</i>	97	
<i>Amaranthus edulis</i>	120	(Kiirats et al., 2002)
Terrestrial single cell C₄ species		
<i>Bienertia cycloptera</i>	63	(Edwards et al., 2007)
C₃ species		
<i>Arabidopsis thaliana</i>	5	(Tazoe et al., 2011)
<i>Nicotiana tabacum</i>	2.5	
<i>Oryza sativa</i>	0.8	(Scafaro et al., 2011)

^aThe values shown are from analysis of the data of Brown and Byrd, 1993 using a non-linear regression on A versus C_a response curves which is considered to give a better estimate of diffusive resistance; values obtained by Brown and Byrd, 1993 using a linear regression were, 425, 510 and 885 m² s mol⁻¹ for *P. maximum*, *P. miliaceum*, and *S. bicolor*, respectively.

Figure 1

Fig. 2

Figure 3

Fig. 5