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SINGLE-CELL C_4 PHOTOSYNTHESIS VERSUS THE DUAL-CELL (KRANZ) PARADIGM

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■ **Abstract** The efficiency of photosynthetic carbon assimilation in higher plants faces significant limitations due to the oxygenase activity of the enzyme Rubisco, particularly under warmer temperatures or water stress. A drop in atmospheric CO_2 and rise in O_2 as early as 300 mya provided selective pressure for the evolution of mechanisms to concentrate CO_2 around Rubisco in order to minimize oxygenase activity and the resultant loss of carbon through photorespiration. It is well established that a carbon-concentrating mechanism occurs in some terrestrial plants through the process of C_4 photosynthesis. These plants are characterized as having Kranz-type leaf anatomy, with two structurally and biochemically specialized photosynthetic cell types, mesophyll and bundle sheath, that function coordinately in carbon assimilation. C_4 photosynthesis has evolved independently many times with great diversity in forms of Kranz anatomy, structure of dimorphic chloroplasts, and biochemistry of the C_4 cycle. The most dramatic variants of C_4 terrestrial plants were discovered recently in two species, *Bienertia cycloptera* and *Borszczowia aralocaspica* (family Chenopodiaceae); each has novel compartmentation to accomplish C_4 photosynthesis within a single chlorenchyma cell. This review discusses the amazing diversity in C_4 systems, how the essential features of C_4 are accomplished in single-cell versus Kranz-type C_4 plants, and speculates on why single-cell C_4 plants evolved.

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INTRODUCTION

A central feature of photosynthetic carbon assimilation is the enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco; E.C. 4.1.1.39). It has a remarkably low efficiency as a catalyst due to its low turnover rate for CO_2 fixation, as well as a lack of specificity for reacting only with CO_2. Thus, photosynthetic organisms have had to develop means to overcome its inefficiency in carbon fixation. As a consequence, plants produce large amounts of Rubisco to compensate for its low turnover rate, resulting in its being the single most abundant soluble protein on Earth. The dual specificity of the enzyme in using CO_2 and O_2 as substrates presents a different problem, requiring more complex solutions. Rubisco functions both as a carboxylase and an oxygenase using the substrate ribulose-1,5-bisphosphate (RuBP), with relative rates depending on the concentrations of CO_2 and O_2. Reaction of RuBP with CO_2 leads to photosynthetic carbon reduction through the C_3 cycle, providing the carbon skeleton needed for plant growth and function. In contrast, reaction of RuBP with O_2 is counterproductive, leading to CO_2 release through a photorespiratory process that involves the glycolate pathway. The reason why a mechanism for photosynthesis developed via this enzyme with dual catalytic activity is considered to be partly, if not totally, related to conditions existing when photosynthesis first evolved.

When photosynthesis evolved in bacteria ~3 billion years ago, carbon dioxide levels were high (circa 100-fold higher than current levels) and there was little or no oxygen (4, 61). Oxygenase activity, which is suggested to be an unavoidable consequence of the reaction mechanism, would therefore have been restricted by the atmospheric conditions. During the Carboniferous Period, about 300 mya, a large decline in CO_2 levels and an increase in O_2 levels occurred, according to geochemical mass balance models (7). This change provided conditions for significant levels of photorespiration, such that a CO_2 concentrating mechanism (CCM), which enhances the ratio of CO_2 to O_2 around Rubisco, would be advantageous in both terrestrial and aquatic environments. It is suggested that there were multiple origins of CCMs among microalgae during the Carboniferous period (5). Although there is no supporting evidence, if land plants independently evolved CCMs during this period, these concentrating mechanisms may have been lost during the great extinction, or plants may have reverted to photosynthesis without a CCM because of a subsequent rise in CO_2. Alternatively, these plants may have escaped recognition by scientists studying CCMs. During the late Tertiary Period, approximately

65 mya, CO_2 levels were again thought to be low enough for conditions to be favorable for evolutionary selection of CCMs and C_4 photosynthesis.

Many aquatic photosynthetic organisms evolved CCMs in response to the limiting inorganic carbon and the 10^4 higher diffusive resistance of CO_2 in water compared with that in air. In the aquatic environment, cyanobacteria, algae, and some angiosperms evolved multiple mechanisms to actively accumulate inorganic carbon around Rubisco by use of membrane transporters and carbonic anhydrases (1a, 4, 5, 14a, 49a, 53a). In contrast, terrestrial plants, and a few aquatic macrophytes, evolved a biochemically and anatomically complex organic carbon pump, called the C_4 pathway, along with the entire carbon fixation process commonly referred to as C_4 photosynthesis. The earliest fossil records of C_4 plants date circa 12 mya, although fossil records are sparse because most C_4 plants grow in ecosystems where they are oxidized if buried (13). During the past 420,000 years, prior to industrialization, the average atmospheric CO_2 concentration was only ~220 ppm (20), a level that can clearly be limiting, with high photorespiratory carbon loss in the oxygen-rich environment (55). The general requirement for C_4 photosynthesis to function is the spatial separation of initial fixation of atmospheric CO_2 via phosphoenolpyruvate carboxylase (PEPC) and formation of C_4 acids malate and aspartate in one compartment close to the entry point of atmospheric CO_2, and the utilization of these C_4 acids by decarboxylases to concentrate CO_2 around Rubisco in another compartment which is distal to the entry point of CO_2 into photosynthetic tissue (Figure 1). From the discovery of C_4 photosynthesis in the 1960s until recently, the spatial compartmentation in terrestrial C_4 plants was consistently linked to the occurrence of Kranz-type anatomy. The term Kranz anatomy is commonly used to describe the dual-cell system associated with C_4 photosynthesis, consisting of mesophyll cells containing PEPC and initial reactions of C_4 biochemistry, and bundle sheath cells containing enzymes for generating CO_2 from C_4 acids and the C_3 carbon reduction pathway, including Rubisco. However, the anatomy associated

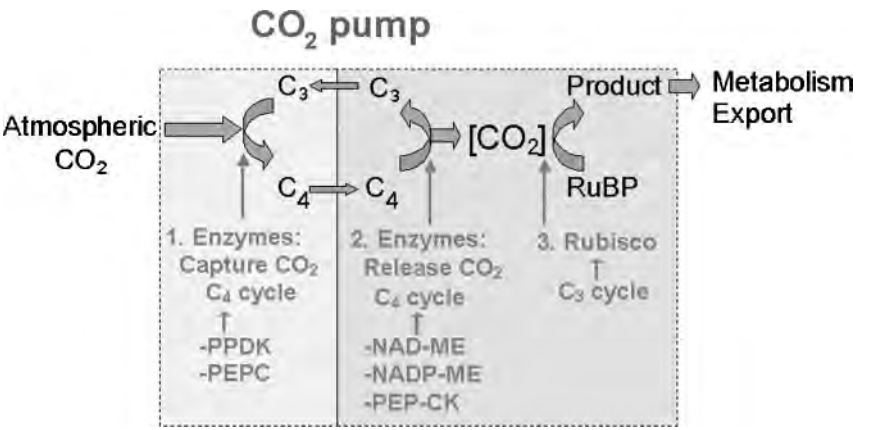

Figure 1 Compartmental depiction of the CO_2 pump for C_4 photosynthesis.

with C_4 plants shows considerable variation from the initial description of Kranz anatomy by Haberlandt (31, 32), which was made long before its association with C_4 photosynthesis was discovered. A study of the structure-function relationships in the diverse C_4 systems is important both for understanding of the operational parameters required for C_4 photosynthesis and for exploring the evolution of the C_4 syndrome.

DIVERSITY IN KRANZ-TYPE C_4

Occurrence and Distribution

Among land plants, C_4 species have been found to date only in angiosperms, where they have been reported in 17 of the ~450 families and in one aquatic family, Hydrocharitaceae (Table 1). Of these 18 families, the monocot family Poaceae has the largest number of C_4 species (about half of the 10,000 species in the family

TABLE 1 List of families having C_4 species, estimates of current number of C_4 species identified, and photosynthetic types according to C_4 acid decarboxylase (59, 60)

Family	# C_4 species: Current estimates	C_4 Photosynthetic types identified in family
Acanthaceae	20	
Aizoaceae	35	
Amaranthaceae	250	NAD-ME, NADP-ME
Asteraceae	150	NADP-ME
Boraginaceae	60	
Capparidaceae (now in the Brassicaceae)	10–20	
Caryophyllaceae	30	
Chenopodiaceae	550	NAD-ME, NADP-ME
Cyperaceae	1350	NAD-ME, NADP-ME
Euphorbiaceae	250	NADP-ME
Hydrocharitaceae	2	NADP-ME
Molluginaceae	3–5	NAD-ME
Nyctaginaceae	25	
Poaceae	4600	NAD-ME, NADP-ME, PEP-CK
Polygonaceae	100	
Portulacaceae	100	NAD-ME, NADP-ME
Scrophulariaceae	6–10	
Zygophyllaceae	45	NADP-ME

are C_4), followed by family Cyperaceae. Among the dicots, family Chenopodiaceae has the largest number of C_4 species. Compared with the more common C_3 species, the total number of known C_4 species is very low, currently about 3% of angiosperm species. However, C_4 species have an effect on global productivity that is disproportionate to the number of species exhibiting C_4 photosynthesis (discussed below). Interestingly, C_4 species are primarily herbaceous or shrubby and C_4 trees are rare. In contrast, although C_3 herbs and shrubs are abundant, C_3 trees also represent a major component of terrestrial plant biomass.

Physiological parameters that affect photosynthesis have been studied extensively in a wide range of plant species, and an understanding of these is relevant to a discussion of evolution and architecture of C_4 systems. A combination of factors can contribute to CO_2-limited photosynthesis in C_3 plants, including low atmospheric levels of CO_2, high temperature (which increases activity of RuBP oxygenase relative to carboxylase), and stomatal limitations induced by drought and high salinity (46). The importance of temperature is particularly evident from surveys of grasses at different latitudes in countries as far-ranging as Argentina, Russia, North America, and Australia (12, 33, 51, 66, 74). These surveys all come to a remarkably similar conclusion: The percentage of C_4 species increases linearly with decreasing latitude, with C_4 species being dominant among the grasses 30° north and south of the equator. The efficiency of C_4 photosynthesis in warmer climates and the importance of C_4 grasses in tropical and semitropical savannas account in large part for their productivity. C_4 plants are estimated to contribute $\sim$20–30% to global terrestrial productivity (25, 45), even though they make up only about 3% of angiosperm species.

Evolutionary Considerations

Selective pressures from decreasing supply of CO_2 to Rubisco driven by decreasing atmospheric CO_2, high temperatures, and limitations on water availability in high light environments probably all contributed to the evolution of CCMs and C_4 photosynthesis in terrestrial plants. Given that most extant C_4 plants studied require a dual-cell system coupled with biochemical specialization, this complex adaptation must represent the endpoint of a stepwise mechanism of development of the system. This view is supported by studies in extant plants, where some examples of CCMs with anatomical intermediates of Kranz anatomy have been found (17, 49). There must have been a strong, selective pressure for the C_4 type of CCM, since it is estimated that C_4 photosynthesis evolved independently more than 40 times (59). This figure is likely to increase as information is provided from ongoing studies on phylogeny in families known to have C_4 species. Angiosperms evolved $\sim$120 mya, and families in which the most C_4 species are found (e.g., Poaceae, Chenopodiaceae), may have evolved earlier which, over time, became more diversified in their leaf structure (facilitating evolution of Kranz anatomy) and adaptation to different habitats (56). This phase of radiation or adaptation to more varied environments where C_4 still provided an advantage is thought to account

for the variation in anatomical and biochemical features related directly to C_4 photosynthesis (i.e., Kranz anatomy). Part of the anatomical variation seen in Kranz anatomy is also a reflection of leaf morphological and anatomical adaptations to other environmental pressures such as advantages of reduced leaf surface areas to volume ratios, water storage tissues, mechanical tissues, etc.

Despite evidence that C_4 photosynthesis has evolved many times, why it has evolved in only a few families is not clear. Important factors for evolution of C_4 are suggested to be conditions associated with climate, ecological disturbances (fire, grazing by mammals providing open high-light habitats), and differences in evolutionary diversification among plant families (19, 56). Furthermore, C_4 photosynthesis requires duplication of many genes followed by mutations in one of the duplicates to function in a novel role. Monson (48) suggested this duplication could result in genetic constraints where differences in population attributes (population size, generation time, frequency of recruitment of sexually produced individuals) would control the probability for evolution of C_4.

The Link Between Anatomical and Biochemical Features

Plants expressing C_4 photosynthesis can be categorized based on biochemistry or anatomy. C_4 species have been classified biochemically according to the primary type of C_4 acid decarboxylase used in C_4 photosynthesis: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and PEP carboxykinase (PEP-CK) (37). Although malic enzyme subtypes have often evolved independently, evidence indicates that PEP-CK type grasses are derived from ancestors having the NAD-ME subtype (41). Immunolocalization studies show clearly the selective compartmentation of key enzymes between mesophyll cells and bundle sheath cells in each of the three subgroups, which is a requirement for C_4 to function. This compartmentation is illustrated in Figure 2 for Rubisco, PEPC, and the decarboxylase for species representing the three subtypes: *Halothanmus glaucus*, NADP-ME type (family Chenopodiaceae), *Salsola laricina*, NAD-ME type (family Chenopodiaceae), and *Spartina anglica*, PEP-CK type (Poaceae, the only family known to have PEP-CK-type C_4 species). In each case, PEPC is located in mesophyll cells whereas Rubisco and the decarboxylases are localized to the bundle sheath cells. With respect to intracellular compartmentation, in each of the three biochemical types, the decarboxylases are in a different intracellular compartment in bundle sheath cells: NADP-ME in chloroplasts, NAD-ME in mitochondria, and PEP-CK in the cytosol. Rubisco is located in the chloroplasts of bundle sheath cells; pyruvate Pi dikinase (PPDK), which generates PEP for the C_4 cycle, is located in the mesophyll chloroplasts; and PEPC is located in the cytosol of mesophyll cells. Thus, chloroplasts of the bundle sheath and mesophyll are different with respect to enzyme content, as well as to ultrastructural features such as relative amounts of grana and thylakoids which we will refer to as dimorphic. [See Edwards et al. (16) for a review of earlier in vitro methods for separating dimorphic chloroplasts and Kranz cell types, and recent advances using immunolocalization methods that have been instrumental in characterizing the C_4 mechanism.]

Among the families known to have C_4 species, Poaceae and Chenopodiaceae have the most diversity in biochemistry and anatomy relevant to C_4 photosynthesis. In the Poaceae, C_4 photosynthesis is currently estimated to have evolved independently at least 10 times across three subfamilies: Panicoideae, Arundinoideae, and Chloridoideae (28). All three biochemical subtypes have been found in the grasses, and three classical types of Kranz leaf anatomy are associated with each, based on the structure of dimorphic chloroplasts and the position of chloroplasts in bundle sheath cells (18, 30). However, other well-known anatomical variants occur with less frequency, and sometimes are described as separate, with up to eight different Kranz-type anatomies in the family (see 15). These anatomical classes are based on the arrangement of mesophyll cells relative to the bundle sheath and other leaf cell types.

Family Chenopodiaceae has five major types of Kranz anatomy (Atriplicoid, Kochioid, Salsoloid, Kranz-Suaedoid, and Conospermoid) if arrangements of mesophyll and bundle sheath cells versus other tissues (vascular, and water storage, if present) are considered (11, 22, 36). Also, some variants occur as anatomical subtypes with differences in the presence or absence of hypodermis and spongy parenchyma, the layout and number of vascular bundles, among other features (36). Examples of subfamilies and tribes in which these types of Kranz anatomies are found are Atriplicoid, mainly in Chenopodioideae in the tribe Atripliceae; Kochioid, also in Chenopodioideae but in the tribe Camphorosmeae; Salsoloid in Salsoloideae in the tribe Salsoleae; and Kranz-Suaedoid and Conospermoid in Suaedoideae in the tribe Suaedeae (11, 36, 62). The chenopod C_4 species are classified into two C_4 cycle biochemical subtypes, NAD-ME and NADP-ME, which usually form aspartate or malate as the predominant initial photosynthetic product, respectively (24, 26, 52, 53, 72). Only NAD-ME-type C_4 plants are found in members of subfamily Chenopodioideae having Atriplicoid leaf anatomy, and in members of subfamily Suaedoideae having Kranz-Suaedoid-type anatomy. Only NADP-ME species are found in Chenopodiaceae belonging to the tribe Camphorosmeae of family Chenopodioideae having Kochioid leaf anatomy, whereas in tribe Salsoleae in Salsoloideae, both biochemical subtypes are found (24, 27, 50, 53). C_4 members of the subfamilies Salsoloideae and Suaedoideae are predominant in biodiversity and biomass in deserts and semideserts of central Asia, where they grow under xerophytic and/or halophytic conditions with tolerance to high temperatures, drought, and salinity.

The Role of Dimorphic Chloroplasts in C_4 Photosynthesis

In addition to the anatomical and biochemical differences of the Kranz cell types, the chloroplasts in mesophyll and bundle sheath cells of C_4 plants are dimorphic due to differences in enzymes, location of starch (predominantly in bundle sheath chloroplasts), and in chloroplast ultrastructure. Differences in chloroplast ultrastructure are related to variations in energy requirements between mesophyll and bundle sheath cells between subgroups. High grana-containing chloroplasts have higher PSII activities and linear electron flow (producing NADPH and ATP),

whereas low grana-containing chloroplasts are richer in PSI-mediated cyclic electron flow-producing ATP [18; also, see discussion of Anderson (3) on compartmentation of linear and cyclic electron flow between grana and stromal lamella]. This dimorphism is very apparent in NADP-ME type species, where the mesophyll chloroplasts have well-developed grana, whereas the bundle sheath chloroplasts are deficient in grana. NADP-ME type C_4 species are predominantly malate formers, whereby malate is shuttled from mesophyll to bundle sheath cells. Analyses of the energetics of carbon assimilation of the two cell types in representative species show the high grana-containing mesophyll chloroplasts of NADP-ME type species to have a greater demand for reductive power (utilized in part for synthesis of malate) (18, 72).

NAD-ME and PEP-CK species generally have grana in both mesophyll and bundle sheath chloroplasts. However, extensive studies of NAD-ME-type species in family Chenopodiaceae show that mesophyll chloroplasts are deficient in grana compared to bundle sheath chloroplasts (24, 27, 73). In these NAD-ME type species, aspartate is the primary product of CO_2 fixation (52), and support of an aspartate cycle requires only production of ATP to drive conversion of pyruvate to PEP by the mesophyll chloroplasts (18, 72). This ATP may be provided via PSI cyclic electron flow in the low grana-containing chloroplasts. Thus, in general, the dimorphic nature of chloroplasts in the Kranz anatomy cells is correlated with the energy requirements of the biochemical systems that are operating.

TERRESTRIAL SINGLE-CELL C_4 PHOTOSYNTHESIS

In studies on plant anatomy in the late 1800s, Haberlandt observed that some species of Cyperaceae and Poaceae have two very distinctive photosynthetic cell types that form wreaths around the vascular tissue, a formation described as Kranz anatomy (31, 32). When the C_4 pathway of photosynthesis was discovered and the first species studied in the 1960s, plants such as maize and sugarcane were soon recognized as having Kranz anatomy. Over the past 35 years, many C_4 species have been identified (Table 1) among terrestrial plants, and where leaf anatomy has been examined, Kranz-type leaf anatomy has been synonymous with the identification of C_4 photosynthesis.

Although the C_4 pathway of photosynthesis was being defined in the 1960s, it was not until the early 1970s, when the role of Rubisco in photorespiration was discovered in C_3 plants, that its function was understood. It became clear that the function of the C_4 pathway is to concentrate CO_2 around Rubisco in bundle sheath cells to prevent photorespiration (16). As noted above, for C_4 photosynthesis to function, there needs to be spatial separation between the trapping of atmospheric CO_2 into C_4 acids by PEPC (not affected by O_2) and the donation of CO_2 from C_4 acids to Rubisco, with sufficient diffusive resistance between the two processes to prevent CO_2 leakage and futile cycling. The bundle sheath cell wall itself has been considered to be an important barrier to CO_2 leakage, because of either a

thickened cell wall or a suberin lamella in the wall (68). Kranz anatomy is an elegant evolutionary solution to separating the processes, and for more than three decades it was considered a requirement for the function of C_4 photosynthesis in terrestrial plants.

Recently, this paradigm was broken when two species, *Borszczowia aralocaspica* and *Bienertia cycloptera*, both representing monotypic genera of the family Chenopodiaceae, were shown to have C_4 photosynthesis within a single cell without the presence of Kranz anatomy (58, 70, 71). Previously, these two species were reported to have C_4/Crassulacean acid metabolism (CAM)-type carbon isotope composition and unusual chlorenchyma without Kranz anatomy (2, 22, 23, 75), which suggested they are either non-Kranz C_4 plants, CAM, or that they have a previously unknown type of photosynthesis mechanism. Subsequent microscopic, biochemical and physiological studies have shown these species to function as C_4 plants (69–71). Although they are succulent like other related species in subfamily Suaedoideae, analyses of leaf titratable acidity at dawn and dusk, and dark CO_2 exchange, show that they do not perform CAM (69–71). In fact, no CAM plants have been identified in family Chenopodiaceae; but some succulent C_4 species in tribes Salsoleae and Suaedeae have been reported to have low CAM with nighttime fixation of CO_2, a few percent of that fixed by photosynthesis during the day (9, 78).

Borszczowia grows in central Asia from northeast of the Caspian lowland east to Mongolia and western China, whereas *Bienertia* grows from east Anatolia eastward to Turkmenistan and Pakistani Baluchestan [see (1) for distribution of *Bienertia* relative to climate, soil, and other species]. From molecular and morphological studies, *Borszczowia* is classified in subfamily Suaedoideae with *Suaeda* species, forming a monophyletic group (62). From early studies on morphology, *Bienertia* was classified in subfamily Salsoloideae, either in tribe Suaedeae or as a separate tribe called Bienertieae. Akhani (1), who notes its evolution is still a matter of confusion, proposed that *Suaeda* sect. *Schanginia*, which has C_3 species, is a possible ancestor to *Bienertia*. In molecular studies, *Bienertia* was shown to be sister to Suaedoideae in the chloroplast DNA trees; surprisingly, it was sister to subfamily Salicornioideae in a nuclear ribosomal ITS tree, despite having large morphological differences (62). The systematics of these unusual plants require further resolution.

Borszczowia and *Bienertia* are able to perform C_4 photosynthesis within a single chlorenchyma cell by intracellular partitioning of enzymes and organelles in two compartments. However, they have two very different, and novel, means of partitioning the functions of C_4 photosynthesis between two cytoplasmic compartments. Leaves of both species are terete, but their internal anatomy differs somewhat and their chlorenchyma tissue is very different. *Borszczowia* has a single layer of elongate, cylindrical chlorenchyma cells below the epidermal and hypodermal layers, which surround the veins and internal water storage tissue. The cells are tightly packed together with intercellular space restricted to the end of the cells closest to the epidermis. Figure 3*A* is a confocal fluorescence mi-

croscopy image from a *Borszczowia* leaf showing red autofluorescence of chloroplasts in a living cell. There is a dense layer of chloroplasts in the proximal part of the cell, which is closest to the vascular bundles and the center of the leaf, and fewer chloroplasts are located around the periphery at the distal end of the cell, which is close to the leaf surface where atmospheric CO_2 enters. The anatomy of *Bienertia* leaves with respect to photosynthetic tissue is very different in that there are two to three layers of shorter chlorenchyma cells that surround the centrally located water-storage and vascular tissue in the leaf. The cells are loosely arranged, with considerable intercellular space around them. Confocal imaging of chloroplast autofluorescence in living cells shows a large, central cytoplasmic compartment packed with chloroplasts, and a peripheral layer of chloroplasts around the cells (Figure 3*B*). Scanning and transmission electron microscopy show that the cytosolic and the peripheral compartments are connected by cytoplasmic channels through the vacuole (70). This type of anatomy in *Borszczowia* and *Bienertia*, with respect to strict chloroplast partitioning, has not been reported in other plant species. Mitochondria also show strict partitioning in these two species. In *Borszczowia*, they are concentrated at the proximal end of the cell among the chloroplasts, and in *Bienertia*, they are concentrated within the central cytoplasmic compartment.

The unusual chloroplast and mitochondria partitioning and the two cytoplasmic compartments defined by this partitioning are critical components of the C_4 mechanism in these species. Studies on cell structure by light, transmission electron, and scanning electron microscopy, in situ localization of key photosynthetic enzymes by light and electron microscopy, and activity and western blot assays of photosynthetic enzymes all indicate that these two species have dimorphic chloroplasts located between their two cytoplasmic compartments, which have photosynthetic functions analogous to mesophyll and bundle sheath cells in Kranz NAD-ME type C_4 plants. A single *Borszczowia* chlorenchyma cell has the equivalent of a Kranz mesophyll compartment at the distal end and a bundle sheath compartment at the proximal end. In contrast, the *Bienertia* chlorenchyma cell has the equivalent of a bundle sheath cell embedded within, and surrounded by, a mesopyll cell. In both cases, there is no intervening wall between the compartments, but the connecting cytoplasmic compartments have design features that mimic the proposed function of the wall and liquid diffusion path between Kranz anatomy cell types (discussed below).

Figure 4*A* is a model for the operation of C_4 photosynthesis in a single *Borszczowia* chlorenchyma cell. Atmospheric CO_2 enters the chlorenchyma cell at the distal end, which is surrounded by intercellular air space. Here, the carboxylation phase of the C_4 pathway assimilates atmospheric CO_2 into C_4 acids. Two key enzymes in the process are PPDK, located in chloroplasts at the proximal part, which converts pyruvate to PEP, and PEPC, located in the cytosol, which converts PEP and bicarbonate to oxaloacetate, the precursor for forming malate and aspartate. The C_4 acids diffuse to the proximal part of the cell through a thin, cytoplasmic space at the periphery of the middle of the cell, which is devoid of organelles. In the

proximal end, the C_4 acids are decarboxylated by NAD-ME in mitochondria that appear to be localized exclusively in this part of the cell. The CO_2 is captured by Rubisco that is localized exclusively in chloroplasts surrounding the mitochondria in the proximal part of the cell.

Bienertia uses a similar concept of organelle partitioning in a single cell to operate the C_4 process, but it has a very different compartmentation scheme. Figure 4*B* is a model for C_4 photosynthesis in *Bienertia*. Atmospheric CO_2 enters the cell around the periphery, which is exposed to considerable intercellular air space, and here the carboxylation phase of the C_4 pathway functions to convert pyruvate and CO_2 into oxaloacetate through the combined action of PPDK in the chloroplast and PEPC in the cytosol. C_4 acids diffuse to the central cytoplasmic compartment through cytoplasmic channels and are decarboxylated by NAD-ME in mitochondria, which are specifically and abundantly located there. Chloroplasts in the central cytoplasmic compartment surround the mitochondria and fix the CO_2 by Rubisco, which is only present in the chloroplasts of this compartment, through the C_3 cycle.

The specialized organelle and enzyme compartmentation established in individual *Borszczowia* and *Bienertia* chlorenchyma cells clearly mimic the organization of bundle sheath and mesophyll cells in NAD-ME type C_4 species with Kranz anatomy. This compartmentation also extends to other processes in C_4 photosynthesis to reduce photorespiration. In Kranz-type C_4 plants, both Rubisco, which can also function as an oxygenase, and mitochondrial glycine decarboxylase (GDC) are confined to bundle sheath cells (6), where photorespiration functions as a part of the CO_2 pump under limiting CO_2 (16a, 43). Likewise, in these single-cell C_4 plants, it is considered critical to the reduction of photorespiration that GDC, as well as the C_4 acid decarboxylase NAD-ME, are located in mitochondria that occur specifically in the compartment where CO_2 is captured by Rubisco in chloroplasts (69, 70).

The chloroplasts in the single-cell C_4 system provide a particularly remarkable example of the ability to control organelle differentiation at a spatial level within an individual plant cell. In both *Borszczowia* and *Bienertia*, the chloroplasts at the site of entry of atmospheric CO_2 into the C_4 pathway and the site of donation of CO_2 to the C_3 pathway are dimorphic in three ways: enzymes (PPDK versus Rubisco and ADP glucose pyrophosphorylase, respectively), ultrastructure (grana-deficient versus high grana content, respectively), and starch (absent versus present, respectively) (69–71). These features are characteristic of dimorphic chloroplasts in mesophyll and bundle sheath cells in Kranz-type NAD-ME species in genus *Atriplex*, and also species in tribes Suadeae and Salsoleae (24, 27, 73). The mechanisms regulating and maintaining this dimorphism in the single-cell system are currently under investigation and should provide important information on general mechanisms of organelle specialization and partitioning within cells as well.

Whereas partitioning of organelle-based enzyme systems can be envisioned, regardless of the complexity of the mechanisms, partitioning within a single cell

of a major cytosolic protein such as PEPC is probably not possible. In both *Borszczowia* and *Bienertia*, PEPC is located in the cytosol, where it is probably one of the most abundant soluble proteins, but selective function of the enzyme where atmospheric CO_2 enters the cell is necessary to prevent a futile C_4 cycle. There are several ways by which these cells may prevent a futile cycle in the Rubisco-containing cellular compartment. PEPC activity in this compartment may be restricted by less PEPC protein, as there is lower cytosolic space due to the high density of mitochondria and chloroplasts. PEPC activity may be limited by lack of substrate PEP, because PPDK (required to generate PEP) is essentially absent from chloroplasts in this region. There might also be selective allosteric control of PEPC activity in this compartment; e.g., regulation of activity in C_4 plants by phosphorylation/dephosphorylation is well established (14). Whatever the actual mechanism is for regulating PEPC activity in the compartment where the C_3 cycle functions, immunolocalization, gas exchange, $\delta^{13}C$, and initial products studies all indicate that the single-cell system works effectively in the C_4 mode.

Redefining How to Determine if a Terrestrial Plant Is C_4

Since the 1960s, C_4 photosynthesis in terrestrial plants has been linked to Kranz anatomy, although the leaf anatomy of many species classified as C_4 has not been examined (16). The discovery of single-cell terrestrial C_4 plants indicates that mechanisms of concentrating CO_2 around Rubisco in plants may be more diverse than previously thought. Kranz anatomy is a very strong indicator of C_4 photosynthesis since C_3 plants lack Kranz anatomy. However, this can no longer be used as an absolute primary screen because it would not detect single-cell C_4 plants. In addition, plants that have been identified as C_4 based on other criteria, such as carbon isotope composition, can no longer be assumed to have Kranz anatomy.

Carbon isotope composition differs significantly between C_3 and C_4 or CAM plants, and this analysis provides useful information about the potential mechanism of carbon fixation in a plant (45, 76). Dry matter can be analyzed for $^{13}CO_2$ discrimination and if a species has a C_3-type isotope composition, that characteristic would indicate unequivocally that it is functioning as a C_3 plant. In C_3 plants, when the rate of CO_2 fixation is limited by Rubisco, there is high discrimination against assimilating $^{13}CO_2$ versus $^{12}CO_2$. In contrast, PEPC shows little discrimination against $^{13}CO_2$, and where initial fixation of atmospheric CO_2 is by this enzyme, the carbon discrimination value will be low. Thus, when C_4 and CAM plants fix atmospheric CO_2 by PEPC, and the CO_2 is effectively donated via C_4 acids to Rubisco, there is no discrimination against $^{13}CO_2$. However, when photosynthesis in C_3 plants is limited, primarily via diffusive resistance to CO_2 (e.g., low stomatal conductance induced by drought or low humidity), these plants also show less discrimination against assimilating $^{13}CO_2$ (21, 77). Hence, carbon isotope values that show reduced discrimination against $^{13}CO_2$ do not, without additional information, indicate the mode of photosynthesis. For example, if carbon isotope

TABLE 2 Distinguishing between terrestrial single-cell C_4, CAM, and C_3 plants

Parameter	Single-cell C_4	CAM	C_3
Anatomy	Non-Kranz	Non-Kranz	Non-Kranz
Carbon isotope discrimination against $^{13}CO_2$	Low	Low to intermediate	High
C_4 pathway enzymes	High	High	Low
Chlorenchyma	Two cytoplasmic compartments	Large cells with large vacuoles	Palisade or spongy mesophyll
Chloroplasts within a single chlorenchyma cell	Dimorphic	Monomorphic	Monomorphic
Initial products of $^{14}CO_2$ fixation	C_4 major during day	C_4 major during the night	C_3 major during day
Diurnal fluctuation in leaf acidity	No	Yes	No
CO_2 compensation point	Low	High, late afternoon uptake by the C_3 pathway	High
O_2 inhibition of CO_2 fixation measured in the light	No	Yes, late afternoon uptake by the C_3 pathway	Yes

values indicate low discrimination against $^{13}CO_2$, and microscopy indicates non-Kranz anatomy, additional tests (Table 2) are needed to definitively determine if the species performs CAM, single-cell C_4 photosynthesis, or diffusion-limited C_3 photosynthesis.

Are There Single-Cell C_3-C_4 Intermediates?

Besides C_4 photosynthesis, another means of partially overcoming the inefficiency of Rubisco, without employing a C_4 cycle, is to recapture the photorespired CO_2. Certain photosynthetic C_3-C_4 intermediate species have Kranz-like anatomy and reduce photorespiration by refixation of photorespired CO_2 without employing C_4 photosynthesis (17, 49). In these plants, the mesophyll functions as in C_3 plants, where chloroplasts fix CO_2 via the C_3 cycle and produce glycolate via RuBP oxygenase. However, the release of CO_2 in photorespiration occurs by transport of glycine to bundle sheath cells for metabolism via GDC, which is specifically expressed in bundle sheath mitochondria. The photorespired CO_2 is then fixed by chloroplasts in the bundle sheath cells. A low diffusive conductance to CO_2 from the bundle sheath compartment is thought to favor its refixation by RuBP carboxylase. This refixation reduces photorespiration, as is evident from the lower CO_2 compensation points in intermediates, but has little effect on the carbon isotope composition

compared to C_3 plants (17). The discovery of single-cell C_4 systems also indicates that mechanisms of reducing photorespiration by refixing photorespired CO_2 may have evolved in plants via spatial separation of functions within chlorenchyma cells, resulting in a photosynthetic intermediate. In this case, most chloroplasts, containing Rubisco and the C_3 cycle, would be partitioned at the periphery of the cell where CO_2 enters from the atmosphere, whereas other chloroplasts, along with mitochondria containing GDC, would be partitioned to a distal part of the cell away from the entry of CO_2. Either the positioning of chloroplasts around mitochondria and/or a long liquid-phase diffusion path could provide low conductance to CO_2, favorable for refixation and lowering photorespiratory losses. Such intermediates would have lower CO_2 compensation points than is typical for C_3 plants, but they would not be recognized through searches for Kranz-like anatomy or screening of carbon isotope values. Evolution of single-cell photosynthetic intermediates, without C_4 photosynthesis, should be less complex than single-cell C_4 plants (i.e., not having dimorphic chloroplasts, or the C_4 pathway), but more difficult to identify.

Effective Trapping of CO_2 by Rubisco in Single-Cell C_4 Photosynthesis

In C_4 plants, a low conductance for diffusion of CO_2 out of the bundle sheath cells relative to the conductance of Rubisco for assimilating CO_2 is required for efficient operation of C_4 photosynthesis. Kranz anatomy has been recognized as an efficient means of providing a physical constraint on conductance of CO_2 away from Rubisco in the bundle sheath cell, although the magnitude of this diffusive resistance has been difficult to measure (42, 68). The bundle sheath cell wall (due to its thickness, or presence of a suberin lamella in some cases) has been considered an important component of the low diffusive conductance to CO_2 and provides one explanation for the evolution of C_4 photosynthesis via Kranz anatomy. Recent analyses and modeling by von Caemmerer & Furbank (68), indicate large differences in the relative contribution of different components to this resistance in different Kranz-type C_4 plants, considering bundle sheath walls, membranes, bundle sheath chloroplast position, the site of C_4 acid decarboxylation, and the liquid-phase diffusion path. Considering these factors, their calculated bundle sheath resistances on a leaf area basis for different Kranz C_4 subgroups ranges from circa 50 to 150 $m^2\ s\ mol^{-1}$ (68).

Single-cell C_4 plants can capture CO_2 effectively from Rubisco without Kranz anatomy and the bundle sheath cell wall barrier. Photosynthesis in the single-cell systems is not inhibited by O_2, even under low atmospheric levels of CO_2, and their carbon isotope values are the same as in Kranz-type C_4 plants (70, 71), whereas the values would be more negative if there were leakage of CO_2 and overcycling through the C_4 pathway (34). In *Borszczowia*, the elongated cells obviously provide a long liquid-phase diffusion path from sites of donation of CO_2 from C_4 acids to Rubisco in the proximal ends of the chlorenchyma cells to the intercellular

air space at the distal ends (mean distance circa 50 μm). The thickness of the plasma membrane and cell wall is similar at the proximal and distal ends (69). The calculated diffusive resistance to CO_2 in these cells through the liquid phase on a leaf area basis of 110 $m^2\ s^{-1}\ mol^{-1}$ considering area of chlorenchyma exposed to intercellular space at the distal ends (69) is similar to values measured in the Kranz-type NAD-ME species *Amaranthus edulis* by utilizing plants in which the C_4 cycle is inactivated (by mutation or chemically) (42). This resistance value for *Borszczowia* is within the range of those predicted for Kranz-type C_4 plants based on analysis of physical barriers to CO_2 diffusion (68); and C_4 models indicate CO_2 leakage and the quantum requirement per CO_2 fixed increases dramatically with decreasing resistance below this value (16a, 68). In both *Bienertia* and *Borszczowia*, the chloroplasts containing Rubisco are often positioned "external" to the mitochondria where CO_2 is generated from malate by NAD-ME and from glycine by GDC, which may facilitate further the capture of CO_2 by Rubisco. The released CO_2 likely has to cross several membranes to escape from the CCM, a process that could contribute to the diffusive resistance. Just as there are alternatives in the type of C_4 cycle and type of C_4 anatomy, there are also alternative means of generating diffusive resistance to prevent CO_2 loss from the system.

Development of Single-Cell C_4 Photosynthesis

As noted above, single-cell C_4 plants have two different cytoplasmic compartments. One compartment has chloroplasts containing PPDK and functions in the carboxylation phase of the C_4 cycle (equivalent to the mesophyll cells in Kranz anatomy). The other compartment has chloroplasts containing Rubisco and functions in the capture of CO_2 donated from C_4 acids, along with mitochondria containing NAD-ME (equivalent to the bundle sheath in NAD-ME Kranz-type C_4 plants). An obvious question is how this partitioning develops in single-cell C_4 plants. In very young cotyledons of *Borszczowia*, there is one chloroplast type, which contains Rubisco, whereas PPDK is not detected and cytosolic PEPC is low (Figure 5). As the chlorenchyma develop, light-dependent spatial compartmentation for C_4 photosynthesis occurs, and chloroplasts become dimorphic and are polarized to opposite ends of the cells, with PPDK in chloroplasts at the distal end and Rubisco in chloroplasts at the proximal end. Thus, during development the chloroplasts differentiate from a C_3 Rubisco-containing default expression to dimorphic chloroplasts (Figure 5), the same process of differentiation as occurs in Kranz-type C_4 plants (8, 15, 64). In Kranz C_4 species, where photosynthetic genes are present in both cell types, genes of the C_4 pathway and the Rubisco Rbcs gene are located in the nucleus, and the Rubisco RbcL gene is located in the chloroplast (47, 64). If analogous, the single-cell C_4 system may develop from genetically identical chloroplasts rather than from two genetically different populations of chloroplasts. Unlike the Kranz system, in the single-cell C_4 system there may be selective targeting of nuclear-encoded mRNA (e.g., genes encoding PPDK and the Rubisco small subunit) to different

cytoplasmic compartments to initially form, and then maintain, the dimorphic chloroplasts.

Part of the previous reasoning as to why C_4 photosynthesis evolved with Kranz anatomy is that it allows for cell-specific expression of photosynthetic genes at multiple levels, including transcriptional control (8, 64, 65). Thus, differential control of transcription of nuclear genes encoding photosynthetic proteins is important in developing cell-specific functions in Kranz-type anatomy. However, the occurrence of single-cell C_4 plants indicates that an alternative means of spatial expression of enzymes has developed, i.e., posttranscriptional control, possibly through mRNA targeting to specific cytoplasmic compartments.

Why Did Single-Cell C_4 Plants Evolve?

Single-cell C_4 photosynthesis could simply be an alternative mechanism to Kranz-type C_4 photosynthesis although this seems to be a rare occurrence compared with the predominance of Kranz C_4 plants. Perhaps evolution of spatial compartmentation within a single cell is more difficult than separation of functions between two cell types. However, the extent to which this is a rarity among terrestrial plants is not known. Techniques to screen systematically for single-cell C_4 plants, as discussed above, are yet to be utilized.

The single-cell C_4 system, although it may be equally complex in its control of compartmentation of functions, is less complex in that it does not require the cooperative function of two cell types, nor does it require development of Kranz anatomy. Kranz anatomy in planar laminate leaves requires close spacing of veins, where most, or all, mesophyll cells are connected to a bundle sheath cell. Sage (56) suggested that the limited number of families containing species with Kranz anatomy may be linked, in part, to older families that developed more diverse types of leaf anatomy, thereby facilitating evolution of Kranz-type anatomy. Anatomical preconditions that may have facilitated evolution of Kranz anatomy are reduction in mesophyll number, increase in bundle sheath size, and increased vein density, which may have evolved in some families to maintain hydraulic integrity in hot, arid environments (56). Thus, such preconditions required for evolution of Kranz anatomy may not have developed in some families.

With respect to the two single-cell C_4 systems, *Borszczowia* has some constraints in shape and packaging of chlorenchyma cells. Part of the chlorenchyma cell is exposed to intercellular air space where atmospheric CO_2 enters, whereas the other part lacks intercellular air space, a condition that we propose helps prevent CO_2 leakage from sites of C_4 acid donation of CO_2 to Rubisco. This may be a precondition for evolution of *Borszczowia*-type C_4 photosynthesis. *Bienertia* appears to have no anatomical constraints, as it has multiple layers of chlorenchyma cells surrounded by intercellular air space. Thus, C_3 plants, with a variety of leaf anatomies, could potentially function as C_4 plants if they had *Bienertia*-type chlorenchyma cells. There are many families including rushes, onions, legumes, rose, mustard and potato, as well as gymnosperms and ferns, in which Kranz-type C_4 plants have not been found, yet they contain species that grow under

conditions where C_4 photosynthesis should be advantageous (56). While there may be unknown constraints in evolution of single-cell C_4 photosynthesis, with these recent discoveries it is reasonable to search for alternatives to Kranz-type CO_2 concentrating mechanisms among terrestrial plants.

Rather than being simply another means for achieving C_4 photosynthesis, single-cell C_4 might, in addition, allow more flexibility in mode of photosynthesis than Kranz-type C_4 plants by, for example, shifting from C_3 to C_4 depending on environmental conditions (also see *Hydrilla*, next section). With few exceptions (29, 44a, 67), C_4 plants with Kranz anatomy are obligate C_4. *Bienertia* shows some potential for a C_3 to C_4 transition, because under artificial growth conditions young leaves have intermediate carbon isotope values, whereas mature leaves have C_4-type isotope values (23, 70). Though this property has not been studied systematically in natural habitats, samples collected from different geographical areas have C_4 carbon isotope values (23, 70), indicating that they function as C_4 plants. Currently, *Borszczowia* appears to be an obligate C_4 plant, in that samples from plants growing under both natural and artificial conditions, and young versus mature leaf tissue, have C_4 carbon isotope values (22, 71).

AQUATIC SINGLE-CELL C_4 PHOTOSYNTHESIS

There are obvious differences in photosynthesis between aquatic and terrestrial plants. Whereas terrestrial plants take CO_2 from the atmosphere, both CO_2 and bicarbonate are available in the aquatic environment. Leaf polarity occurs in some aquatic macrophytes such as *Hydrilla*, facilitating uptake of CO_2 by acidification at the adaxial side of the leaf as it shifts the equilibrium from bicarbonate to CO_2. Nevertheless, if the concentration of inorganic carbon is low, the high diffusive resistance to inorganic carbon in water could limit the internal concentration in the cytosol (analogous to a high stomatal resistance in terrestrial plants as stomata close), making it beneficial to have C_4 photosynthesis. In this case, the C_4 pump in an aquatic plant may be beneficial even with a low intracellular diffusive resistance to CO_2 and substantial overcycling of the C_4 pathway.

Several freshwater monocots can perform C_4 photosynthesis under submerged conditions without Kranz anatomy. These include the submerged species *Hydrilla verticillata* and *Egeria densa* in family Hydrocharitaceae, *Sagittaria subulata* in family Alismataceae (10, 44), and species of *Orcuttia* in family Poaceace when growing submerged (38–40). Extensive studies have been made with *Hydrilla*, a facultative C_4 species, which is induced from C_3 to C_4 photosynthesis when exposed to low CO_2. There is evidence that it performs C_3 photosynthesis in the winter under cooler conditions and higher concentrations of inorganic carbon, and C_4 photosynthesis in the summer under warmer temperatures and lower levels of inorganic carbon. *Hydrilla* is NADP-ME-type C_4, where the proposed cycle is fixation of atmospheric CO_2 in the cytosol via PEPC, import of oxaloacetate or asparate into the chloroplast, followed by reduction of oxaloacetate to malate by NADP-malate dehydrogenase, and decarboxylation of malate via NADP-ME,

generating CO_2 and pyruvate. The released CO_2 is fixed by Rubisco, the pyruvate is converted to PEP via PPDK, and PEP is exported from the chloroplast to the cytosol to complete the cycle. Although there is evidence that CO_2 is concentrated in *Hydrilla* cells during C_4 photosynthesis, it is not known how it could be concentrated in the chloroplast, and what prevents high CO_2 leakage and futile C_4 pathway cycling. There is no evidence for dimorphic chloroplasts in *Hydrilla* analogous to Kranz-type NADP-ME species. Modeling a single-cell C_4 system such as *Hydrilla* with C_4 acid decarboxylation in the chloroplast shows that the low resistance normally expected of the chloroplast envelope to CO_2 ($1.25\ m^2\ s^{-1}\ mol^{-1}$) would result in a very limited CCM (68).

Another interesting, but mechanistically different, type of inducible CCM occurs in some species of *Eleocharis* (Cyperaceae), which perform C_3 photosynthesis when submerged and are induced to perform C_4 or C_3-C_4 intermediate photosynthesis when emerged (i.e., above the water surface). *Eleocharis* accomplishes C_4 photosynthesis, not through a single-cell system, but through development of Kranz-like anatomy and expression of C_4 enzymes in the emerged part of the plant (67).

C_4 photosynthesis in the genus *Orcuttia* is particularly interesting. *Orcuttia* species grow in seasonal pools formed by rain in California. They germinate and produce terete leaves when submerged, and then form laminate leaves when floating on the water and during continued growth as the pools dry up. Pulse-chase experiments with $^{14}CO_2$-$^{12}CO_2$ with *O. viscida* and *O. californica* indicate that these species perform C_4 photosynthesis without Kranz anatomy when growing under submerged conditions, and C_4 photosynthesis with Kranz anatomy when foliage is terrestrial (38–40). *O. viscida* is an NADP-ME species that has mesophyll-like cells when growing submerged, with chloroplasts in a centripetal position. The proposed mechanism of photosynthesis is like that of *Hydrilla*, with CO_2 fixation via PEPC in the cytosol, and donation of CO_2 from malate via chloroplastic NADP-ME to Rubisco. Interestingly, the location of chloroplasts in the centripetal position may provide liquid-phase diffusive resistance to CO_2, enabling it to be concentrated and fixed by Rubisco. According to this proposal, chloroplasts that develop in mesophyll-like chlorenchyma cells in submerged leaves may be functionally more like the bundle sheath chloroplasts in Kranz-type leaves that develop under terrestrial conditions. However, a CCM has not been established for photosynthesis in submerged *Orcuttia*, and the intracellular mechanism of photosynthesis needs to be elucidated through studies on enzyme compartmentation. Species of *Orcuttia* are rare and endangered owing to loss of habitat, whereas *Hydrilla* is a serious invasive species.

There is also substantial evidence for function of a CCM through a C_4 cycle in the marine macroalga *Udotea flabellum*, indicating C_4 type photosynthesis has evolved outside of the angiosperms (10). Finally, it is important to make the distinction that microalgae have CCMs, not through C_4 photosynthesis, but by other mechanisms. Although C_4 photosynthesis has been suggested in the marine diatom *Thalassiosira weissflogii* under low zinc nutrition and CO_2 (54), whether it functions as a CCM has been questioned (35).

CONCLUSIONS: FUTURE PROSPECTS

Much is known about terrestrial C_4 plants with Kranz anatomy, including their occurrence, significance, and mechanism of photosynthesis. Until recently, it was thought that all terrestrial C_4 plants have Kranz anatomy. With the discovery of terrestrial single-cell C_4 species, new questions have arisen. Are these species rare, or have single-cell systems for reducing photorespiration gone undetected until now? A broader search is needed among terrestrial plants for modifications in photosynthesis within a single cell that reduce photorespiration, whether by development of a C_4 cycle, or by refixation of photorespired CO_2 at sites in the cell distal to the entry of atmospheric CO_2. What are the advantages and disadvantages of the single-cell systems with respect to dual-cell C_4 photosynthetic systems? Is the single-cell system more efficient under certain environmental conditions, or does it impart more flexibility, such as allowing for C_3 photosynthesis under certain conditions?

Single-cell C_4 photosynthesis in terrestrial chenopods is of the NAD-ME type and occurs through compartmentation of structurally and biochemically dimorphic chloroplasts, and polarization of mitochondria containing NAD-ME and GDC. The dimorphic chloroplasts in the single cell serve the same functions as the dimorphic chloroplasts in Kranz-type NAD-ME C_4 species. Many facets of single-cell C_4 photosynthesis in terrestrial plants are yet to be elucidated. What are the mechanisms for development of spatial separation of functions, including ultrastructural and biochemical changes in the organelles, partitioning of organelles in different compartments within an individual cell, phased expression of certain C_4 photosynthesis genes, and targeting of the mRNA from these genes or the gene product to the correct compartments of the cell?

Other interesting mechanistic variations of single-cell C_4 may well be found through further targeted searches. For example, in the aquatic monocots *Hydrilla* and *Orcuttia*, single-cell C_4 photosynthesis is classified as NADP-ME type. In these plants, the functions of dimorphic chloroplasts found in terrestrial Kranz-type NADP-ME species, including PPDK in mesophyll chloroplasts and NADP-ME and Rubisco in bundle sheath chloroplasts, are proposed to be combined into one chloroplast type. This chloroplast would function to donate carbon from malate to Rubisco and to generate PEP, the substrate for cytosolic PEPC, through the combined actions of NADP-ME, Rubisco, and PPDK. Further biochemical and immunolocalization studies will be needed to confirm this proposed mechanism.

In any single-cell C_4 system, an important consideration is the diffusive resistance for CO_2 from sites of C_4 acid donation to Rubisco. How is this achieved, and is it sufficient to function as efficiently as Kranz-type C_4 plants? The effectiveness of concentrating CO_2 around Rubisco from donors of C_4 acids depends on the rate of CO_2 generation by C_4 acid decarboxylation (turnover of the C_4 cycle), diffusive resistance to leakage away from sites of decarboxylation, and the rate of carboxylation by Rubisco. If some single-cell C_4 species have a low diffusive resistance to CO_2 leakage, CO_2 could still be concentrated around Rubisco under special

circumstances, i.e., provided there is a high rate of C_4 acid decarboxylation and low Rubisco capacity. This might be advantageous by preventing photorespiration and increasing photosynthesis when the supply of external CO_2 to the leaf is very limited, despite an increased expense of C_4 pathway overcycling, which would be reflected in lower quantum yields and more negative carbon isotope values. In general, there are currently two types of single-cell C_4 systems. One is monomorphic (one chloroplast type that contains Rubisco, the C_3 cycle, and a C_4 acid decarboxylase), represented by the aquatic single-cell C_4 plants, and by current genetic engineering efforts to introduce C_4 photosynthesis into C_3 crops. The other is dimorphic (two chloroplast types with spatial compartmentation within the cell analogous to that in Kranz-type anatomy) as found in the single-cell C_4 chenopods. Modeling studies show that the monomorphic system has limited ability to concentrate CO_2 in the chloroplast and a high energetic cost when considering the low diffusive resistance that exists between the chloroplast and intercellular space as in C_3 plants. However, it could be valuable when intercellular levels of CO_2 are low (67a, 68), e.g., due to drought and low stomatal conductance in terrestrial plants, or high boundary–layer resistance in aquatic plants.

These systems, terrestrial and aquatic, demonstrate the remarkable plasticity inherent in the plant cell, and they provide unusual examples of the exquisite control of subcellular processes that can be exerted to accomplish very complicated biochemical functions. With interest in genetic engineering of important C_3 crop plants such as rice to perform C_4 photosynthesis (63), the invention by nature of single-cell C_4 photosynthesis provides evidence that this may be an alternative to engineering C_3 plants to perform Kranz-type C_4 photosynthesis without having to engineer a dual-cell system.

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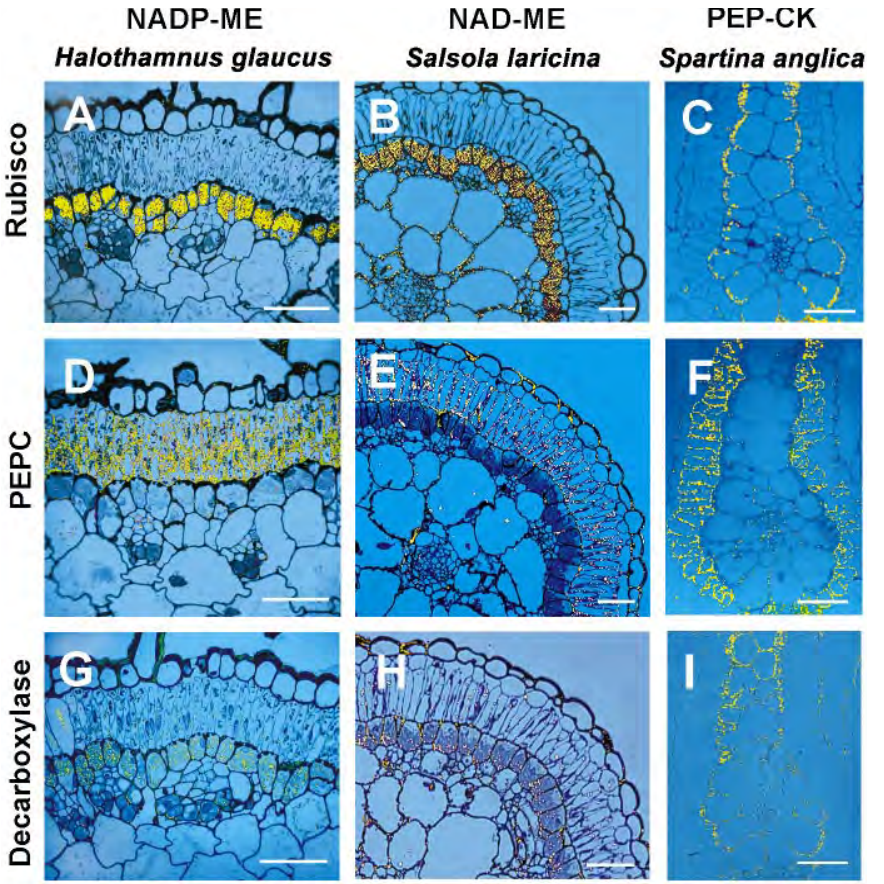

Figure 2 Illustrations of localization of three key enzymes of photosynthesis, Rubisco, PEPC, and C_4 acid decarboxylating enzymes, in representative Kranz-type C_4 plants: *Halothanmus glaucus*, NADP-ME type; *Salsola laricina*, NAD-ME type; and *Spartina anglica*, PEP-CK type. Bars = 50 μm.

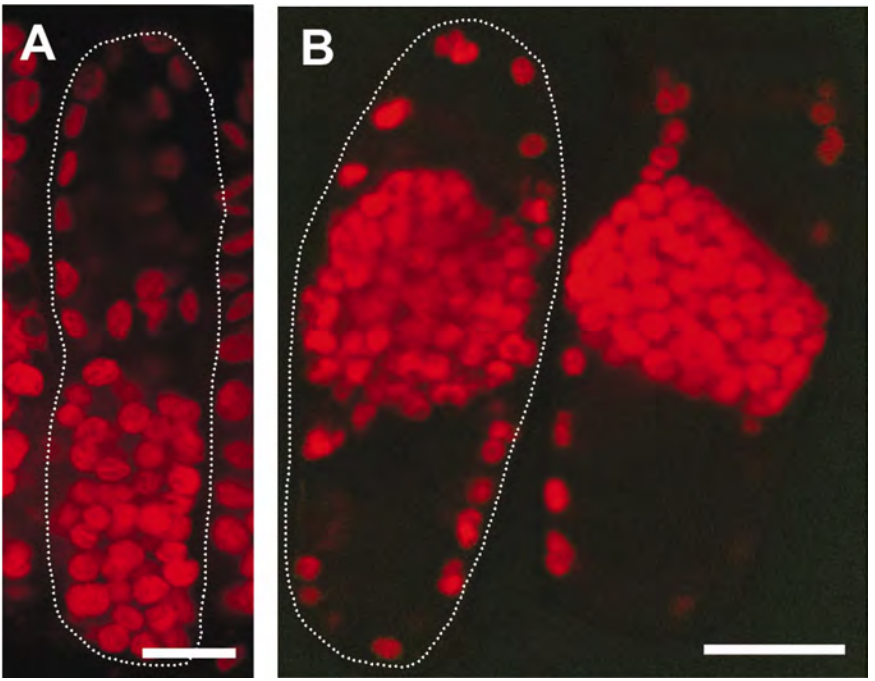

Figure 3 Confocal fluorescence of a chlorenchyma cell of *Borszczowia aralocaspica* (*A*) and *Bienertia cycloptera* (*B*) illustrating the chloroplasts in the two cytoplasmic compartments. The broken white lines show the outline of a single cell. Scale bars = 20 μm.

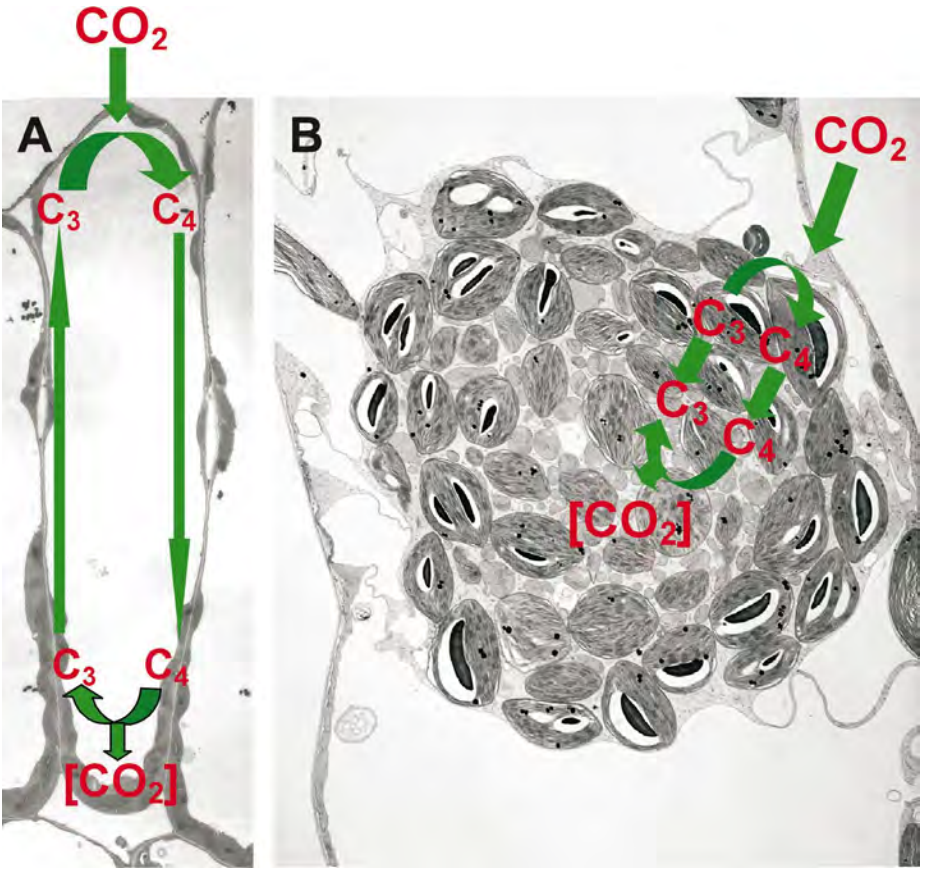

Figure 4 Electron microscopy of *Borszczowia aralocaspica* (*A*) and *Bienertia cycloptera* (*B*) with overlayed schemes of the C_4 cycle.

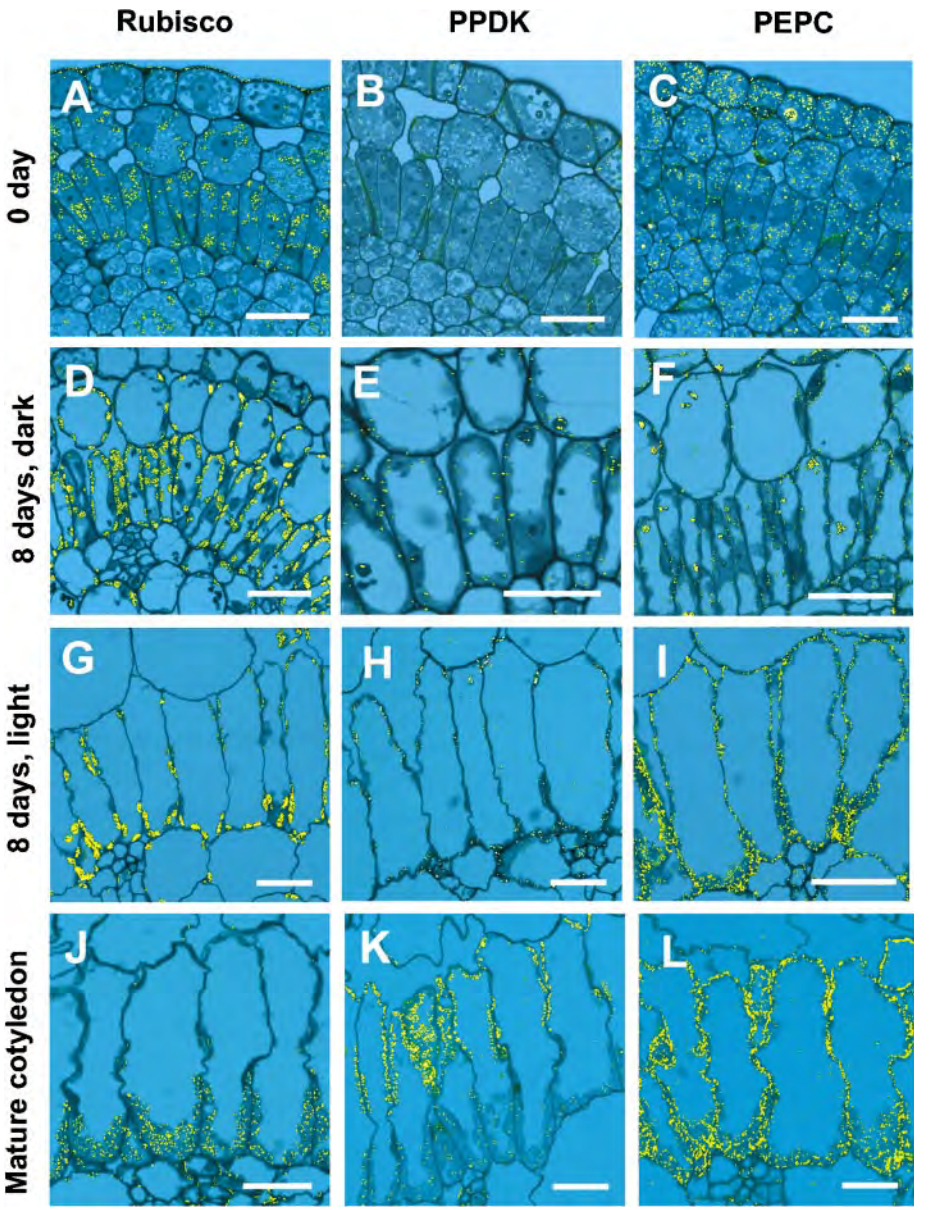

Figure 5 Reflected/transmitted confocal imaging of in situ immunolocalization of photosynthetic enzymes (Rubisco, PPDK, and PEPC) during developmental changes in chlorenchyma cells of *Borszczowia aralocaspica* cotyledons. Label appears as yellow dots. (*A–C*) Cotyledon, 0 d; (*A*) Rubisco; (*B*) PPDK; (*C*) PEPC; (*D–F*) Cotyledon, 8 d, dark; (*D*) Rubisco; (*E*) PPDK; (*F*) PEPC; (*G–I*) Cotyledon, 8 d, light; (*G*) Rubisco; (*H*) PPDK; (*I*) PEPC; (*J–L*) Mature cotyledon; (*J*) Rubisco; (*K*) PPDK; (*L*) PEPC. Scale bars = 20 μm. Figure 5 is reproduced with modifications, with permission from *Annals of Botany* (69a).

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