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Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis

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An important adaptation to CO_2-limited photosynthesis in cyanobacteria, algae and some plants was development of CO_2-concentrating mechanisms (CCM)[1]. Evolution of a CCM occurred many times in flowering plants, beginning at least 15–20 million years ago, in response to atmospheric CO_2 reduction, climate change, geological trends, and evolutionary diversification of species[2]. In plants, this is achieved through a biochemical inorganic carbon pump called C_4 photosynthesis, discovered 35 years ago[3]. C_4 photosynthesis is advantageous when limitations on carbon acquisition are imposed by high temperature, drought and saline conditions. It has been thought that a specialized leaf anatomy, composed of two, distinctive photosynthetic cell types (Kranz anatomy), is required for C_4 photosynthesis[4]. We provide evidence that C_4 photosynthesis can function within a single photosynthetic cell in terrestrial plants. *Borszczowia aralocaspica* (Chenopodiaceae) has the photosynthetic features of C_4 plants, yet lacks Kranz anatomy. This species accomplishes C_4 photosynthesis through spatial compartmentation of photosynthetic enzymes, and by separation of two types of chloroplasts and other organelles in distinct positions within the chlorenchyma cell cytoplasm.

CO_2-concentrating mechanisms (CCM) have evolved that increase the level of CO_2 at the site of fixation by the C_3 photosynthetic pathway via the enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco); these CCMs also negate the counterproductive oxygenase activity of Rubisco. Plants that lack a CCM directly fix atmospheric CO_2 in their photosynthetic cells via Rubisco. They are called C_3 plants[5] because the initial product of fixation is a three-carbon compound, and they show high rates of photorespiration owing to the oxygenase activity of Rubisco. CCM in terrestrial plants occur via a C_4 dicarboxylic acid pathway; thus they are called C_4 plants. C_4 plants actively take up CO_2 from the atmosphere and concentrate it around Rubisco for assimilation into organic matter. This requires spatial separation of fixation of atmospheric CO_2 (via phosphoenolpyruvate carboxylase) into C_4 acids, and donation of CO_2 from C_4 acids (via C_4 acid decarboxylases) to RuBP carboxylase of the C_3 pathway.

Photosynthesis has been thought to occur in all terrestrial C_4 plants by the cooperative function of two types of photosynthetic tissue: an inner layer called Kranz or bundle sheath cells, and an outer layer of palisade cells[4,6]. Whereas the CCM in terrestrial plants occur via a C_4 dicarboxylic acid pathway, cyanobacteria and algae employ different mechanisms[1]. Besides C_3 and C_4 plants, some vascular plants fix atmospheric CO_2 at night through a C_4 pathway and further process the carbon via the C_3 pathway during the day (called crassulacean acid metabolism or CAM)[7]. This results in a temporal separation of the process rather than a spatial separation, such as in Kranz anatomy, as occurs in C_4 plants. The process of photosynthesis in C_3 and CAM plants is achieved within a single photosynthetic cell, without Kranz anatomy.

Our evidence that Kranz anatomy is not essential for C_4 plant photosynthesis in terrestrial species is based on studies with the monotypic genus *Borszczowia*. *Borszczowia aralocaspica* Bunge (subfamily Salsoloideae, family Chenopodiaceae) grows in salty depressions of Central Asian semi-deserts. It is a succulent species with unusual chlorenchyma, and its carbon isotope composition is like that of C_4 or obligate CAM plants[8]. The Chenopodiaceae family has the largest number of C_4 species among dicotyledonous plants[4]; it has high diversity in evolution of C_4 photosynthesis, including five variants of Kranz anatomy[8,9] and two variants of C_4 biochemistry[10–12].

Figure 1 shows the leaf anatomy of *B. aralocaspica*, *Salsola laricina* and *Suaeda heterophylla*, all in subfamily Salsoloideae, family Chenopodiaceae. The general leaf anatomy of *B. aralocaspica* (Fig. 1a), looks similar to the C_4 salsoloid type as demonstrated by the C_4 plant *S. laricina* Pall (Fig. 1b)[10]. *S. laricina* has a central, main vein surrounded by water storage parenchyma, and Kranz anatomy with distinctive peripheral layers of palisade and Kranz cells. The Kranz cell chloroplasts have grana and accumulate starch, whereas the palisade chloroplasts have reduced grana and lack starch[10].

However, in contrast, *B. aralocaspica* has only a single layer of unusual palisade-shaped chlorenchyma cells, which are located between the central water storage tissue and the hypodermal cells (Fig. 1a, also see ref. 8). These radially elongated chlorenchyma cells have a large, central vacuole and a layer of peripheral cytoplasm with few chloroplasts in the distal (from the vascular bundle) part of the cell and a high density of cytoplasm with numerous chloroplasts and large mitochondria (the latter observed by electron microscopy) in the proximal position (see arrows in Fig. 1a). Chloroplasts

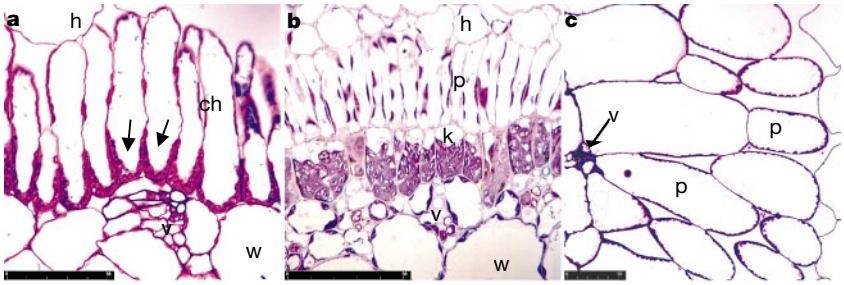

Figure 1 Light microscopy of leaves (transverse sections). **a**, *Borszczowia aralocaspica*; **b**, *Salsola laricina*; and **c**, *Suaeda heterophylla*. ch, chlorenchyma cell; h, hypodermal cell; k, Kranz cell; p, palisade cell; v, vascular tissue; w, water storage cell. Scale bars, 50 μm.

in the distal part of the cell lack grana, and are without starch, while those in the proximal part have grana and contain starch (Fig. 1a, also see ref. 8; observations on degree of grana formation were made by electron microscopy, not shown). There are intercellular air spaces between the distal parts, but not the proximal parts, of the chlorenchyma cells.

Thus, this plant has leaf anatomy similar to C_4 *Salsola*, with one notable exception: it has dimorphic chloroplasts in a single photosynthetic cell instead of in two cell types with Kranz anatomy. For comparison, Fig. 1c shows the leaf anatomy of *Su. heterophylla* (Kar. & Kir.) Bunge, a C_3 species that is also in subfamily Salsoloideae. It lacks Kranz anatomy and has two to three layers of large, palisade-like mesophyll cells with a large central vacuole and a thin layer of cytoplasm with chloroplasts along the cell periphery, which is common for C_3 photosynthetic cells.

The unique cytological features of *B. aralocaspica* suggest that its photosynthetic cells may be biochemically compartmentalized for carbon assimilation, and represent a new photosynthesis mechanism. We have tested this hypothesis with a number of corroborative techniques. Using immunolocalization techniques, we have demonstrated the compartmentation of important photosynthetic enzymes in the chlorenchyma cells. Rubisco is concentrated in chloroplasts in the proximal part of the cell (Fig. 2a and c). With respect to enzymes of the C_4 pathway, PEP carboxylase is abundant

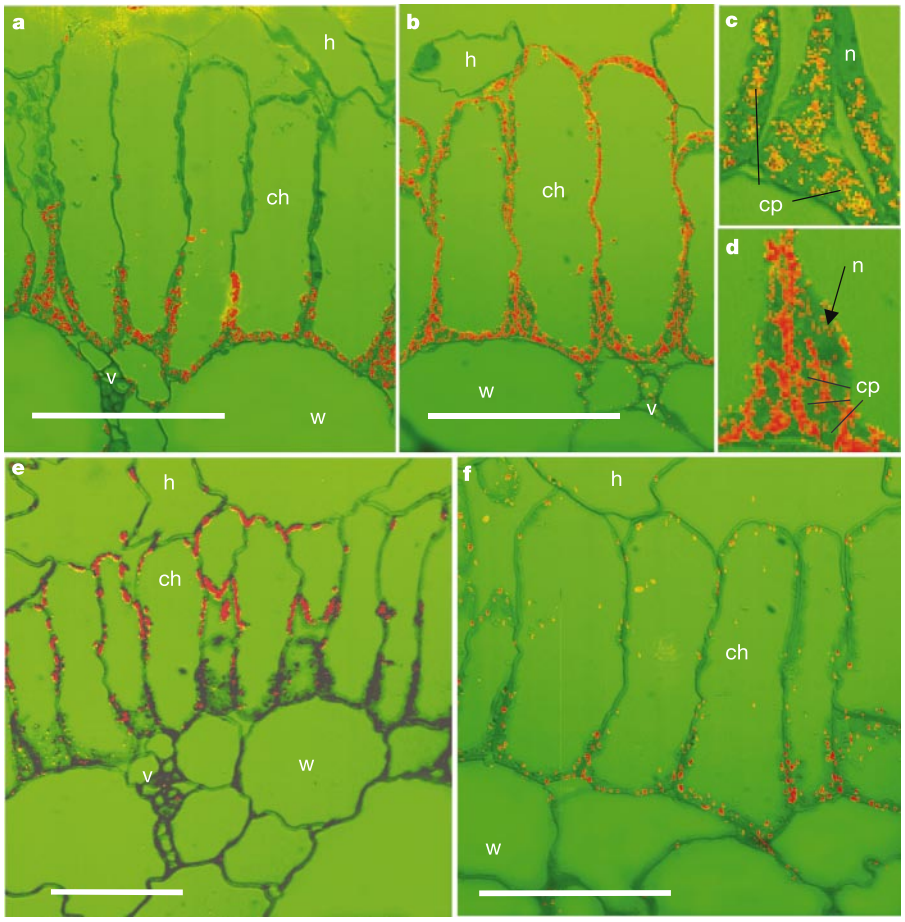

Figure 2 Immunolocalization of photosynthetic enzymes in leaves of *Borszczowia aralocaspica* by confocal laser scanning microscopy. Immunolocalization of Rubisco (**a**), PEP carboxylase (**b**), higher magnification showing Rubisco in chloroplasts in proximal end of cell (**c**), higher magnification showing PEP carboxylase in cytosol (**d**), pyruvate,Pi dikinase (**e**) and NAD-malic enzyme (**f**). Red dots indicate where the enzyme is present. cp, chloroplast; ch, chlorenchyma cell; h, hypodermal cell; n, nucleus; v, vascular tissue; w, water storage cell. Scale bars, 50 μm.

Table 1 Activities of photosynthetic enzymes

Species	Enzyme* activity (μmol mg chlorophyll^{-1} min^{-1})				
	Rubisco	PPDK	PEPC	NAD-ME	NADP-ME
Suaeda heterophylla (C_3)	7.07 ± 0.33	0.25	0.3 ± 0.03	2.80 ± 0.45	n.d.
Salsola laricina (C_4)	1.13	10.33	20.5	12.7 ± 3.1	0.39 ± 0.12
Borszczowia aralocaspica	2.17 ± 0.15	8.52 ± 0.42	12.8 ± 0.5	3.76 ± 0.45	2.42 ± 0.53

* PPDK, pyruvate,Pi dikinase; PEPC, PEP carboxylase; NAD-ME, NAD-malic enzyme; NADP-ME; NADP-malic enzyme.
Temperature of assay was 25 °C. n.d., not detected. For methods for assay of enzymes see refs 12 and 24. Standard errors where shown were for two independent replications.

throughout the cytosol (Fig. 2b and d), and pyruvate,Pi dikinase (PPDK) is located in chloroplasts in the distal part of the cell (Fig. 2e), whereas NAD-ME (NAD-malic enzyme) is located proximally in the cell (Fig. 2f) in mitochondria (immunogold labelling by electron microscopy, not shown). Water storage and hypodermal cells have a few, small chloroplasts that show labelling for Rubisco, but not for enzymes of C_4 photosynthesis. This indicates the succulent water storage cells are not performing CAM[7].

Borszczowia aralocaspica has activities of photosynthetic enzymes that are characteristic of C_4 plants[13], as seen by comparison with the C_4 *S. laricina* (NAD-ME subtype) (Table 1). The activities of enzymes of the C_4 cycle, PPDK, PEP carboxylase, and malic enzyme, are high and more than sufficient to support the measured rates of CO_2 assimilation (Fig. 3). As we already noted, the immunolocalization results show that NAD-ME is located proximally in the mitochondria in photosynthetic cells in *B. aralocaspica*.

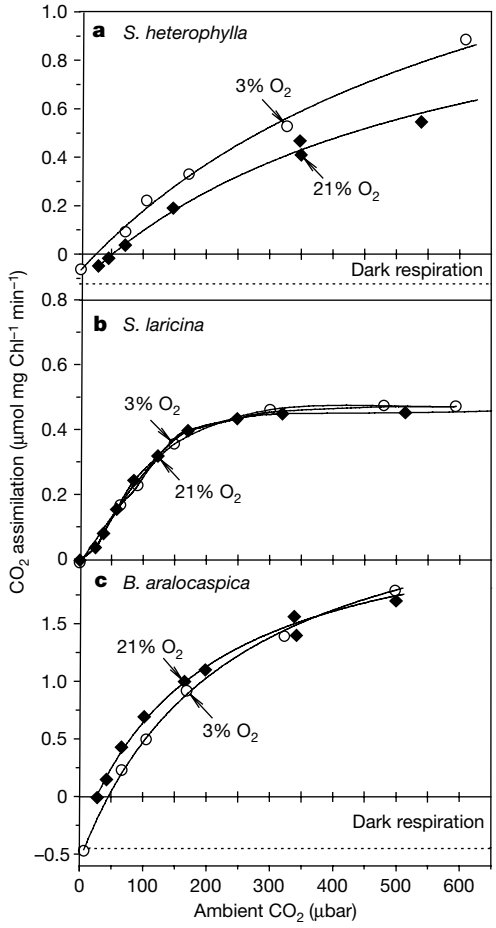

Figure 3 The response of photosynthesis to varying atmospheric levels of CO_2. **a**, *Suaeda heterophylla* (C_3); **b**, *Salsola laricina* (C_4); and **c**, *Borszczowia aralocaspica*. The rates of photosynthesis were measured as described[26]. Conditions were 25 °C, a light intensity of 1,200 μmol quanta m^{-2} s^{-1}, and varying levels of CO_2 (C_a) and O_2, as shown.

The mitochondrial NAD-ME may react with both NAD^+ and (to a lesser extent) $NADP^+$ as substrate (Table 1), because no NADP-ME protein was detected on western blots or by immunolocalization (using antibody of NADP-ME from maize). In comparison, *Su. heterophylla* has activities of Rubisco, PEP carboxylase, PPDK and NADP-ME characteristic of C_3 species. It has significant activity of NAD-ME, a constitutive mitochondrial enzyme that is readily detected in C_3 tissue[12,14].

The carbon isotope composition ($\delta^{13}C$) of the three species is given in Table 2. This diagnostic feature can be used to distinguish types of photosynthetic mechanisms based on the carboxylation enzymes operating. C_3 plants have a more negative value of $\delta^{13}C$ because atmospheric CO_2 is fixed by Rubisco, which discriminates against $^{13}CO_2$. C_4 plants have a more positive value because atmospheric CO_2 is fixed by PEP carboxylase, which does not discriminate against $^{13}CO_2$. The CO_2, which is subsequently donated to Rubisco, is compartmentalized and largely fixed with minimal leakage[15]. The high $\delta^{13}C$ value for *S. laricina* is typical of that of C_4 plants, while the low value for *Su. heterophylla* is typical of that for C_3 plants. Of significance to the hypothesis of operation of a single-cell C_4 mechanism, *B. aralocaspica* has $\delta^{13}C$ values that are clearly of C_4 origin.

Rubisco is a bifunctional enzyme, where CO_2 and O_2 are competitive substrates for reaction with RuBP[16]. Whereas reaction of RuBP with CO_2 results in carbon assimilation, reaction with O_2 results in photorespiration. This is an apparently counterproductive process that is a significant component of photosynthetic activity in C_3 plants, but not in C_4 plants, owing to the localized CO_2-concentrating effect of the C_4 mechanism. Thus, the response of photosynthesis to varying CO_2 and O_2 is also diagnostic for the C_3 versus C_4 mechanism of carbon assimilation. Ambient levels of O_2 (21%) inhibit photosynthesis in *Su. heterophylla* at varying levels of CO_2, a characteristic of C_3 plant photosynthesis, whereas photosynthesis in *S. laricina* is insensitive to O_2, as is typical of C_4 plants (Fig. 3a, b). The response of *B. aralocaspica* is also like that of C_4 plants, in that O_2 does not inhibit photosynthesis (Fig. 3c); in fact, there is some stimulation by O_2 under limiting CO_2, which has been previously observed in some C_4 species[17]. Even at the CO_2 compensation point, where there is no net CO_2 fixation, O_2 does not affect photosynthesis. This suggests that photorespiration is restricted, and that, at this point, the rate of CO_2 fixation by Rubisco equals the rate of dark respiration. *B. aralocaspica* also has substantial rates of respiration in the dark, ruling out the possibility that its growth occurs through fixation of atmospheric CO_2 in the dark through PEP carboxylase via crassulacean acid metabolism (CAM).

Table 2 Comparison of carbon isotope composition ($\delta^{13}C$) of leaf tissue

Species	Carbon isotope value ($\delta^{13}C$)
Suaeda heterophylla (C_3)	–25.34*, –27.28†
Salsola laricina (C_4)	–14.80‡
Borszczowia aralocaspica	–12.37*, –13.78§

The $\delta^{13}C$ isotope values were determined as described[25].
* Original data on leaves from plant material used in current study.
† Original data from leaves kindly provided by W. Stichler, average of two replications.
‡ Data from ref. 12.
§ Data from ref. 8.

Thus our results prove that C_4 photosynthesis exists in a terrestrial plant without the dual-cell Kranz anatomy system. C_4 photosynthesis is accomplished within a single cell by novel cytological features that allow spatial separation of the biochemical events necessary for operation of the C_4 mechanism. Until now, the separation of functions in terrestrial C_4 plants has been associated only with Kranz-type leaf anatomy. *B. aralocaspica* has evolved a unique solution for the requirement of spatial separation of these biochemical functions within a single cell. PPDK is positioned at the distal part of the cell, where it can generate PEP, the substrate for PEP carboxylase. PEP carboxylase fixes atmospheric CO_2 supplied to the cell through the adjoining intercellular air spaces. NAD-ME and Rubisco are compartmentalized to the interior of the cells, where CO_2 can be donated from C_4 acids to the C_3 pathway. The C_4-type $\delta^{13}C$ values and lack of inhibition of photosynthesis by O_2 demonstrate that CO_2 can be concentrated sufficiently around Rubisco through this specialized compartmentation to minimize photorespiration. The physical requirements for C_4 photosynthesis may be met by the existence of a sufficiently high diffusive resistance in the aqueous phase between sites of CO_2 donation to Rubisco in the proximal ends of the cells and sites of fixation of atmospheric CO_2 by PEP carboxylase at the distal ends.

Our results are relevant to the discussion of evolution of C_4 photosynthesis in plants, because the first land plants were C_3 species[2,4]. C_4 species are an important component of global ecosystems and there is interest in their evolution and the consequences to evolution of mammals[2,4,18,19]. Palaeorecords have been used to study how long C_4 plants have existed on Earth by finding well preserved fossils that have Kranz anatomy and C_4-type isotope composition. Now our results indicate that it is possible that plant fossils with a C_4 isotope composition but without Kranz anatomy may be C_4 species (rather than CAM species).

C_4 plants are also of considerable interest because this mechanism of photosynthesis has an advantage over C_3 plants for conversion of solar energy into biomass in hot, dry and/or saline habitats. Maize, sugarcane and sorghum are important C_4 crop plants, but most agricultural crops, including rice and wheat, are C_3 plants. This has led to interest in genetically engineering C_3 crops to perform C_4 photosynthesis in order to increase productivity[20,21]. Although this may require alterations in anatomy as well as biochemistry, our results indicate that it would not require development of two photosynthetic cell types. □

Methods

Plants were grown under controlled growth conditions with day/night temperatures of 25/18 °C, and a 14/10 h photoperiod, with a stepwise increase and decrease in light intensity during the day to a maximum photosynthetic quantum flux density of 1,100 $\mu mol\ m^{-2}\ s^{-1}$. For immunolocalization studies, samples were prepared as described[10]. Antibodies used were anti-spinach Rubisco (LSU) IgG (courtesy of B. McFadden), anti-maize PPDK IgG (courtesy of T. Sugiyama), anti-maize PEPC IgG (Chemicon), anti-maize NADP-ME IgG with relative molecular mass (M_r) 62,000 (courtesy of C. Andreo[22]), and anti-*Amaranthus hypochondriacus* mitochondrial NAD-ME IgG, which was prepared against the α subunit with M_r65,000 (courtesy of J. Berry[23]). For details of techniques used for immunolocalization by light microscopy see ref. 10. The background labelling with preimmune serum was non-specific and low to nonexistent (results not shown but see ref. 10).

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Interactive memory systems in the human brain

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Learning and memory in humans rely upon several memory systems, which appear to have dissociable brain substrates[1,2]. A fundamental question concerns whether, and how, these memory systems interact. Here we show using functional magnetic resonance imaging (FMRI) that these memory systems may compete with each other during classification learning in humans. The medial temporal lobe and basal ganglia were differently engaged across subjects during classification learning depending upon whether the task emphasized declarative or nondeclarative memory, even when the to-be-learned material and the level of performance did not differ. Consistent with competition between