Proof of C₄ photosynthesis without Kranz anatomy in Bienertia cycloptera (Chenopodiaceae)

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Summary

Kranz anatomy, with its separation of elements of the C₄ pathway between two cells, has been an accepted criterion for function of C₄ photosynthesis in terrestrial plants. However, Bienertia cycloptera (Chenopodiaceae), which grows in salty depressions of Central Asian semi-deserts, has unusual chlorenchyma, lacks Kranz anatomy, but has photosynthetic features of C₄ plants. Its photosynthetic response to varying CO₂ and O₂ is typical of C₄ plants having Kranz anatomy. Lack of night-time CO₂ fixation indicates it is not acquiring carbon by Crassulacean acid metabolism. This species exhibits an independent, novel solution to function of the C₄ mechanism through spatial compartmentation of dimorphic chloroplasts, other organelles and photosynthetic enzymes in distinct positions within a single chlorenchyma cell. The chlorenchyma cells have a large, spherical central cytoplasmic compartment interconnected by cytoplasmic channels through the vacuole to the peripheral cytoplasm. This compartment is filled with mitochondria and granal chloroplasts, while the peripheral cytoplasm apparently lacks mitochondria and has grana-deficient chloroplasts. Immunolocalization studies show enzymes compartmentalized selectively in the CC compartment, including Rubisco in chloroplasts, and NAD-malic enzyme and glycine decarboxylase in mitochondria, whereas pyruvate, Pi dikinase of the C₄ cycle is localized selectively in peripheral chloroplasts. Phosphoenolpyruvate carboxylase, a cytosolic C₄ cycle enzyme, is enriched in the peripheral cytoplasm. Our results show Bienertia utilizes strict compartmentation of organelles and enzymes within a single cell to effectively mimic the spatial separation of Kranz anatomy, allowing it to function as a C₄ plant having suppressed photorespiration; this raises interesting questions about evolution of C₄ mechanisms.

Keywords: C₄ plants, C₄ plant anatomy, photosynthetic enzymes, carbon isotope composition, immunolocalization.

Introduction

Beginning in the early 1970s, it was recognized that Kranz anatomy is one of the major distinguishing features of plants with C₄ photosynthesis (Hatch et al., 1971). Spatial separation between the fixation of atmospheric CO₂ by phosphoenolpyruvate carboxylase (PEPC), and donation of CO₂ by decarboxylation of C₄ acids to Rubisco, is required; this has been demonstrated to occur in many terrestrial C₄ plants through co-operative function of two biochemically and ultrastructurally distinct photosynthetic cells, i.e. Kranz anatomy (Edwards et al., 2001a,b).

The family Chenopodiaceae, which has the most C₄ species among any of the dicot families (Sage and Monson, 1999), provides a valuable source of species to explore the variation and evolution of C₄ anatomy and biochemical mechanisms in plants. Four variants of Kranz anatomy were earlier described in this family, namely Atriplicoid, Kochioid, Salsoloid, and Kranz-Suaedoid types.
(Carolin et al., 1975), and, recently, another type was described in the genus *Suaeda*, named Conospermoid (Freitag and Stichler, 2000). There are also two basic C₄ subtypes in Chenopodiaceae that are characterized by differences in biochemistry of the C₄ pathway and the ultrastructure of chloroplasts and mitochondria. One
subtype has NADP-malic enzyme (NADP-ME) as the primary decarboxylase, with palisade chloroplasts having high grana development and Kranz cell chloroplasts having reduced grana. The other subtype has NAD-malic enzyme (NAD-ME) as the primary decarboxylase, with palisade chloroplasts having reduced grana development and Kranz cell chloroplasts having well-developed grana (Pyankov et al., 2000; Voznesenskaya and Gamaley, 1986; Voznesenskaya et al., 1999). In the tribes Salsoleae and Suaeaeae, there are many species that have been classified either as $C_4$ with Salsoloid, Suaeaeoid or Conospermoid-type Kranz anatomy, or as $C_3$ plants, which lack Kranz anatomy. Leaves of species in this subfamily are often succulent, with water storage tissue containing few chloroplasts surrounded by the main photosynthetic tissue. Thus, there has been great diversity in evolution of $C_4$ photosynthesis with respect to Kranz anatomy within Chenopodiaceae.

More diversity in structure/function of $C_4$ photosynthesis in family Chenopodiaceae has been discovered in two species of tribe Suaeaeae. In Borszczowia aralocaspica, an unusual leaf anatomy was described as 'Borszczowiioid type'; this species was suspected to carry out $C_4$ photosynthesis in a single chlorenchyma cell (Freitag and Stichler, 2000). Later, it was shown to function as a $C_4$ plant (Voznesenskaya et al., 2001b). Another Chenopodiaceae species, Bienertia cycloptera, was reported earlier to have $C_4$ type carbon isotope composition in surveys of species from arid regions in Asia (Akhani et al., 1997; Winter, 1981). Based on current knowledge of photosynthetic pathways in terrestrial plants, this suggests it is fixing atmospheric CO$_2$ via PEPC, either as a $C_4$ or CAM plant. However, Glagoleva et al. (1992) suggested it was a $C_3$ species based on lower labelling in $C_4$ acids than in other $C_4$ species from analysis of $^{13}$CO$_2$ fixation. Sage et al. (1999) noted that this raises questions about its mechanism of photosynthesis and that diagnosis by carbon isotope composition can be problematic. Recently, Freitag and Stichler (2002) identified a new type of anatomy in Bienertia, which they named 'Bienertoid', and suggested it functions as a $C_4$, facultative $C_4/C_3$ species. In light microscopy studies they showed it lacks Kranz anatomy, but instead the chlorenchyma cells exhibit a unique separation of cytoplasm into a globular, starch-containing central compartment, and a peripheral compartment without starch. They also obtained $C_4$/CAM type carbon isotope composition in plants from natural habitats, and suggested the peripheral and central cytoplasmic compartments may be functionally equivalent in photosynthesis to palisade and Kranz cells in Kranz-type $C_4$ plants. In this study, we have used a number of physiological, biochemical and cytological methods to determine the mechanism of photosynthesis in this species. We demonstrate that Bienertia has evolved a novel mechanism to carry out $C_4$ photosynthesis without Kranz anatomy.

Results

Anatomy and ultrastructure

Succulent leaves of Bienertia (Figure 1a) have 1–3 layers of chlorenchyma under the epidermis, and internal to the chlorenchyma are very large, roundish cells with characteristics of water storage tissue. These latter cells have only a few chloroplasts. The chlorenchyma consists of short, semicylindrical cells with extensively developed intercellular spaces between them (Figure 1a,b). These cells have a thin, peripheral cytoplasm layer and a large spherical cytoplasmic compartment in the centre of the cell. [Figures 1 and 2a; also see Freitag and Stichler (2002)]. This central cytoplasmic compartment (CC compartment) is interconnected with peripheral cytoplasm by cytoplasmic channels traversing the vacuole (Figure 1c-f). These cytoplasmic channels radiate mainly between the peripheral cytoplasm and the CC compartment along the medial transverse plane of the chlorenchyma cell.

The CC compartment contains numerous chloroplasts that have starch [Figures 1g and 2a,b, also see Freitag and Stichler (2002) and well-developed grana (Figure 2b)]. Many large mitochondria are present (Figure 2b), and the internal membrane structure of these mitochondria consists of an extensive system of tubules and lamellae. They are concentrated mainly in the central part of the compartment and are encircled by the chloroplasts (Figure 2b). Rather large microbodies are also located in the CC compartment (Figure 2b). In contrast to CC compartment chloroplasts, peripheral cytoplasm chloroplasts are nearly agranal and lack starch, and their internal structure consists.

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**Figure 1.** Light micrographs of Bienertia cycloptera leaf and chlorenchyma structure.

(a) Part of a leaf showing chlorenchyma (cl) directly beneath the epidermis (e) surrounding the water storage tissue (ws) and vascular bundles (vb) in the middle of the leaf.

(b) Enlarged view of chlorenchyma showing ball of cytoplasm (central cytoplasmic compartment, CCC), which contains chloroplasts and mitochondria, in the middle of the cell. Some chloroplasts also occur in the periphery of the cell (pcp).

(c-f) Sections through the CCC and its periphery showing the network of cytoplasmic channels (arrows) that connect it to the peripheral cytoplasm. Note nucleus (n) in (e).

(g) PAS staining for polysaccharides showing that starch grains (sg) are restricted to the chloroplasts in the CCC (arrows). Bars: 100 μm in (a), 10 μm in (b-g).

of stromal thylakoids with very few grana (Figure 2c). No mitochondria were observed in the peripheral cytoplasm.

Quantitative analysis of the structural characteristics of CC compartment and peripheral cytoplasm chloroplasts also showed rather clear differences (Table 1). In the CC compartment, the granal index (24%) is 1.5 times higher, and the ratio of appressed to nonappressed thylakoids is 1.75 times higher, than that of the chloroplasts in the peripheral cytoplasm. The density of appressed thylakoids is also 1.7 times higher in chloroplasts in the peripheral cytoplasm, while the density of nonappressed thylakoids is similar in the two types of chloroplasts. The main

difference between these peripheral and centrally located chloroplasts is in the size of grana stacks. Most of the grana in peripheral chloroplasts are represented by two appressed thylakoids, with only a few grana having up to 6–7 (or, rarely, more) thylakoids. In centrally located chloroplasts, most grana consist of 3–5 thylakoids, but in some cases up to 8–9, and sometimes more.

A few chloroplasts occur within the interconnecting cytoplasmic channels close to the peripheral cytoplasm. These chloroplasts have a development of grana intermediate between that of the central and peripheral chloroplasts (Table 1).

In situ immunolocalization

*Bienertia* represents a unique case not only in the structure of its chlorenchyma, but also in the distribution of photosynthetic enzymes in these cells (Figure 3). Rubisco is essentially confined to chloroplasts in the CC compartment, as shown by immunocytochemical labelling (Figure 3a). Only some peripheral chloroplasts show a weak immunological response for Rubisco. In contrast, the labelling for pyruvate Pi dikinase (PPDK) is more intensive in peripheral chloroplasts (Figure 3f). The labelling for PEPC shows that it is distributed in the cytosol of both the peripheral cytoplasm and the CC compartment (Figures 3b and 4a,b). However, the most intensive labelling of PEPC occurs in the peripheral cytoplasm. Immunolabelling of NAD-ME occurs in the CC compartment (Figure 3c), whereas there is no labelling for NADP-ME in these cells (Figure 3d). Glycine decarboxylase was also labelled more intensively in the middle part of the CC compartment (Figure 3e). Electron microscopy level immunocytochemistry shows that both NAD-ME and glycine decarboxylase are localized to the mitochondria of this central domain of the cell (Figure 4c,d).

**Enzyme activity and Western blots**

Activities of Rubisco and several enzymes of C₄ photosynthesis in *Bienertia* and, for comparison, the C₃ species *Salsola laricina* and the C₃ species *Suaeda heterophylla* are shown in Table 2. *Bienertia* has activities of PPDK, PEPC, and NAD-ME, enzymes of the C₄ cycle, which are characteristic of C₄ plants, including the C₄ species *S. laricina* of the same subfamily. The C₃ species *S. heterophylla* has low activities of these enzymes, which is typical of C₃ plants. Also, in *Bienertia* and *S. laricina*, the activity of PEPC is much greater than that of Rubisco, which is typical of C₄ species, while the C₃ *S. heterophylla* has high Rubisco activity and very low PEPC activity. *Bienertia* has high NAD-ME activity and low NADP-ME activity.

The results of immunoblotting studies for C₄ pathway enzymes and Rubisco in *Bienertia* compared to the C₃ species *S. heterophylla*, and the C₄ species *S. laricina*, are shown in Figure 5. The immunoblots for Rubisco showed significant reactive protein bands in all three species. *Bienertia* showed high immunoreactivity with antibodies to the C₄ enzymes PEPC, PPDK, and NAD-ME, similar to that with *S. laricina*. *S. laricina* is an NAD-ME type C₄ species; previous studies showed the higher molecular mass band of NAD-ME is the major isoform in this species (Pyankov et al., 2000). In the C₃ *S. heterophylla*, no reactive band was detected for PPDK, very low reactivity for PEPC and low reactivity for NAD-ME. There was no reactive protein band with NADP-ME antibody for any of the species.

**Carbon isotope composition (δ¹³C)**

The carbon isotope values of mature leaves and stems of *Bienertia* under our growth conditions were ~13.5 and ~14.7‰, respectively. The younger leaves and stems had slightly more negative values. The isotope value in the mature tissue was similar to that reported for plants collected from natural habitats. The values of the C₃ species *S. heterophylla* and the C₄ species *S. laricina* are typical for values of C₃ and C₄ plants, respectively, and are shown for comparison (Table 3).

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Table 1 Structural features of chloroplasts in the central cytoplasmic compartment, in the peripheral cytoplasm and in the cytoplasmic channels interconnecting the compartments in *Bienertia cycloptera*. The standard error was from 5 to 10%.

<table>
<thead>
<tr>
<th>Position of chloroplasts</th>
<th>Granal index (b %)</th>
<th>Appressed thylakoid density (a)</th>
<th>Non-appressed thylakoid density (a)</th>
<th>Total thylakoid density (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC compartment</td>
<td>36</td>
<td>0.56</td>
<td>7.03</td>
<td>12.80</td>
</tr>
<tr>
<td>Peripheral cytoplasm</td>
<td>24</td>
<td>0.32</td>
<td>4.20</td>
<td>13.41</td>
</tr>
<tr>
<td>Cytoplasmic channels</td>
<td>27</td>
<td>0.37</td>
<td>6.38</td>
<td>17.37</td>
</tr>
</tbody>
</table>

*CC Compartment: central cytoplasmic compartment. Granal index — the length of all appressed thylakoid membranes as a percentage of the total length of all thylakoid membranes in a chloroplast. Thylakoid density in this case means the length of thylakoid membranes (in μm) per 1 μm² of chloroplast stroma area (analysed for the total area of the chloroplast, excluding starch grains).*
Figure 4. Immunolocalization of photosynthesis-related enzymes in *Bienertia cycloptera* chlorenchyma cells as seen at the TEM level shows organelle or cytosol-specific labelling. Black dots are gold particles indicating antibody binding.
(a) PEP carboxylase is present in the cytosol of the central cytoplasmic compartment (CCC).
(b) PEP carboxylase is present in cytosol of peripheral cytoplasm.
(c) NAD-malic enzyme is associated with the large mitochondria in the CCC. Note the abundant cristae in the mitochondria.
(d) Glycine decarboxylase is present in the mitochondria of the CCC. Bars: 0.5 µm.

Figure 3. Immunolocalization of photosynthesis-related enzymes in *Bienertia cycloptera* chlorenchyma cells. Transmitted-reflected images of immunogold labelled sections, with label appearing as orange deposits.
(a) Rubisco is selectively localized in the chloroplasts of the central cytoplasmic compartment (CCC).
(b) PEP carboxylase is enriched in the peripheral cytoplasm, with lower amounts in the central cytoplasmic compartment.
(c) NAD-malic enzyme is localized in the central cytoplasmic compartment.
(d) NADP-malic enzyme is not present in this species.
(e) Glycine decarboxylase is also concentrated in the CCC.
(f) Pyruvate, Pi dikinase is high in peripheral chloroplasts and low in the chloroplasts of the CCC. Bars: 10 µm.

During steady-state photosynthesis in the light, a pulse of $^{14}$CO$_2$ was given and the initial products determined. After a 3-sec exposure of *Bienertia* leaves to $^{14}$CO$_2$, about 50% of the initial products were in the C$_4$ acids malate and aspartate, and 13% in 3-phosphoglycerate (PGA). In the C$_4$ plant *S. laricina*, after an 8-sec exposure to $^{14}$CO$_2$ about 90% of the label was in C$_4$ acids and only 4% in PGA, while in *S. heterophylla*, after an 8-sec exposure less than 10% of the label was in C$_4$ acids, and 26% of the label was in PGA (Table 4).

### Table 2 Activities of several photosynthetic enzymes in *Bienertia cycloptera*, tribe Suaedeae compared to the C$_4$ species *Salsola laricina*, tribe Salsoleae and the C$_3$ species *Suaeda heterophylla*, tribe Suaedeae. For data with standard errors $n = 3$, other data are from one measurement

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme activity, $\mu$mol mg chlorophyll$^{-1}$ min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rubisco</td>
</tr>
<tr>
<td><em>Bienertia cycloptera</em></td>
<td>4.3 ± 0.55</td>
</tr>
<tr>
<td><em>Salsola laricina</em> (C$_4$)*</td>
<td>1.13</td>
</tr>
<tr>
<td><em>Suaeda heterophylla</em> (C$_3$)*</td>
<td>7.07 ± 0.33</td>
</tr>
</tbody>
</table>

ND, not detected.*Data from (Voznesenskaya et al., 2001b).

**Response of photosynthesis to varying CO$_2$**

The response of photosynthesis in *Bienertia* to varying levels of CO$_2$, with 21% versus 3% O$_2$, is like that of C$_4$ plants (Figure 6). Ambient O$_2$ does not inhibit photosynthesis even at low CO$_2$ concentrations. Rather, the rates at limiting CO$_2$ are slightly stimulated by O$_2$. Even at the CO$_2$ compensation point (1'), where there is no net fixation of CO$_2$, there was no difference between 21% and 3% O$_2$. However, there is a substantial rate of respiration in the dark, which is similar to the rate of respiration in the light in the absence of CO$_2$. Since photosynthesis was measured on a branch, stem tissue, as well as leaves contributed to respiration. This accounts for the relative high 1' of 40–50 μbar, which is insensitive to O$_2$ between 21 and 3% O$_2$. The rate of photosynthesis was near saturating at atmospheric levels of CO$_2$ (approximately 350 μbar).

**Discussion**

It is well established that plants that have Kranz leaf anatomy function as C$_4$ species. In these plants, the chlorenchyma is composed of two biochemically and anatomically distinct photosynthetic cell types: an outer layer of palisade mesophyll cells and an inner layer called bundle sheath or Kranz cells, see Sage and Monson (1999). From extensive studies of the biochemistry of C$_4$ photosynthesis and the inter- and intra-cellular compartmentation of the main photosynthetic enzymes [see Edwards and Walker (1983), Hatch (1987), Edwards et al. (2001a)], it was determined that, in the C$_4$ pathway, atmospheric CO$_2$ is fixed by PEPC, which is localized in the cytosol of mesophyll cells, and the product, oxaloacetate, is converted to the C$_4$ products, aspartate or malate. Aspartate and malate are transported to the adjacent bundle sheath (Kranz) cells via plasmodesmata, where these C$_4$ products are decarboxylated-regenerating CO$_2$. The CO$_2$ is then fixed by Rubisco, which is restricted to the bundle sheath cells. C$_4$ plants are characterized by a set of features that include spatial compartmentation of reactions in the C$_4$ and C$_3$ cycles, high activities and quantities of C$_4$ cycle enzymes.
enzymes, C_{4}-type carbon isotope composition, daytime uptake of CO_{2} into the C_{4} cycle, and lack of O_{2} inhibition of photosynthesis or effect of O_{2} on \( G \) (atmospheric versus low O_{2}). Although CAM plants also fix CO_{2} via PEPC, and they are characterized by high activities of C_{4} cycle enzymes, they are distinguished by temporal separation of CO_{2} fixation in the C_{4} cycle into organic acids at night, donation of CO_{2} from C_{4} acids to the C_{3} cycle within one photosynthetic cell during the day behind closed stomata, and a C_{4}-type isotope signature dependent on the fraction of atmospheric CO_{2} fixed at night.

The chlorenchyma of Bienertia is very unusual and unlike that of any previously identified photosynthetic type. The results of the present study show it functions as a C_{4} plant, with all the essential physiological and biochemical features, but that it achieves spatial compartmentation within a single photosynthetic cell, i.e. without Kranz anatomy. The high enzyme activity and immunoreactivity with NADP-ME and low activity and immunoreactivity for NAD-ME indicates Bienertia is an NAD-ME type C_{4} species like S. laricina.

The primary photosynthetic tissue in Bienertia is made up of chlorenchyma cells, in which the cytoplasm is separated into two compartments, a central compartment and a peripheral compartment, joined by cytoplasmic channels. An analysis of the organelle ultrastructure of the compartments and enzyme distribution between them indicates the mechanism by which the C_{4} pathway can be made to operate within a single cell, without the temporal separation used in CAM. The central compartment has one type of chloroplast, which has greater development of grana, contains Rubisco, and stores starch. The peripheral chloroplasts are deficient in grana and Rubisco, contain PPDK of the C_{4} cycle, and do not store starch. Electron microscopy shows that mitochondria are concentrated in the CC compartment. Immunolocalization with light microscopy demonstrates that NAD-ME and glycine decarboxylase are located in the CC compartment, and

| Table 3 Carbon isotope composition (\( \delta^{13}C \)) of leaves of Bienertia cycloptera, tribe Suaedeae, compared to the C_{4} species Salsola laricina, tribe Salsoleae, and the C_{3} species Suaeda heterophylla, tribe Suaedeae |
|----------------|----------------|----------------|---------------------------------|
| Species        | Carbon isotope value (\( \delta^{13}C \)) % | Country | Reference                        |
| Bienertia cycloptera (from current study) |                      |         |                                 |
| Mature leaves   | - 13.5          |         |                                 |
| Mature stems    | - 14.7          |         |                                 |
| Young leaves    | - 17.6          |         |                                 |
| Young stems     | - 16.3          |         |                                 |
| Bienertia cycloptera (from natural habitats) |                      |         |                                 |
| Iran            | - 14.3          |         | (Akhani et al., 1997)            |
| Pakistan        | - 13.4          |         | (Freitag and Stichler, 2002)     |
| Iran            | - 15.5          |         | (Freitag and Stichler, 2002)     |
| Iraq            | - 13.8*         |         | (R. Sage, personal communication)|
| Iraq            | - 14.5*         |         | (R. Sage, personal communication)|
| Iraq            | - 11.5*         |         | (R. Sage, personal communication)|
| Salsola laricina | - 14.8          |         | (Pyankov et al., 2000)           |
| Suaeda heterophylla (from current study) | - 25.3          |         |                                 |

*Kew Botanical Garden Herbarium, collector Rawi and Serhabriel, collection number 6300.
*Kew Botanical Garden Herbarium, collector Khatib and Alizzi, collection number 32648.
*Harvard University Herbarium, collector Weinert, Hilly and Mousawi, 31-10-74, loamy clay soil

| Table 4 Initial products of \(^{14}CO_{2}\) fixation in Bienertia cycloptera |
|----------------|----------------|----------------|-------|
| Species        | \(^{14}CO_{2}\) pulse | Aspartate | Malate | PGA | Other |
| Bienertia cycloptera | 3 sec             | 21.3       | 26.3   | 12.7 | 39.7  |
| Salsola laricina   | 8 sec             | 61.7       | 27.8   | 3.8  | 6.7   |
| Suaeda heterophylla | 8 sec             | 5.7        | 4.0    | 26.3 | 64.7  |

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immunolocalization by electron microscopy shows that these enzymes are located in the mitochondria. Immunolocalization by light microscopy shows PEPC is enriched in the peripheral cytoplasm, and, by electron microscopy, demonstrates that it is located in the cytosolic space. Since PEPC is a cytosolic enzyme, the more intense labelling in the peripheral cytoplasm may be explained by the presence of fewer organelles and more cytosolic space. Conversely, in the CC compartment, much of the space is occupied by organelles, leaving less relative cytosolic space where PEPC is located (Figures 2b and 4a). Catalysis by PEPC in the central compartment may be limited by availability of phosphoenolpyruvate, since PPDK is selectively localized in the peripheral chloroplasts. The peripheral cytoplasm of *Bienertia* has compartmentation of photosynthetic enzymes like that in mesophyll cells, and the CC compartment like that in bundle shear cells of Kranz-type, NAD-ME-type C₄ species. PPDK in the peripheral chloroplasts of the chlorenchyma cells can generate phosphoenolpyruvate, the substrate for PEPC. The latter enzyme can fix atmospheric CO₂, supplied to the cell through the adjoining intercellular air spaces. NAD-ME, which is responsible for the decarboxylation of C₄ acids and regeneration of CO₂ for the C₃ cycle, is localized in the large mitochondria, and Rubisco is localized in the chloroplasts surrounding the mitochondria in the CC compartment, where CO₂ can be donated from C₄ acids to the C₃ pathway.

Mitochondria and chloroplasts in the central part of chlorenchyma cells of *Bienertia* have an internal structure characteristic of those in bundle shear cells of NAD-ME-type C₄ plants. The mitochondria are large and have tubular and lamellated cristae like those in bundle shear cells of some other Chenopodiaceae species with NAD-ME-type C₄ photosynthesis, for example as in *Atriplex* species (Gamaley, 1985; Laetsch, 1968; Osmond *et al*., 1969; Troughton and Card, 1974; Voznesenskaya, 1976a) or *Suelda microphylla* (Voznesenskaya, 1976b). The granal index in the peripheral chloroplasts is lower than that in the chloroplasts in the central cytoplasm in *Bienertia*, which is analogous to the granal index in mesophyll chloroplasts being lower than that in bundle shear chloroplasts of NAD-ME-type Chenopodiaceae species having Kranz anatomy (i.e. *Halocharis gossypina*, *S. laricina*, and *S. gemmascens*, Pyankov *et al*., 2000; Voznesenskaya *et al*., 1999). Less grana stacking suggests an increased demand for production of ATP relative to NADPH by chloroplasts in the mesophyll cells or, in the case of *Bienertia cycloptera*, in the peripheral cytoplasm. It was suggested that, in NAD-ME-type species of the tribe Salsoleae (Voznesenskaya *et al*., 1999), the primary role of mesophyll chloroplasts is the generation of ATP, which is used by PPDK for the synthesis of the substrate phosphoenolpyruvate for PEPC, which is required for synthesis of aspartate from alanine and CO₂. The primary role of the granal chloroplasts in bundle shear (Kranz) cells in plants of this group is fixation of CO₂ via Rubisco and generation of NADPH and ATP to support the C₃ cycle (Calvin cycle).

In C₄ plants and C₃-C₄ intermediates, the specific localization of glycine decarboxylase in mitochondria of bundle shear cells is considered an important evolutionary development for reducing photorespiration. As was shown in genera *Moricandia*, *Panicum*, *Flaveria* and *Mollugo*, in the C₄ and C₃-C₄ species this enzyme is confined to the mitochondria in the Kranz cells, while in C₃ species it is located in mitochondria of both mesophyll and non-Kranz type cells (Hyton et al., 1988; Rawsthorne *et al*., 1988). The occurrence of the enzyme in the prominent mitochondria in bundle shear cells increases carbon gain under limiting CO₂, as photorespired CO₂ is refixed by the C₃ cycle (Hyton *et al*., 1988; Rawsthorne *et al*., 1988). Recently, the same pattern of immunolabelling for glycine decarboxylase, which was confined to bundle shear mitochondria, was shown for two representatives of the family Chenopodiaceae, the *C₄* *Salsola arbuscula* and the C₃-C₄ intermediate species *Salsola arbusculiformis* (Voznesenskaya *et al*., 2001a). Thus, localization of glycine decarboxylase in large mitochondria in the CC compartment in *Bienertia* is consistent with it functioning to minimize loss of any photorespired CO₂. Interestingly, in this species mitochondria are located in the centre of the CC compartment and are encircled by the chloroplasts, which may facilitate the capture of both CO₂ generated from NAD-ME in the C₄ cycle, and any photorespired CO₂ released from glycine decarboxylase. The results suggest that the bundle shear cell wall in C₄ plants having Kranz anatomy may not be as important a barrier to diffusion and leakage of CO₂ as previously thought.

The carbon isotope composition of *Bienertia cycloptera*, based on analyses of specimens from natural habitats, is C₄ or CAM-type (Table 3). In the present study with plants grown under high light, the isotope composition of mature tissues also indicates C₄ photosynthesis, while young tissues had a slightly more negative value. A number of factors could contribute to more negative isotope values in young tissue, including the p/i ratio (ratio of CO₂ in the intercellular air space/CO₂ in the atmosphere), fraction of leakage of CO₂ from the site of donation to Rubisco, some initial fixation of CO₂ by Rubisco, and discrimination during respiration (Farquhar, 1983; Henderson *et al.*, 1992; Henderson *et al.*, 1998). Freitag and Stichler (2002) obtained carbon isotope values ranging from −16.9 to −21.1‰ in leaves of greenhouse grown *Bienertia*, collected at different times of the year and different leaf positions. They suggested the more negative values might be due to unsuitable conditions relative to the natural habitat or that under some conditions it may function as a facultative C₃-C₄ plant.

The response of the rate of photosynthesis in *Bienertia* versus CO₂ concentration under 21 versus 3% O₂ is like that of C₄ plants in that O₂ does not inhibit photosynthesis, nor does it increase Γ. Rather, there is some stimulation of photosynthesis by O₂ under limiting CO₂, which has been observed previously in some C₄ species (Glagoleva *et al.*, 1978; Maroco *et al.*, 2000). One explanation is that the O₂-dependent Mehler peroxidase reaction provides some of the ATP needed in C₄ photosynthesis to support the C₄ cycle. The rate of photosynthesis in *Bienertia* is near saturating at atmospheric levels of CO₂, which is characteristic of C₄ plants, whereas C₃ plants require higher concentrations due to competition by photorespiration. The comparative species, C₄ *S. laricina* and the C₃ *S. heterophylla*, have photosynthetic responses to CO₂ and O₂ which are typical for C₄ and C₃ plants, respectively (Voznesenskaya *et al.*, 2001b).

As discussed above, the selective intracellular compartmentation of chloroplasts, mitochondria, microbodies and certain photosynthetic enzymes, the lack of O₂ inhibition of photosynthesis in CO₂ response curves, and the C₄ carbon isotope values of mature tissue in this study and of 7 collections of plant material from natural habitats, indicates *Bienertia* is functioning as a C₄ plant. According to the mechanism of C₄ photosynthesis atmospheric CO₂ is initially fixed into C₄ acids. Fixation of 14CO₂ by *Bienertia* in a 3-sec pulse resulted in about 50% of the products labelled as C₄ acids and 12% label in PGA, compared to 90% label in C₄ acids in *S. laricina* after labelling for 8 sec. In a previous study of initial products of photosynthesis in species of Chenopodiaceae growing in a natural habitat, after a 10-sec pulse with 14CO₂, the percentage labelling appearing in C₄ acids malate and aspartate in *Bienertia* was 30%, compared to an average of 73% for six C₄ Chenopodiaceae species (Glagoleva *et al.*, 1992). The rate of movement of label from 14CO₂ to C₄ acids to C₃ products in C₄ plants will depend on several factors, including the size of the C₄ acid pool, the diffusive resistance from sites of formation of C₄ acids and their utilization, and the rate of photosynthesis. The results suggest that, in the single cell C₄ system in *Bienertia*, the label moves more rapidly from C₄ acids to donation of CO₂ to Rubisco than in C₄ plants with Kranz anatomy. Thus, *Bienertia* may have either a lower pool of C₄ acids (allowing maximum specific activity of the pool to be reached more rapidly) and/or a shorter distance for diffusion of C₄ acids from their synthesis in the peripheral cytoplasm to the site of donation to Rubisco in the CC compartment. From light microscopy of S. laricina (Voznesenskaya *et al.*, 2001b) the estimated distance for diffusion from sites of synthesis in the mesophyll to sites of utilization in the bundle sheath is about 50 μm, compared to an average distance of 10–15 μm in *Bienertia* from the peripheral cytoplasm to the CC compartment (from examination of light micrographs). Detailed pulse-chase experiments with *Bienertia* are needed to gain further insight into its mechanism of C₄ photosynthesis.

Some C₄ species in tribes Salsoleae and Suaedeae have been reported to have low CAM, with night time fixation of CO₂, a few percent of that fixed by photosynthesis during the day (Bil’ *et al.*, 1982; Zalenskii and Glagoleva, 1981). C₄-type isotope composition and C₄-type enzymatic activities with lack of Kranz anatomy could potentially be accounted for by CAM. However, in the present study there was no net carbon uptake by *Bienertia* in the dark, and the rate of respiration in the dark versus that in the light in the absence of CO₂ was the same, indicating its growth was not occurring via CAM. Recently, it was shown that *Borszczowia aralocaspica*, another species in tribe Suaedeae, is a C₃ plant without Kranz anatomy (see Introduction). It has spatial separation of functions in photosynthesis accompanied by differential compartmentation of key photosynthetic enzymes and differentiation of chloroplasts (Voznesenskaya *et al.*, 2001b). However, the evolution of cytoplasmic organization and C₄ photosynthesis has taken distinctly different paths in *Bienertia* and *Borszczowia*. In *Borszczowia*, the cytoplasm is subdivided into two parts, corresponding to mesophyll and bundle sheath cells, at different ends of one cell while in *Bienertia*, the same result was reached with organization of the CC compartment. Based on initial results from studies on the comparative anatomy and molecular taxonomy of Chenopodiaceae (Freitag, Schütze, Clausing and Weising, unpublished data), it was suggested that these species are more distantly related and that their C₃ leaf types have evolved along independent evolutionary lines from the basal Austrobasoidioid type which is still present in C₃ species of *Suaeda* (Freitag and Stichler, 2002).
These results are significant to the discussion of evolution of C₄ photosynthesis in plants, since the first land plants were C₃ species, and, thus, C₄ plants evolved from C₃ plants (Sage and Monson, 1999). Our results show that this can occur without a requirement for Kranz anatomy. In addition, these observations are relevant to the ongoing attempts to enhance photosynthesis in important C₃ crop plants by the introduction of C₄ photosynthetic metabolism, since they indicate engineering of Kranz anatomy is not essential to the process.

Experimental procedures

Plant material and growth conditions

Plants were grown in a growth-chamber (Enconair) from seeds originating from the Kavir Protected Area near Mobarakiyeh, Iran, in November, 2000. Usually this plant grows in salty depressions of desert areas from eastern Anatolia to Turkmenistan and Pakistani Baluchestan. Seeds were stored at 3±5°C in November, 2000. Usually this plant grows in salty depressions of desert areas from eastern Anatolia to Turkmenistan and Pakistani Baluchestan. Seeds were stored at 3±5°C. These results are significant to the discussion of evolution of C₄ photosynthesis in plants, since the first land plants were C₃ species, and, thus, C₄ plants evolved from C₃ plants (Sage and Monson, 1999). Our results show that this can occur without a requirement for Kranz anatomy. In addition, these observations are relevant to the ongoing attempts to enhance photosynthesis in important C₃ crop plants by the introduction of C₄ photosynthetic metabolism, since they indicate engineering of Kranz anatomy is not essential to the process.

Light and electron microscopy

Light microscopy observations. Cross sections, 0.8–1 μm thick, were placed onto gelatin coated slides and blocked for 1 h with TBST + BSA (10 mM Tris-HCl, 150 mM NaCl, 0.1% v/v Tween 20 plus 1% w/v bovine serum albumin, pH 7.2). They were then incubated for 3 h with either the pre-immune serum diluted in TBST + BSA (1:100), anti-Rubisco (1:500 dilution), anti-PEPC (1:100 dilution), anti-NAD-ME (1:100), anti-NAD-ME (1:20), anti-PDK (1:100) or antiglycine decarboxylase (1:400) antibodies. The slides were washed with TBST + BSA and then treated for 1 h with protein A-gold (10 nm particles diluted 1:100 with TBST + BSA). After washing, the sections were exposed to a silver enhancement reagent for 20 min according to the manufacturer’s directions (Amersham Pharmacia Biotech, Piscataway, NJ, USA), stained with 0.5% (w/v) Safranin O, and imaged in a reflected/transmitted mode using a Bio-Rad MRC 1024 confocal system (Biorad, Hercules, CA, USA) with Nikon Eclipse TE 300 inverted microscope and Lasergraph image program 3.10. The background labelling with pre-immune serum was very low or non-existent, as shown previously with these same antibodies (results not shown).

TEM. Thin sections on coated nickel grids were incubated for 1 h in TBST plus 1% (w/v) BSA to block non-specific protein binding on the sections. They were then incubated for 3 h with either the pre-immune serum diluted in TBST + BSA, or anti-PEPC (1:50 dilution), anti-NAD-ME (1:100), anti-NAD-ME (1:20), or antiglycine decarboxylase (1:400) antibodies. After washing with TBST + BSA, the sections were incubated for 1 h with Protein A-gold (10 nm) (Amersham) diluted 1:100 with TBST + BSA. The sections were washed sequentially with TBST + BSA, TBST, and distilled water, and then post-stained with a 1:4 dilution of 1% (w/v) potassium permanganate and 2% (w/v) aqueous uranyl acetate. Images were collected using a JEOL-1200 EX transmission electron microscope.

Staining for polysaccharides. Sections, 0.8–1 μm thick, were made from the same samples dried onto gelatin coated slides, incubated in periodic acid (1% w/v) for 30 min, washed and then incubated with Schiff’s reagent (Sigma, St Louis, MO, USA) for 1 h. After rinsing, the sections were ready for analysis by light microscopy.

Enzymes

Extraction and assay. Enzymes were extracted from illuminated leaves harvested during the photoperiod. Leaves were frozen in liquid N₂ in a small mortar and ground to a fine powder at liquid N₂ temperature. The powder was then transferred to a Ten-Broek grinder with cold extraction buffer (1 ml per 50 mg FW) and ground for about 30 sec. The extraction buffer contained 100 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 2 mM EDTA and 2% PVPP. The extract was then centrifuged in an Eppendorf microcentrifuge (1 min, 14,000 r.p.m) and immediately assayed. For Rubisco and PEPC assay the reaction was started by adding 50 μl of extract to 450 μl of media containing 1 mM ribulose-1,5-bisphosphate (RuBP) for rubisco assay or 5 mM PEP in the case of the PEPC assay, and 10 mM NaHCO₃. The reaction was stopped...
after 30 sec and acid-stable radioactivity was counted. NAD-ME, NADP-ME and PPDK activities were measured spectrophotometrically in a 1-ml reaction mixture. The assay medium for NAD-ME contained 25 mM HEPES/KOH (pH 8.0), 0.5 mM NADP, 20 mM MgCl2, 5 mM DTT, 0.1 mM EDTA, and 5 mM malate (reaction starter). The assay medium for PPDK contained 50 mM HEPES/KOH (pH 8.0), 10 mM MgCl2, 3 mM DTT, 0.1 mM EDTA, 15 mM NaHCO3, 1.25 mM pyruvate, 0.2 mM NADH, 2.5 mM KH2PO4, 1.25 mM ATP, 10 U malate dehydrogenase, and 1 U PPDK. The reaction was started with 50 μl of extract.

For Western blot analysis, proteins were extracted from leaves in 200 mM Bicine-KOH (pH 9.5), 25 mM DTT, and 1% (v/v) SDS. Extracts were boiled for 90 sec with equal volumes of solubilization buffer (62.5 mM Tris, 20% (v/v) glycerol, 2.5% (v/v) SDS, and 5% (v/v) 2-mercaptoethanol, pH 6.8). 20 μg of protein was loaded per lane. Proteins were resolved by SDS-PAGE (Laemmli, 1972) using a linear 7.5-15% acrylamide resolving gel and 5% acrylamide stacking gel. Each gel carried pre-stained SDS-PAGE molecular weight markers (Bio-Rad). After electrophoresis, proteins on the gel were electro-transferred to nitrocellulose membrane (Towbin et al., 1979) and probed with an appropriate antibody overnight. For antibodies used, see the section on in situ immunolocalization. Goat antirabbit IgG-alkaline phosphatase conjugate (Bio-Rad) was used as the secondary antibody for detection of the enzymes. All blots were air dried and used for image analysis.

**Exposure of leaves to 14CO2 and identification of 14C products**

For the pulse-chase experiments, 1 branch (approximately 3-5 cm in length) was cut from the plant 4-6 h into the light period. The branch was immediately placed into a glass vial containing 25 ml of water, and the base of the branch submerged in distilled water, with a stream of humidified, CO2-free air. Before initiating the experiment, 14CO2 was generated in a separate vial by the addition of NaH14CO3 to HCl. Immediately after flushing the plant cuvette with CO2-free air, 2 ml of 14CO2 gas, containing 4 μCi of 14CO2, was injected, resulting in a final CO2 concentration of 356 μl l−1.

After giving a short pulse of 14CO2, the plant material was killed by flushing into boiling 80% ethanol (v/v). Tissue was boiled an additional 2-3 min, ground thoroughly with a mortar and pestle with the addition of a small amount of acid-washed sand, and extracted again with 96, 80, 60, 40% ethanol and twice with water. All extracts were pooled and concentrated to approximately 1 ml. The leaf extract was partitioned with CHCl3. Separation and identification of the labelled photosynthetic products were accomplished using two-dimensional thin-layer electrophoresis and chromatography methods (Moore and Seeman, 1980; Schurmann, 1989). Recovery of radioactivity from the plates was >90%.

**Δ13C carbon isotope determination**

Carbon isotope fractionation values were determined on dried leaves and stems, using a standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard (Bender et al., 1973).

**Rates of CO2 exchange**

Rates of CO2 assimilation were measured on a branch of an intact plant with a Bingham Bi-2-dp mini cuvette controller (Bingham, Hyde Park, UT, USA), an MK3-225 IR gas analyzer (ADC, Hoddesdon, Hertfordshire, UK) and data obtained with a chart recorder (Linear, Tekmar Co., Cincinnati, OH, USA). Gas exchange was measured by CO2 depletion in the differential mode with an open system where a given gas mixture was flowing through the reference cell and sample cell (in line with the plant enclosed in a cuvette). The temperature-controlled plant cuvette contained a copper-constantan thermocouple, which was placed in contact with the lower epidermis of a leaf for monitoring plant temperature. Water vapour leaving the chamber was measured with a digital hygrometer (Fisher Scientific, Federal Way, WA, USA). Photosynthetic photon flux density (PPFD) was measured with a Li-Cor-185 quantum sensor (Li-Cor, Lincoln, NE, USA). Relative humidity was maintained at 60% to 80% in the leaf chamber. The leaf temperature was 25°C and the PPFD was 1300 μmol m−2 s−1.

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**References**


