Chapter 4

C_4 Photosynthesis: Kranz Forms and Single-Cell C_4 in Terrestrial Plants

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Summary

Plants identified as having C_4 photosynthesis have a C_4 metabolic cycle with phosphoenolpyruvate carboxylase as the initial catalyst for fixation of atmospheric CO_2, and a C_4 acid decarboxylase (NADP-malic enzyme, NAD-malic enzyme, or phosphoenolpyruvate carboxykinase), which releases CO_2 for fixation by the C_3 cycle. Effective donation of CO_2 to Rubisco minimizes competition by O_2 and photorespiration, and thus increases photosynthesis under conditions where CO_2 is limiting. To achieve this, fixation of atmospheric CO_2 in the cytosol by phosphoenolpyruvate carboxylase must be separated from the donation of CO_2 to Rubisco by the decarboxylation of C_4 acids. In most documented C_4 plants, this is accomplished through evolution of various forms of Kranz anatomy, with fixation of atmospheric CO_2 in mesophyll cells and donation of CO_2 from C_4 acids to Rubisco in bundle sheath cells. In the family Chenopodiaceae, two alternative means of accomplishing this spatial separation evolved within individual photosynthetic cells, whereby one cytoplasmic compartment specializes in fixation of atmospheric CO_2 in the carboxylation phase of the C_4 cycle, and the other cytoplasmic compartment specializes in donating CO_2 from C_4 acids to Rubisco. In this chapter, biochemical and structural variations of Kranz anatomy in three major C_4-containing families, Poaceae, Cyperaceae, and Chenopodiaceae, as well as other known forms for dicots, are summarized. Then, the phylogeny, biogeography, development, and structure-function relationships of the single-cell C_4 systems are discussed in comparison to Kranz type C_4 plants.

I. Introduction

A. What Does It Take to Be C_4?

This question was posed in a short commentary on the history of C_4 by (Edwards et al., 2001) who noted that the minimum requirements for the CO_2 concentrating mechanism in C_4 photosynthesis are "(a) cell-specific amplification of enzymes of C_4 photosynthesis; i.e. phosphoenolpyruvate carboxylase (PEPC) in mesophyll, and C_4 acid decarboxylases and Rubisco in bundle sheath cells, with complementary adjustments of photosystem and electron transport activities; (b) novel cell-specific organelle metabolite translocators; (c) symplastic connections of the spatially separated sources and sinks of 4C-dicarboxylic acid transport metabolites; and (d) barriers to CO_2 diffusion between the site of CO_2 fixation by PEPC in mesophyll cells and sites of CO_2 release and refixation by Rubisco in bundle-sheath cells". These requirements have been met through the multiple, independent evolution of C_4 photosynthesis in different groups of terrestrial plants. Until the recent discovery of two alternative means of performing C_4 photosynthesis within individual chlorenchyma cells (single-cell C_4), all terrestrial C_4 plants were presumed to have Kranz anatomy.

B. Occurrence of C_4 Among Terrestrial Plants

The earliest studies which led to the identification of C_4 plants were on maize and sugarcane, members of family Poaceae (see review by Hatch, 1999). Since then, C_4 plants have been found in 19 families with the largest number of species appearing in families Poaceae, Cyperaceae and Chenopodiaceae. C_4 is estimated to have evolved independently over 50 times (Muhaidat et al., 2007), resulting in three biochemical subtypes (see Chapter 14 by Drincovich et al.) based on the mechanism of C_4 acid decarboxylation: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEP-CK).

Abbreviations: BS – Bundle sheath(s); Kranz cells – An inner layer of chlorenchyma cells specialized for C_4 photosynthesis, irrespective of whether there is contact with vascular bundles (sometimes referred to as BS cells in C_4 plants); M – Mesophyll; MS – Mestome sheath(s); NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PEPC – Phosphoenolpyruvate carboxylase; PEP-CK – Phosphoenolpyruvate carboxykinase; PGA – 3-Phosphoglyceric acid; PPDK – Pyruvate, Pi dikinase; PSI – Photosystem I; PSII – Photosystem II; RuBP – Ribulose 1,5-bisphosphate; SL – Suberin lamella

II. Structural and Biochemical Diversity in Kranz Type Anatomy

The occurrence of Kranz anatomy (Kranz means wreath in German) has been known since its initial characterization by Haberlandt (1884). In a broad sense, Kranz anatomy can now be functionally defined to accommodate all known structural variants of Kranz type C_4 plants. A double concentric layer of chlorenchyma cells together form the Kranz tissue with the outer layer capturing atmospheric CO_2 in the C_4 cycle, and the inner layer donating CO_2 from C_4 acids to Rubisco in the C_3 cycle. The outer layer is commonly referred to as mesophyll (M) cells (usually consisting of palisade parenchyma) and the inner layer as specialized bundle sheath (BS) cells or Kranz cells. The M cells are always closer to the atmosphere than the BS cells, and the BS cells, as a rule, have limited contact with intercellular air space. The cells of chlorenchymatous M and BS layers are usually adjacent to one another, but in some cases they are separated by an additional layer of cells. The ratio of M/BS cells is lower than in C_3 plants, and most of the M cells are in direct contact with BS cells. There are two common structural forms, a double concentric layer of chlorenchyma around individual veins, and a double concentric layer which surrounds all the vascular tissue in the leaf. Since C_4 evolved multiple times from different C_3 leaf anatomies, there are various structural types. Specific types are described below which represent striking examples of evolutionary convergence on a common suite of anatomical features.

A. Structural Diversity

Among C_4 plants, there is considerable variation in features relevant to the C_4 mechanism (Carolin et al., 1973, 1975, 1977, 1978; Laetsch, 1974; Brown, 1975, 1977; Ellis, 1977; Edwards and Walker, 1983; Dengler et al., 1985; Voznesenskaya and Gamaley, 1986; Prendergast and Hattersley, 1987; Prendergast et al., 1987; Ueno et al., 1988a; Hattersley and Watson, 1992; Dengler and Nelson, 1999; Sage, 2004; Muhaidat, et al., 2007). While distinct biochemical and anatomical types have been catalogued since C_4 photosynthesis was first described more than three decades ago (see Hatch, 1971), new structural subgroups continue to be discovered, providing further insight into the evolution of the syndrome. Usually, several characteristics are taken into account to distinguish between different structural and biochemical subtypes. The most important among them are: (1) number of BS layers; (2) presence or absence of a mestome sheath (MS) and its positioning in relation to other parenchyma sheaths (notably in grasses); (3) presence or absence of a suberin lamella (SL) in BS cell walls (in grasses); (4) position of BS organelles (mainly chloroplasts); and (5) chloroplast differentiation between M and BS: BS cells in NADP-ME species have grana-deficient chloroplasts with a few, small mitochondria, while NAD-ME species have chloroplasts with well-developed grana and numerous, large mitochondria with specialized cristae. In both subtypes, M chloroplasts have a reversed pattern of grana development to that expressed in the BS, with abundant, large grana in NADP-ME species, and a deficiency of grana in chloroplasts of M cells in NAD-ME species. Some other features, such as the shape (outline) of the outer parenchyma BS, have been mentioned as being useful in characterizing certain subtypes, for example an uneven outline of the outer BS in PEP-CK type grasses; however, this does not seem to be especially important phylogenetically in relation to C_4 photosynthetic subtypes, as there are many exceptions (Prendergast et al., 1987). Only features of organelle differentiation provide an easy means to predict biochemical subtypes, while other structural characters only give additional information for considering evolutionary development of Kranz anatomy. The main structural forms of Kranz that are known to occur among C_4 species are illustrated in Figs. 1, 2 and 3. Historically, different forms of Kranz anatomy have been referred to either by taxonomic names, or by names descriptive of their structure. We have used taxonomic names to be consistent and concise, recognizing that they are used descriptively and do not always imply phylogenetic identity. An exception is description of the three classical forms of Kranz anatomy associated with the three biochemical subtypes in family Poaceae, which accounts for many C_4 grasses.

1. Poaceae

For C_4 grasses, important distinguishing characteristics include the presence or absence of a

MS, and, when present, the size of MS cells and thickness of their cell walls, the positioning of chloroplasts in BS cells, the presence or absence of a SL and, when present, its distribution in the Kranz cell walls (Carolin et al., 1973; Brown, 1975, 1977; Ellis, 1977; Hattersley and Browning, 1981; Hattersley, 1992; Hattersley and Watson, 1992). At least nine structural subtypes have been distinguished on the basis of these features, and most of the known C_4 grasses fit into these subtypes (described below and illustrated in Fig. 1 and Table 1). It is suggested from phylogenetic analyses that C_4 evolved from C_3 a minimum of 17 times in family Poaceae (Christin et al., 2008, 2009). Certain forms of Kranz anatomy evolved multiple times in the family (Table 1).

In the Poaceae, C_4 species occur in subfamilies Panicoideae, Chloridoideae, Aristidoideae, and Micrairoideae (Sanchez-Ken et al., 2007; Vicentini et al., 2008; Christin et al., 2008, 2009). While most C_4 species in subfamily Panicoideae are NADP-ME type, NAD-ME and PEP-CK type species also occur in the subfamily as discussed below. In subfamily Chloridoideae, most C_4 species are NAD-ME type, while a few genera have PEP-CK type species. C_4 species identified in subfamilies Aristidoideae and Micrairoideae are NADP-ME type (Table 1). Structural forms of Kranz anatomy among these biochemical subtypes are discussed below.

Classical NADP-ME Type Anatomy

This type of anatomy was originally the so-called Panicoid (Carolin et al., 1973). Among the three major biochemical C_4 subgroups first identified in Poaceae (Gutierrez et al., 1974; Hatch et al., 1975; Brown, 1977), classical NADP-ME type species have a single parenchyma BS (the Kranz BS which is derived from provascular tissue and, thus, lacks a MS), with BS chloroplasts in a centrifugal/peripheral position and with a deficiency in their grana development, whereas, M chloroplasts have well-developed grana (Fig. 1) (Voznesenskaya and Gamaley, 1986; Hattersley, 1992; Yoshimura et al., 2004). C_4 species with classical NADP-ME type anatomy in subfamily Panicoideae occur in tribes Paniceae, Arundinelleae, and Andropogoneae (Hattersley and Watson, 1992; Sage et al., 1999; GPWG, 2001; Vicentini et al., 2008). The degree of grana reduction in chloroplasts in the BS cells varies, from having nearly agranal BS chloroplasts (representatives of tribe Andropogoneae), to having numerous, small grana (tribe Paniceae), to having a few rather large grana (*Panicum obseptum* or *Rhynchelytrum repens)*. This subtype has fewer mitochondria in BS cells than in the NAD-ME and PEP-CK subtype (Yoshimura et al., 2004). The SL is present in the outer tangential wall, and partly in the radial cell wall of the BS cells (Hattersley and Browning, 1981).

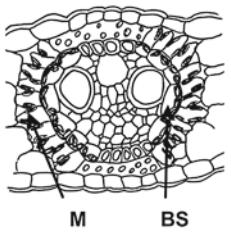
Classical NAD-ME Type Anatomy

This structural type of C_4 grasses has a double sheath: a MS with thick cell walls and a few plastids, surrounded by a Kranz type chlorenchyma sheath (derived from ground tissue). Bundle sheath chloroplasts are in a centripetal position and have well-developed grana, while M chloroplasts show different degrees of grana reduction according to species (Fig. 1). A SL is usually absent in BS cells; but if present, is only in BS cell walls adjacent to sclerenchyma cells which do not contain chloroplasts (Hattersley and Browning, 1981). This subgroup also has abundant, large specialized mitochondria in BS cells, the site of the C_4 acid decarboxylase (Gutierrez et al., 1974; Hatch et al., 1975; Brown, 1977; Hattersley and Watson, 1992; Yoshimura et al., 2004). This form of anatomy was originally named Eragrostoid (Carolin et al., 1973). It occurs particularly in subfamily Chloridoideae (the core Chloridoideae and *Centropodia* lineages); but, also in subfamily Panicoideae, tribe Paniceae, evolving once in the *Panicum*, *Urochloa*, *Setaria* clade, Table 1 (Sage et al., 1999; GPWG, 2001; Aliscioni et al., 2003; Christin et al., 2008, 2009).

in *Arundinella hirta* for Arundinelloid type, of large veins in *Aristida adscensionis* for Aristidoid type, of *Stipagrostis pennata* for Stipagrostoid type, of *Eriachne aristidea* for Eriachneoid type, of *Alloteropsis semialata* ssp. *semialata* for Neurachneoid type, and of *Triodia scariosa* for Triodioid type (Pictures are adapted from Voznesenskaya and Gamaley, 1986; Prendergast and Hattersley, 1987; Dengler and Nelson, 1999). *B*, biochemical subtype; *BS*, bundle sheath; *Chl*, chloroplast; *M*, mesophyll; *Mito*, mitochondria; *MS*, mestome sheath; *OP BS*, outer parenchymatous BS; *SL*, suberin lamella; *VB*, vascular bundle.

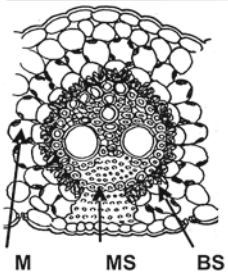
Poaceae

Classical NADP-ME



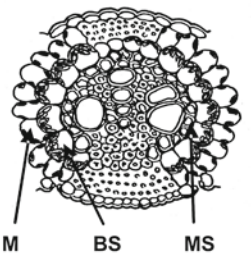
MS:(-). **Chl:** BS with reduced grana in centrifugal position, M with well-developed grana. **Mito:** small and few in BS. **SL:** usually in outer tangential BS cell wall.

Classical NAD-ME



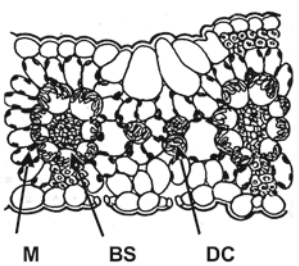
MS:(+). **Chl**: BS granal, in centripetal position, M with reduced grana. **Mito:** numerous specialized in BS cells. **SL:** Absent in BS cell wall.

Classical PEP-CK



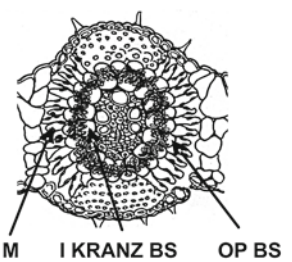
MS:(+). **Chl:** have well-developed grana in M and BS. Centrifugal or scattered/peripheral in BS cells. **Mito**: BS a little larger than in M, with more abundant cristae. **SL**: present.

Arundinelloid



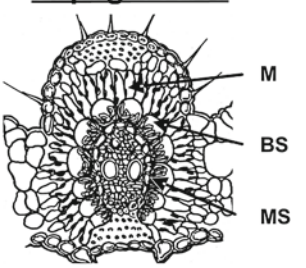
B: NADP-ME. **MS:**(-). **Chl:** agranal in BS and centrifugal. **SL:** present. Presence of distinctive cells (DC) similar to BS cells without VB.

Aristidoid



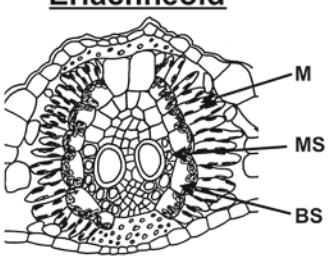
B: NADP-ME. **BS:** two parenchymatous BS, the inner Kranz. **Chl:** agranal, peripheral or centrifugal in Kranz BS, centripetal and granal in outer parenchymatous BS. **SL:** absent.

Stipagrostoid



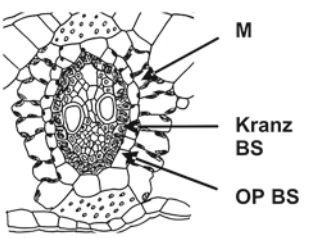
B: NADP-ME. **MS:**(+) enlarged thin-walled cells. **Chl:** BS cells have reduced grana and are centripetal. **SL:** absent.

Eriachneoid



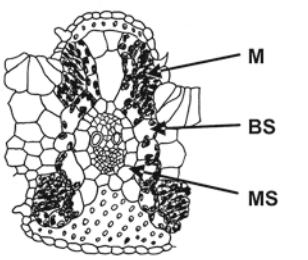
B: NADP-ME. **MS:**(+) enlarged, thin-walled cells. **Chl:** BS centrifugal with well-developed grana, but stacking lower than in M **SL:** absent.

Neurachneoid



MS:(-). **BS:** double parenchyma sheath, inner is Kranz. **SL:** present.
B: PEP-CK. **Chl:** BS with well-developed grana, peripheral.
B: NADP-ME. **Chl:** BS centrifugal, with grana reduced in comparison with M.

Triodioid



B: NAD-ME. **MS:** (+) thin-walled. **BS:** extensions towards chlorenchyma. **Chl:** BS centrifugal or peripheral with well-developed grana. **SL:** absent.

Fig. 1. Illustrations of the forms of Kranz anatomy in family Poaceae. Sketch of vascular bundles in maize for classical NADP-ME, of a large vein in *Eragrostis* sp. for classical NAD-ME, and of species representing PEP-CK type. Sketches of leaf structure

Table 1. Structural and biochemical forms of Kranz anatomy in relation to the number of independent C_4 lineages in family Poaceae. The number of each lineage is according to Christin et al., (2008, 2009)

		Type	
Subfamily	Lineage	Structural	Biochemical
Aristidoideae	1 *Stipagrostis*	Stipagrostoid	NADP-ME
	2 *Aristida*	Aristidoid	NADP-ME
Chloridoideae	3 Core Chloridoideae	Classical	NAD-ME
		Classical	PEP-CK
		Triodioid	NAD-ME
	4 *Centropodia*	Classical	NAD-ME
Micrairoideae	5 *Eriachne*	Eriachneoid	NADP-ME
Panicoideae	6 Arundinelleae	Arundinelloid	NADP-ME
		Classical	NADP-ME
	12 Andropogoneae	Classical	NADP-ME
Panicoideae	7 *Panicum*	Classical	NAD-ME
(tribe Paniceae)	*Urochloa*	Classical	PEP-CK
	Setaria clade	Classical	NADP-ME
	9 *Echinochloa*	Classical	NADP-ME
	11 *Digitaria*	Classical	NADP-ME
	13a *Paspalum* clade	Classical	NADP-ME
	13b *Ophichloa* clade	Classical	NADP-ME
	14 *Anthaenantia*	Classical	NADP-ME[a]
	15 *Oncorachis ramose* (=*Streptostachys*)	Classical	NADP-ME[b]
	17 *Mesosetum* clade	Classical	NADP-ME
	8 *Neurachne munroi*	Neurachneoid	NADP-ME
		Neurachneoid	PEP-CK
	10 *Alloteropsis*	Neurachneoid	PEP-CK
	16 *Panicum prionitis* clade	Neurachneoid	NADP-ME

[a]Dr. Osvaldo Morrone, personal communication, 2009 Argentina

[b]Sede et al., 2009

Classical PEP-CK Type Anatomy

The classical PEP-CK type has a double chlorenchyma sheath similar to the NAD-ME type, with an inner MS and outer Kranz chlorenchyma sheath with grana-containing BS chloroplasts in a centrifugal position, or scattered peripherally around the cell, see Fig. 1 (Gutierrez et al., 1974; Brown, 1977; Dengler and Nelson, 1999; Yoshimura et al., 2004). The level of grana development is very similar in BS and M chloroplasts, and the BS mitochondria are quite small (generally comparable in size to M mitochondria) and are usually more numerous than in NADP-ME species, but less abundant than in NAD-ME species (Yoshimura et al., 2004; Voznesenskaya et al., 2006). Suberin lamella is present in the outer tangential BS cell walls and extends approximately to the middle of the radial cell walls (Hattersley and Browning, 1981). This subtype has been found in subfamily Chloridoideae in *Bouteloua, Eleusine, Muhlenbergia, Spartina*, *Sporobolus*, and *Zoysia*, and in subfamily Panicoideae, evolving once in the *Panicum/Urochloa/Setaria* clade, e.g. in *Brachiaria, Chaetium*, *Eriochloa, Melinis,* and *Urochloa*, see Table 1 (Sage et al., 1999; Guissani et al., 2001; Aliscioni et al., 2003; Christin et al., 2008).

Arundinelloid: Biochemical Subtype NADP-ME

This type of anatomy was studied in detail in genus *Arundinella* (tribe Arundinelleae) subfamily Panicoideae. Like the classical NADP-ME type, species in this genus have NADP-ME type biochemistry, with BS chloroplasts having reduced grana and in the centrifugal position; MS is absent in all vascular bundles. However,

the Kranz anatomy in veins is widely spaced, and there is the unusual occurrence of a row, or rows, of Kranz assemblies between the veins, which sometimes are referred to as distinctive cells because these bundle sheath-like cells are not associated with vascular tissue (Tateoka, 1958), see Fig. 1. A SL is usually present and continuous in the distinctive cells, or interrupted in the radial cell walls in BS cells surrounding the vascular tissue (Hattersley and Browning, 1981). Distinctive cells have structural and biochemical characteristics similar to the BS cells (Crookston and Moss, 1973; Dengler et al., 1990, 1996; Dengler and Dengler, 1990; Wakayama et al., 2002, 2003, 2006). Distinctive cells have also been found in genera *Arthraxon* and *Microstegium* (tribe Andropogoneae), where they have ultrastructural characteristics similar to those shown for *Arundinella* (Ueno, 1995), and in genus *Garnotia*; but, there is no additional biochemical or ultrastructural data for species of this genus (Tateoka, 1958).

Aristidoid: Biochemical Subtype NADP-ME

The Kranz anatomy of species in genus *Aristida*, tribe Aristideae in subfamily Aristidoideae, is unusual in having three distinct layers of chlorenchyma cells surrounding the vascular tissue: an inner BS, an outer BS, and the M cells (Brown, 1958; Johnson, 1964; Bisalputra et al., 1969), see Fig. 1. *Aristida* species have NADP-ME type biochemistry, based on analyses of several species in the genus (Gutierrez et al., 1974; Hattersley, 1987; Prendergast et al., 1987; Voznesenskaya, et al., 2005b). Mestome sheath is absent; both the inner and outer sheaths are chlorenchymatous. However, in the inner sheath, chloroplasts are nearly agranal, while the outer BS contains chloroplasts with well-developed grana similar to the M chloroplasts. In this type, only the inner sheath functions as Kranz cells, while the outer BS functions mainly for storage of starch and, possibly, for refixation of photorespired CO_2 (Voznesenskaya et al., 2005b). In the inner BS, chloroplasts are scattered around the cell or tend to be centrifugal, while in the outer parenchyma BS, chloroplasts are located in a centripetal position. The SL is absent in cell walls of both types of BS (Hattersley and Browning, 1981).

Stipagrostoid: Biochemical Subtype NADP-ME

The genus *Stipagrostis* belongs to tribe Aristideae in subfamily Aristidoideae. Like *Aristida*, *Stipagrostis* species also evolved NADP-ME type photosynthesis, but they have a different type of Kranz anatomy, named Stipagrostoid (Voznesenskaya et al., 2005a). This subtype has an inner MS consisting of enlarged cells with thinner cell walls and few chloroplasts, and an outer layer of Kranz cells with chloroplasts in the centripetal position (Brown, 1975). In the Kranz cells of *Stipagrostis*, mitochondria are few and small, and the chloroplasts are deficient in grana compared to M chloroplasts, which have well-developed grana. In contrast, the classical NADP-ME subtype grasses lack a MS and they have Kranz cells with chloroplasts in a centrifugal position. Also, the Kranz cells of *Stipagrostis* lack a SL in the walls, whereas the classical NADP-ME type grasses have SL in the Kranz cells, which are thought to have originated from the MS.

Eriachneoid: Biochemical Subtype NADP-ME

This subtype, which occurs in genera *Pheidochloa* and *Eriachne* (subfamily Micraioideae, Christin et al., 2008, 2009), has NADP-ME type biochemistry (Prendergast et al., 1987) and an inner MS with an outer Kranz sheath, like the Stipagrostoid type. However, unlike *Stipagrostis,* which has chloroplasts in a centripetal position in Kranz cells, in *Pheidochloa* and in most *Eriachne* species (18 of 21) the chloroplasts in Kranz cells are in a centrifugal position (Hattersley, 1987; Prendergast and Hattersley, 1987; Prendergast et al., 1987; Taniguchi et al., 2003). The BS chloroplasts have well-developed grana with numerous, long intergranal thylakoids; in *Eriachne aristidea*, the degree of grana stacking is lower compared to the M chloroplasts (Taniguchi et al., 2003). For other species, the situation is not very clear. Bundle sheath chloroplasts of *E. glabrata, E. obtusa* and *P. gracilis* have an unexpectedly large number of grana (up to 21 thylakoids in a stack) for an NADP-ME subtype (Prendergast et al., 1987); however, there is no data about the degree of grana differentiation in M chloroplasts of these

species. The SL is absent in cell walls of Kranz cells (Prendergast et al., 1987).

Neurachneoid: Biochemical Subtypes NADP-ME and PEP-CK

Species with this type of anatomy have a double parenchymatous sheath and the MS is absent; however, in this case the inner sheath is Kranz and the outer sheath is a non-specialized parenchyma BS containing only a small number of chloroplasts (see Dengler et al., 1985; Hattersley et al., 1986; Prendergast et al., 1987; Ueno and Sentoku, 2006). It was suggested that the inner Kranz BS in all species having this type of anatomy originated from the MS of C_3 grasses (Brown, 1975, 1977; Dengler et al., 1985). Species with this type of anatomy which perform NADP-ME type photosynthesis are *Neurachne munroi*, *Paraneurachne muelleri* in the Neurachne clade, and also *Panicum petersonii* and *P. prionitis* in section Prionita; all belong to the subfamily Panicoideae. In both *N. munroi* and *P. muelleri*, thick cell walls of the Kranz inner sheath have a SL, which is only continuous in the outer tangential walls and outer parts of radial walls. The outer parenchyma sheath has relatively thin cell walls without SL. Chloroplasts in Kranz cells are distributed evenly in *N. munroi*, but are in a centrifugal position in *P. muelleri.* Kranz cell chloroplasts have granal stacks which are less pronounced than in the M chloroplasts (Hattersley et al., 1986).

Alloteropsis semialata, subfamily Panicoideae, represents a very unique case, where diversity in the form of photosynthesis occurs among subspecies, with ssp. *semialata* being C_4 and ssp. *eckloniana* being C_3 (Frean et al., 1983; Prendergast et al., 1987; Ueno and Sentoku, 2006; Ibrahim et al., 2009). An Australian accession of spp. *semialata* biochemically is PEP-CK type with Neurachneoid type anatomy (Prendergast et al., 1987). The Kranz sheath, unlike the classical PEP-CK type species, is considered to be derived from the MS sheath of C_3 plants. Most anatomical characteristics are similar to those cited above: cell walls of Kranz cells are thicker than in the parenchymatous BS and they have a SL. There are abundant chloroplasts and mitochondria in Kranz cells which do not have a special orientation, and chloroplasts have well-developed grana like those in the M cells (Ueno and Sentoku, 2006). The ssp. *semialata* has high PEP-CK activity and variable amounts of NADP-ME which may be influenced by growth conditions (Prendergast et al., 1987; Ueno and Sentoku, 2006).

Other Forms of NAD-ME Type Anatomy

There are several NAD-ME C_4 species in family Poaceae having some anatomical features that are not characteristic of the classical NAD-ME type C_4 species. Like the classical NAD-ME and PEP-CK types, these species have a double parenchyma sheath, with an inner MS and outer Kranz chlorenchyma sheath with grana-containing BS chloroplasts. Also, the BS cells have abundant mitochondria characteristic of NAD-ME type species. However, the BS chloroplasts are not arranged in the centripetal position, but are located in a centrifugal or peripheral position like the PEP-CK species. Also, the BS cell walls generally have a SL which is usually absent in the classical NAD-ME type species. This includes some species in genera *Eragrostis*, *Enneapogon*, *Triraphis*, and some *Panicum* species of the section Dichotomiflora (Ohsugi and Murata, 1980; Ohsugi et al., 1982; Prendergast et al., 1986). Interestingly, different cultivars of one NAD-ME type species, *P. coloratum*, were found to have different positions of chloroplasts in the Kranz cells, centripetal versus centrifugal (Ohsugi et al., 1982), which further shows that this feature cannot be taken as a criterion for distinguishing between different biochemical types.

A more extreme structural variant of NAD-ME type species is the Triodioid type anatomy. Species with Triodioid anatomy have two BS: an inner, thin-walled MS and an outer, chlorenchymatous Kranz BS which lacks SL in the cell walls. There are two variants of Kranz anatomy in this genus: Kranz BS form (“drape”) extensions between adjacent vascular bundles, as in *Triodia pungens* (Hattersley and Watson, 1992), or BS extensions towards patches of M cells on both the abaxial and adaxial sides of the leaf, which are not associated with vascular bundles, for example *T. irritans* and *T. scariosa*, as illustrated in Fig. 1 (Dengler and Nelson, 1999). The species

which have been studied have NAD-ME type biochemistry; the appearance of mitochondria in Kranz cells is typical for NAD-ME species (Craig and Goodchild, 1977). The chloroplasts in Kranz cells have well-developed grana; but, unlike classical NAD-ME species, they, are in a centrifugal position or peripherally scattered around the cytoplasm, as in PEP-CK type species (Craig and Goodchild, 1977; Prendergast et al., 1987).

2. *Family Cyperaceae*

In family Cyperaceae, there are four types of Kranz anatomy (Fig. 2). As in family Poaceae, C_3 Cyperaceae species have an inner MS and an outer parenchyma sheath around the vascular tissue. In C_4 Cyperaceae species, the Kranz cells are considered to have evolved either internal to the MS (Fimbristyloid, Chlorocyperoid and Eleocharoid) or from the MS (Rhynchosporoid) (Brown, 1975; Carolin et al., 1977; Gilliland and Gordon-Gray, 1978; Bruhl et al., 1987; Bruhl and Perry, 1995; Soros and Dengler, 1998, 2001; Dengler and Nelson, 1999). In the first case, the MS, which is situated between M and Kranz cells, is nonphotosynthetic, thick-walled and generally suberized (Carolin et al., 1977; Ueno et al., 1988b; Ueno and Samejima, 1989; Bruhl and Perry, 1995; Soros and Dengler, 2001). This sheath may contribute to diffusional resistance of gases and help to minimize leakage of CO_2 generated from decarboxylation of C_4 acids in the Kranz cells. Most C_4 representatives in the family are NADP-ME type; NAD-ME species have only been found in genus *Eleocharis* (Bruhl et al., 1987; Ueno et al., 1988a; Murphy et al., 2007).

Fimbristyloid: Biochemical Subtypes NADP-ME and NAD-ME

This type of anatomy was found in C_4 species of the tribe Fimbristylideae, for example in *Bulbostylis* and *Fimbristylis* (see Carolin et al., 1977; Ueno et al., 1988a) and more recently in genus *Eleocharis* (Bruhl et al., 1987; Ueno, 1998a; Murphy et al., 2007). The Kranz cells originated internal to the MS and do not form a continuous wreath; rather, they are interrupted by metaxylem elements. In this type, there are three BS layers around all vascular bundles, even small ones: Kranz BS surrounded by the MS, and parenchymatous BS (external to the MS) having fewer chloroplasts than in M cells. Both NADP-ME biochemical subtype in genera *Fimbristylis* and *Bulbostylis* (Ueno et al., 1986; Ueno, 1998a), and NAD-ME subtype in some species of *Eleocharis* (Ueno, 1998b; Murphy et al., 2007), have been reported to have Fimbristyloid anatomy. The species of *Fimbristylis* have Kranz cells with chloroplasts that are centrifugally located and nearly agranal having numerous small, short grana; mitochondria are small and few, consistent with NADP-ME type photosynthesis (Carolin et al., 1977; Gilliland and Gordon-Gray, 1978). One population of *Eleocharis vivipara* (type 1) and *E. retroflexa* ssp. *chaetaria* are NAD-ME type C_4 species with Fimbristyloid-like anatomy; the latter has Kranz cell chloroplasts with well-developed grana and large mitochondria, typical of NAD-ME type C_4 species (Ueno and Samejima, 1989; Ueno et al., 1989; Ueno, 1996a). Immunolocalization studies show M and parenchymatous BS cells of *E. vivipara* type I (Ueno, 1996b) and *Fimbristylis dichotoma* (Ueno, 1998a) have PEPC and pyruvate, Pi dikinase (PPDK), indicating both cell types function to capture CO_2 by PEPC, with delivery of C_4 acids to the Kranz cells, where Rubisco is located.

Chlorocyperoid: Biochemical Subtype NADP-ME

The Chlorocyperoid type, as a rule, has two layers of BS, with the Kranz cells internal to the MS. Chlorenchyma cells external to the MS include a partial parenchymatous chlorenchyma sheath (occurring in large vascular bundles, it is less developed than in the Fimbristyloid, and may be completely absent in some species) and palisade-like M cells, both of which are considered to function in the carboxylation phase of the C_4 cycle. Kranz cells contain few large centrifugally-arranged chloroplasts having mostly single stroma thylakoids, convoluted in loops. The degree of grana reduction varies in different species. The SL is usually discontinuous in the radial cell wall of the MS and absent from Kranz BS (Ueno et al., 1988a, b; Bruhl and Perry, 1995). In some species (as with the Fimbristyloid), both

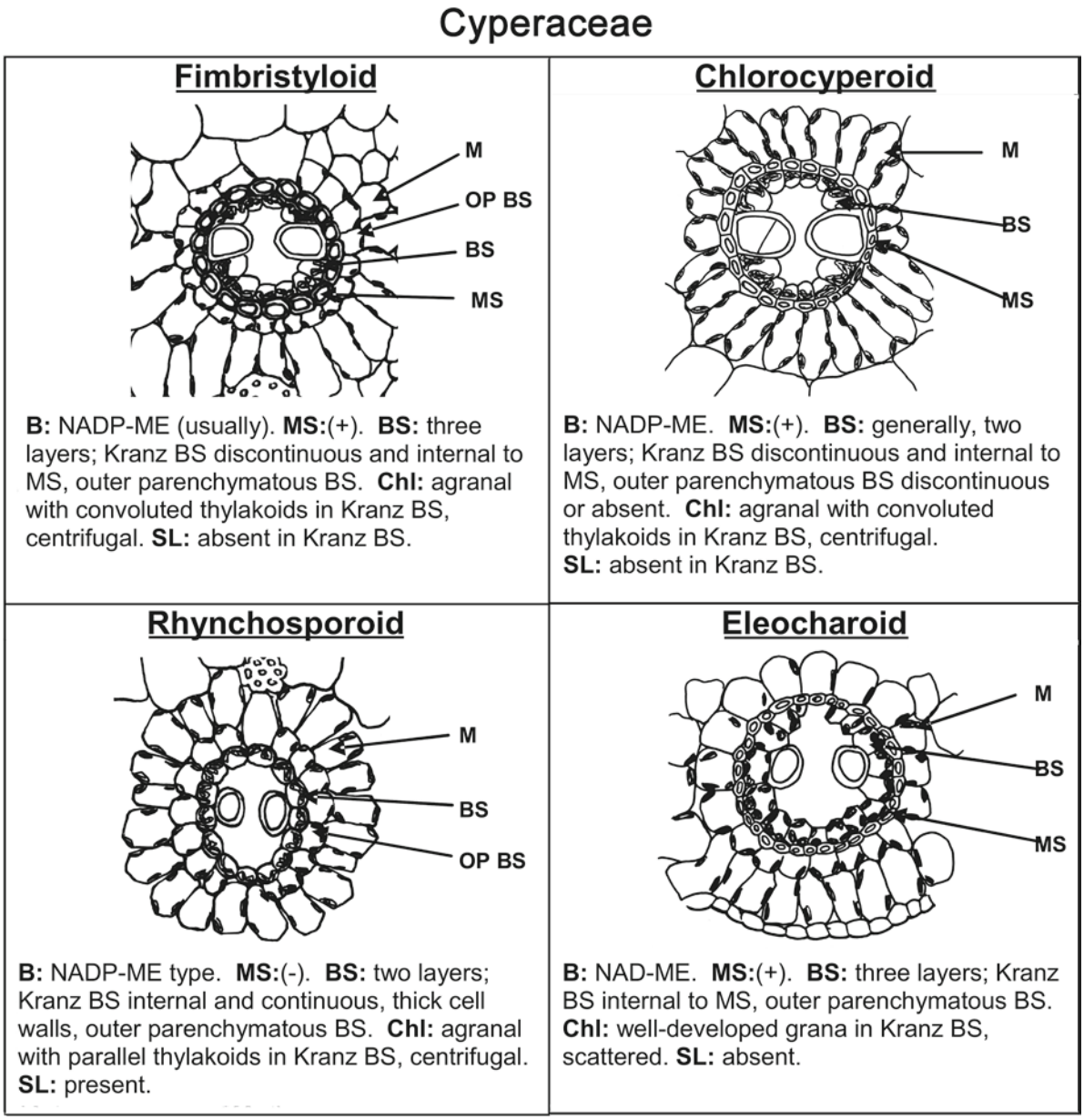


Fig. 2. Illustrations of the forms of Kranz anatomy in family Cyperaceae. Sketches of vascular bundles in *Fimbristylis* sp. for Fimbristyloid type, in *Cyperus* sp. for Chlorocyperoid type, in *Rhynchospora* sp. for Rhynchosporoid type and in *Eleocharis retroflexa* for Eleocharoid type (Drawings to illustrate the anatomy were made from light micrographs, Dengler and Nelson, 1999). For abbreviations, see Fig. 1.

M and parenchymatous BS cells may function to capture CO_2 by PEPC, with delivery of C_4 acids to the Kranz cells, where Rubisco is exclusively localized (Ueno, 1998a). This type of anatomy has been found in genera *Cyperus*, *Kyllinga*, *Pycreus* and *Torulinium* of the tribe Cypereae and *Lipocarpha* in the Lipocarpheae (Carolin et al., 1977; Gilliland and Gordon-Gray, 1978; Ueno et al., 1986, 1988a). Representative species having Chlorocyperoid type anatomy have NADP-ME type C_4 photosynthesis (Ueno et al., 1986; Bruhl et al., 1987). *Eleocharis baldwinii*, which has NAD-ME biochemistry and ultrastructure, has an intermediate type of anatomy called sub-Chlorocyperiod (Ueno and Samejima, 1989; Ueno, 2004).

Rhynchosporoid: Biochemical Subtype NADP-ME

Unlike the other forms of Kranz anatomy in Cyperaceae, in Rhynchosporoid type the Kranz cells evolved from the MS (Takeda et al., 1980). This thick-walled sheath is surrounded by an incomplete chlorenchymatous parenchyma sheath and palisade-like M cells which are considered to function in fixation of atmospheric CO_2 into C_4 acids. Both M and outer parenchyma sheath chloroplasts have similar thylakoid structure with large grana. Kranz cells have numerous, centrifugally-arranged agranal chloroplasts but, unlike the convoluted thylakoids in the previous types, here the thylakoid membranes usually have a parallel arrangement (Gilliland and Gordon-Gray, 1978; Ueno et al., 1988a; Bruhl and Perry, 1995). Mitochondria are comparable in size and number in M and BS cells. The SL is mostly continuous around the cell but can be discontinuous in the radial cell walls. Biochemical analysis indicates NADP-ME type photosynthesis (Ueno et al., 1986; Bruhl et al., 1987).

Eleocharoid: Biochemical Subtype NAD-ME

The Eleocharoid type anatomy was named after C_4 species of *Eleocharis* which have three types of BS, with the innermost Kranz cells forming a continuous wreath. As in Chlorocyperoid and Fimbristyloid types, the Kranz cells originate internal to the MS. The outer parenchyma BS contain some chloroplasts, while the middle MS lacks, or contains only a few, chloroplasts filled with starch; Kranz cells contain numerous organelles typical of NAD-ME species, with no strict orientation in the cell (scattered around the periphery) or tending slightly towards centrifugal. Chloroplasts of Kranz cells have well-developed grana and store starch; chloroplasts of parenchyma BS are smaller than those in M cells, but in both types of cells there are well-developed grana. Usually, a SL is present on both the inner and outer tangential cell walls in the MS, but sometimes it is absent on the radial cell walls (Ueno and Samejima, 1989; Bruhl and Perry, 1995). The Kranz cells have abundant and large mitochondria, typical of NAD-ME type C_4 grasses and dicots (Ueno and Samejima, 1989; Bruhl and Perry, 1995; Ueno, 2004; Ueno and Wakayama, 2004). The genus *Eleocharis* is very diverse in forms of photosynthesis between species (C_3, C_3–C_4, C_4-like). It includes amphibious species which change their mode of photosynthesis between submerged and terrestrial growth; and both Eleocharoid and Fimbristyloid type anatomy with NAD-ME type photosynthesis has been found among its C_4 species (Bruhl et al., 1987; Bruhl and Perry, 1995; Ueno, 2004; Ueno and Wakayama, 2004; Murphy et al., 2007).

3. Dicotyledons

Among dicot families, it is well-established that family Chenopodiaceae has the largest number of C_4 species and also the greatest diversity in leaf anatomy, including C_3, C_4 Kranz and C_4 single-cell types (Carolin et al., 1975; Pyankov et al., 1992; Sage et al., 1999; Edwards et al., 2004; Voznesenskaya et al., 2007). This family has been studied most extensively, resulting in classification of six types of Kranz anatomy (Fig. 3) which has been extended to several other families (Carolin et al., 1975, 1982; Jacobs, 2001). The C_4 types of leaves vary in the structure and arrangement of chlorenchyma tissue, in arrangement of water storage and vascular tissue, and by the presence, or absence, of various specialized hypodermal cells. Within these six main types of Kranz anatomy, additional anatomical differences have been recognized, indicating potential for further subdivision of structural types of Kranz in the family (Kadereit et al., 2003). The C_4 structural types in family Chenopodiaceae are named after the corresponding taxonomic names, as indicated below. These main structural forms were also given descriptive names (Vasilevskaya and Butnik, 1981; Voznesenskaya and Gamaley, 1986) which are also referred to in the descriptions below. In addition to the six Kranz types in the Chenopodiaceae, other forms have been recognized in dicot lineages found in *Cleome* (Cleomaceae), *Isostigma* and Glossocardia (Asteraceae) and *Portulaca* (Portulacaceae).

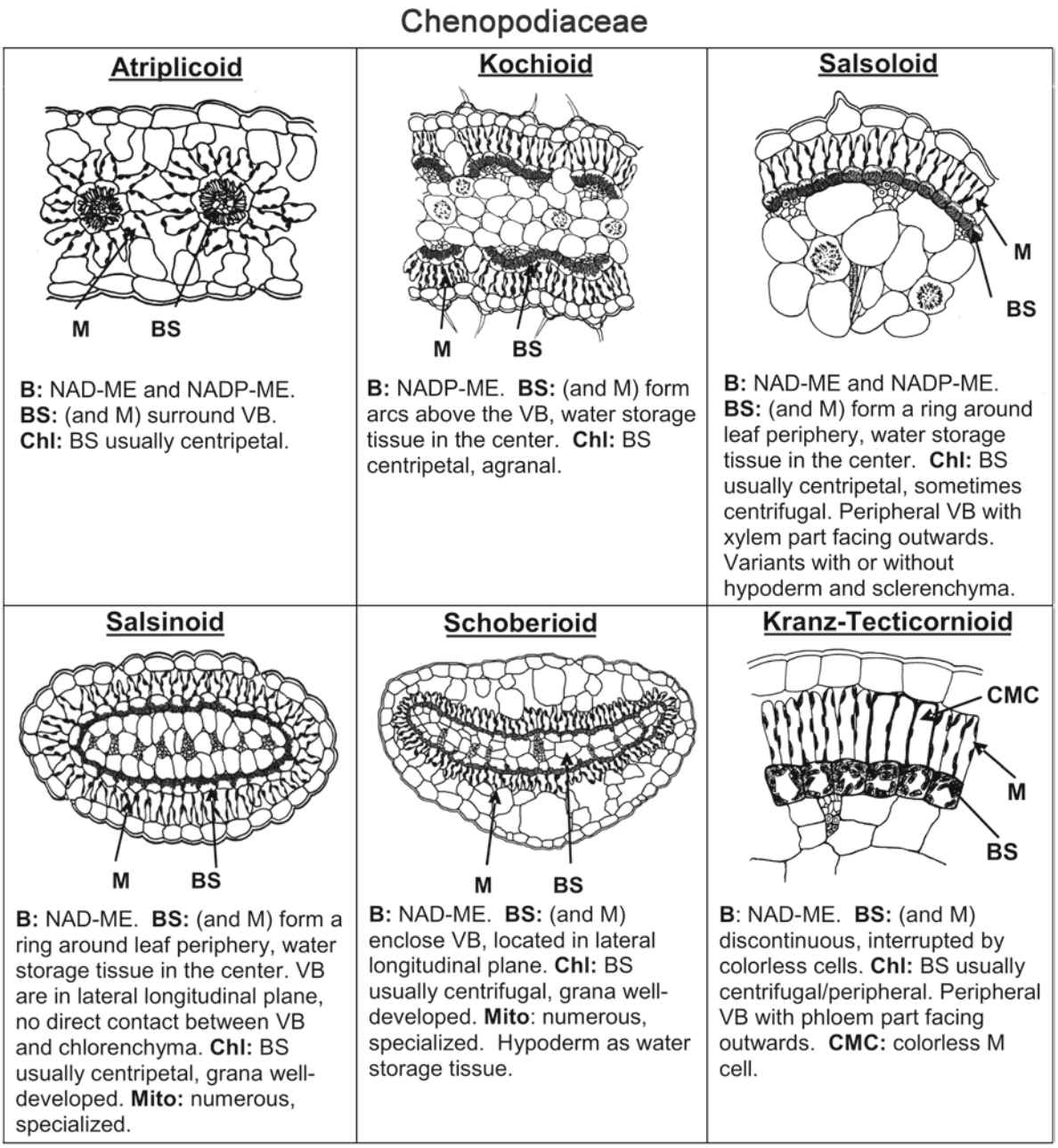


Fig. 3. Illustrations of the forms of Kranz anatomy in family Chenopodiaceae. Sketches of leaf structure in *Atriplex* sp. for Atriplicoid type (tribe Atripliceae), *Bassia hyssopifolia* for Kochioid type (tribe Camphorosmeae), *Salsola collina* for Salsoloid type (tribe Salsoleae), *Suaeda taxifolia* for Salsinoid type (tribe Suaedeae), *Suaeda eltonica* for Schoberioid type (tribe Suaedeae), and *Tecticornia* (=*Halosarcia*) *indica* for Kranz-Tecticornioid type (tribe Salicornieae) (Some pictures are adapted from Voznesenskaya and Gamaley, 1986). For abbreviations, see Fig. 1.

Atriplicoid: Biochemical Subtypes NAD-ME and NADP-ME

In C_3 dicots, all the vascular bundles are surrounded by a parenchyma sheath which is more or less distinguishable from M tissue; this sheath becomes a specialized Kranz BS in C_4 species. In Atriplicoid type of anatomy which occurs in some dicot species having laminate leaves, the Kranz tissue forms a classical wreath-like

structure with concentric layers of chlorenchyma around each vascular bundle. The Kranz BS encloses vascular bundles; although it can become disrupted on the phloem side in larger bundles. There is structural diversity and potential for recognition of additional subtypes where Kranz encloses individual veins in C_4 dicots having flattened leaves. In this type, hypodermal tissue when present usually fulfills the role of water storage tissue. For example, *Portulaca oleracea* has extensively developed water storage hypoderm with variable positioning of the veins between the abaxial and adaxial sides of the leaf. In Atriplicoid type, palisade M cells are usually arranged radially; but this can vary in different species (Rathnam et al., 1976; Dengler and Nelson, 1999; McKown et al., 2005; Muhaidat et al., 2007). For species of families Chenopodiaceae and Amarathaceae, Kadereit et al. (2003) distinguished four different types of anatomy in laminate leaves within the Atriplicoid type, which differ in the presence or absence of hypoderm, the occurrence of parenchyma cells between M cells, or the occurrence of additional layers of spongy parenchyma on the abaxial side of the leaf. Nevertheless, similar features may be found in other taxons. Two biochemical subtypes, NADP-ME and NAD-ME, have been found in species having this leaf structure, each having differences in chloroplast ultrastructure (Laetsch, 1968; Kennedy and Laetsch, 1974; Carolin et al., 1975, 1978; Rathnam et al., 1976; Gamaley and Voznesenskaya, 1986; Voznesenskaya and Gamaley, 1986; Sage et al., 1999; Marshall et al., 2007; Muhaidat et al., 2007; Akhani et al., 2008). In the NADP-ME subtype, BS chloroplasts have reduced grana, as is typical for this subtype. The NADP-ME subtype is present in Acanthaceae, Aizoaceae, Amaranthaceae, Asteraceae, Boraginaceae, Cariophyllaceae, Chenopodiaceae, Euphorbiaceae, Nyctaginaceae, Portulacaceae and Zygophyllaceae. The opposite variant, NAD-ME type, which has well-developed grana in BS chloroplasts and reduced grana in M chloroplasts, is found in Acanthaceae, Aizoaceae, Amaranthaceae, Chenopodiaceae, Cleomaceae, Euphorbiaceae, Gisekiaceae, Molluginaceae and Portulacaceae. As a rule, Kranz BS have thickened cell walls, but the thickness varies; they are usually thinner in NAD-ME species. Organelles are usually arranged centripetally in BS cells, except for *Trianthema triquetra* (Aizoaceae), which has centrifugal positioning of organelles (Carolin et al., 1975).

Kochioid: Biochemical Subtypes NAD-ME and NADP-ME

Kochioid type species, also referred to as Semi-Wreath type, have laminate or semi-terete to terete succulent leaves with water-storage tissue underneath the chlorenchyma. The main vascular bundle is in the center, and the remaining vascular bundles are located in two paradermal planes on the leaf periphery or around the periphery in terete leaves. The chlorenchyma tissue is distributed along the peripheral veins; BS and M cells form arcs above the vascular bundles. Bundle sheath cells have relatively thick cell walls, and organelles are located in the centripetal position. Kadereit et al. (2003) recognized three different types of anatomy with such distribution of chlorenchyma tissues, differing in the presence or absence of hypoderm in two *Kochia* species, while in *Kirilowia* species, vascular bundles with arcs of chlorenchyma are distributed in the lateral plane only on the adaxial side of the leaf, with spongy parenchyma on the abaxial side. Species with this type of anatomy have been found to have NADP-ME type biochemistry and chloroplast ultrastructure (reduction in grana in BS cells is highly pronounced, up to having totally agranal chloroplasts) in genera *Bassia* and *Kochia* of family Chenopodiaceae (Gutierrez et al., 1974; Carolin et al., 1975; Gamaley, 1985; Voznesenskaya and Gamaley, 1986; Pyankov et al., 2000a; Jacobs, 2001), and NAD-ME type biochemistry in C_4 species of the genus *Zygophyllum* with respective granal chloroplasts and numerous specialized mitochondria in BS cells (Crookston and Moss, 1972; Muhaidat et al., 2007). In the latter case, the leaf is cylindrical with the main vascular bundle in the center of water storage tissue. Two layers of Kranz tissue form arcs outside of the small peripheral veins.

Salsoloid: Biochemical Subtypes NAD-ME and NADP-ME

Species with Salsoloid type anatomy, also referred to as Kranz-Centrical type, have cylindrical or

terete leaves (or stems in aphyllous species) with two concentric layers of chlorenchyma, typical of C_4 Kranz anatomy, located around the periphery of assimilating organs. The central part is occupied by water storage tissue with the main vein in the middle. The net of secondary vascular bundles penetrates into the water storage tissue; and the small peripheral veins contacting with BS cells are facing toward the chlorenchyma by their xylem. In some desert species, a scleromorphous variant of this type has been found which has a high volume of sclerenchymatous tissue in the center around the main vein and/or in the peripheral bundles, with only a small amount of water storage tissue, for example, in reduced leaves of *Nanophyton erinaceum*, in the leaves and stems of *Arthrophytum lehmannianum* from family Chenopodiaceae, or in some *Calligonum* species of family Polygonaceae (see Butnik et al., 2001). Kadereit et al. (2003) distinguished five different types of anatomy within this type: *Salsola* type with or without hypoderm, *Nanophyton* type with sclerenchyma, *Climacoptera* type having no contact of peripheral veins with chlorenchyma and *Halothamnus auriculus* type with flattened leaves and several secondary veins distributed in the water storage parenchyma in lateral plane and the net of small peripheral veins adjacent to BS cells. Two biochemical subtypes, NADP-ME (in genera *Salsola*, *Halothamnus*, *Haloxylon*, *Horaninovia and* some others in family Chenopodiaceae) and NAD-ME (for example in genera *Salsola*, *Climacoptera*, *Halocharis* in Chenopodiaceae and *Calligonum* in Polygonaceae), with their respective ultrastructural chloroplast subtypes, have been found in species with this anatomy (Winter et al., 1977; Voznesenskaya and Gamaley, 1986; Pyankov and Vakhrusheva, 1989; Sage et al., 1999; Pyankov et al., 2000c; Muhaidat et al., 2007). Variants occur with or without hypodermal tissue, which, if present, plays the role of additional water storage tissue. Usually BS chloroplasts are in the centripetal position, but species of the genus *Halothamnus* (previously named *Aellenia*) have centrifugally-arranged chloroplasts, see Edwards et al. (2004).

Salsinoid: Biochemical Subtype NAD-ME

Species with Salsinoid type anatomy (also referred to as Kranz-Isopalisade Circular type) occur in genus *Suaeda*, section Salsina (Kadereit et al., 2003; Schütze et al., 2003). They have terete leaves with two concentric layers of chlorenchyma, palisade M and Kranz cells, around the leaf periphery, and water storage tissue in the center of the leaf. The vascular tissue forms a network in the lateral longitudinal plane; there are no peripheral vascular bundles and only the lateral veins may have contact with chlorenchyma. Only one biochemical subtype has been found, NAD-ME, and structural characteristics are typical for this subtype: numerous specialized mitochondria and chloroplasts with well-developed grana in BS cells, and reduced grana in M chloroplasts. Unlike other C_4 subtypes, the Kranz cells have a large vacuole with less abundant organelles which occur in a centripetal position in a relatively thin layer of cytoplasm. This type was originally called Kranz-Suaedoid (Carolin et al., 1975; Jacobs, 2001). However, subsequently this form of Kranz was recognized as Salsina type after the section of *Suaeda* in which it occurs (Schütze et al., 2003), and is called Salsinoid here for consistency in nomenclature. For a description of the structural and functional features, see (Shomer-Ilan et al., 1975, 1979, 1981; Fisher et al., 1997; Voznesenskaya et al., 2007).

Schoberioid: Biochemical Subtype NAD-ME

This is another form of Kranz anatomy in genus *Suaeda* which recently was called Schoberia after the section in which it occurs (Kadereit et al., 2003; Schütze et al., 2003); it is called Schoberioid type here for consistency in nomenclature (also referred to as Kranz-Isopalisade type). Before more recent phylogenetic analyses of the Suaedoideae subfamily, it was referred to as Conospermoid type anatomy by Freitag and Stichler (2000). It is found in semi-terete leaves with positioning of vascular bundles in a lateral plane. This subtype is unique in having the vascular bundles enclosed by two layers of Kranz type chlorenchyma in the central part of the leaf, with continuous BS extensions between the veins. Large hypodermal cells, which are located between the chlorenchyma and epidermis, function as water storage tissue. These are NAD-ME type species with typical ultrastructural features for this subtype: BS cells have

granal chloroplasts and specialized mitochondria, and M cells have reduced chloroplasts with less grana development. Unlike the Salsinoid type, Schoberioid type species have BS chloroplasts located in the centrifugal position. A variant of this type of anatomy has been found in *Suaeda cochlearifolia*, which has only one layer of BS cells between the vascular bundles (Voznesenskaya et al., 2007).

Kranz-Tecticornioid: Biochemical Subtype NAD-ME

This unique structural subtype of Kranz anatomy is found in the genus *Halosarcia* (*H. indica*, Salicornieae, family Chenopodiaceae) (Carolin et al., 1982; Jacobs, 2001), which is now included in the broadly circumscribed genus *Tecticornia* (Shepherd and Wilson, 2007). In general appearance, it is similar to the Kranz-Centrical (Salsoloid) type of leaf anatomy, with peripheral distribution of two chlorenchyma layers in cylindrical assimilating stems and with a net of small peripheral vascular bundles adjoining BS cells. However, in the Kranz-Tecticornioid type, these small veins are oriented with the phloem side facing towards the chlorenchyma. A striking feature of this type is the presence of bands of thick-walled colorless parenchyma cells between groups of chlorenchymatous M cells. Also, in the Kranz cells granal chloroplasts tend to be located centrifugally, or occasionally scattered around the periphery of the cell. Western blot analysis for C_4 acid decarboxylases and immunolocalization studies indicate NAD-ME type C_4 photosynthesis. There are numerous mitochondria in the Kranz cells, which compared to other NAD-ME species in the family are smaller, but they have a similar specialized structure (Voznesenskaya et al., 2008). Mesophyll chloroplasts have reduced grana, characteristic of this biochemical subtype.

Pilosoid: Biochemical Subtype NADP-ME

An interesting variant of Kranz anatomy occurs in species of the clade Pilosa, genus *Portulaca,* family Portulaceae (for example, in *P. grandiflora, P. pilosa, P. villosa, P. sclerocarpa*) which have terete cylindrical leaves with a circular arrangement of the small vascular bundles around the leaf periphery, with main vein and water storage cells in the center. Each peripheral vein is surrounded by BS cells (sometimes less developed on the inner side), with M cells forming a wreath-like structure only on the outer and lateral sides of the vascular bundles as illustrated in Nishioka et al. (1996) and Kim and Fisher (1990). Thus, the structure of the mesophyll-bundle sheath-vascular bundle complex of each vein is similar to one of the variants of Atriplicoid type anatomy, but differs in having a peripheral arrangement of veins around the leaf (Fig. 4). Flattened leaves of *P. amilis* have similar arrangement of VB but only about four layers of water storage tissue in the middle part of the leaf. The whole leaf anatomy can be considered to represent an intermediate stage of evolution from laminate Atriplicoid anatomy to Kochioid or directly to Salsoloid. NADP-ME type of biochemistry is well known for *P. grandiflora* (Gutierrez et al., 1974; Guralnick et al., 2002). It was recently also shown for two other species of this clade, *P. pilosa* and *P. amilis* (Voznesenskaya et al., 2010); and, all other studied species with this type of anatomy have similar ultrastructural features of BS and M chloroplasts characteristic of this biochemical subtype. A similar distribution of vascular bundles was found in *Zygophyllum simplex*; but, with Kranz tissue forming open arcs typical for Kochioid type of anatomy, showing similar evolutionary trends in different families.

Portulacelloid: Biochemical Subtype NADP-ME

Species of the clade/section Portulacella, genus Portulaca (Portulacaceae) have vascular bundles surrounded by two concentric layers of Kranz anatomy distributed only on the adaxial side of the leaf; there are 4–5 layers of water storage tissue on the abaxial side (Voznesenskaya et al., 2010). As in Pilosoid type, palisade M cells are better developed on the upper and lateral sides of vascular bundles. It is NADP-ME biochemical type with centripetal position of grana-deficient chloroplasts in bundle sheath cells.

Glossocardioid: Biochemical Subtype NAD-ME and NADP-ME

Kranz anatomy similar to Salsoloid type was reported for representatives of family Asteraceae *Glossocardia bosvallia* (Das and Raghavendra,

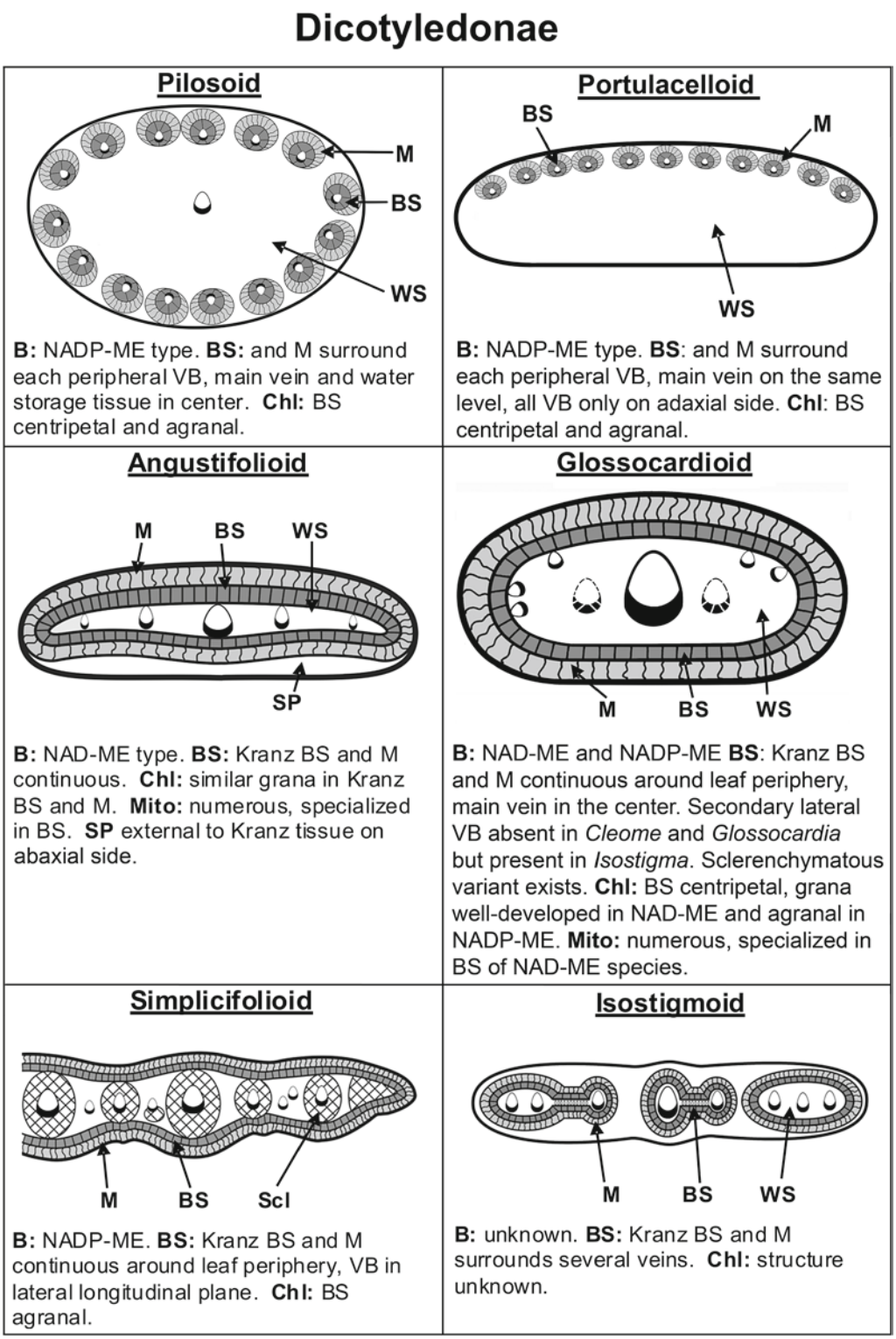


Fig. 4. Illustration of other forms of Kranz anatomy among Dicotyledonae. Sketches of vascular bundles in *Portulaca grandiflora* for Pilosoid type, *Portulaca* cf. *bicolor* for Portulacelloid type, *Cleome angustifolia* cotyledon for Angustifolioid type, *Glossocardia bosvallia* and/or *Cleome angustifolia* leaf for Glossocardioid type, *Isostigma simplicifolium* for Simplicifolioid type, and Isostigmoid type (adapted from Fig. 2, Peter and Katinas, 2003). SP, spongy parenchyma tissue; for other abbreviations, see Fig. 1.

1976) and some *Isostigma* species (Peter and Katinas, 2003), and also for *Cleome angustifolia* leaf (Cleomaceae); all these species have semi-terete to terete leaves with concentric layers of Kranz type chlorenchyma surrounding the leaf on the periphery. Leaf venation consists of the central main vein with or without lateral secondary veins embedded in the water storage parenchyma, with sclerenchyma tissue being either present around veins or absent depending on the species. Small peripheral bundles are in contact with BS cells with their xylem side, characteristic of Salsoloid type anatomy. The main difference from the classic Salsoloid anatomy is the absence of even distribution of small veins around the leaf periphery. The biochemical subtype of *G. bosvallia* based on western blot analysis is NADP-ME, while *C. angustifolia* is NAD-ME, the biochemical subtype for *Isostigma peucedanifolium* is NADP-ME based on deficiency of grana in BS chloroplasts (Voznesenskaya, Koteyeva and Edwards, unpublished). This type of structure was designated as *Eryngiophyllum* (= *Chrysanthellum*) in Peter and Katinas (2003); but it has only been observed in *Isostigma* and *Glossocardia* in family Asteraceae. Therefore, according to the genus where it was first described (Das and Raghavendra, 1976), we define this as Glossocardioid type anatomy.

Simplicifolioid: Biochemical Subtype NADP-ME

Isostigma simplicifolium (family Asteraceae) has a form of Kranz anatomy in which the chlorenchyma tissue forms a continuous layer around flattened leaves (Fig. 4). The major veins, which run in parallel with rare anastomoses, are enclosed in the well-developed sclerenchyma sheaths, with only very small bundles lacking sclerenchyma tissue. There is some water storage parenchyma between major veins. The biochemical subtype is NADP-ME based on deficiency of grana in BS chloroplasts (E. Voznesenskaya, N. Koteyeva and G. Edwards, unpublished).

Isostigmoid: Biochemical Subtype unknown

An unusual form of Kranz anatomy, called Isostigmoid, was reported for several species of the genus *Isostigma* in family Asteraceae having flattened leaves (Peter and Katinas, 2003). In Isostigmoid type, instead of Kranz anatomy encircling individual veins, as occurs in Atriplicoid, the two chlorenchyma layers surround several veins together (illustrated in Fig. 4). It is considered an intermediate form between Atriplicoid type and anatomical forms having a continuous ring of Kranz anatomy around the leaf. The biochemical subtype is unknown.

Angustifolioid: Biochemical Subtype NAD-ME

This form of Kranz anatomy occurs in cotyledons of the C_4 species *Cleome angustifolia* (family Cleomaceae). Kranz tissue continuously surrounds the central part of the blade with vascular bundles distributed in the lateral longitudinal plane and with the main vein in the center of the leaf. Vascular bundles are separated by water storage parenchyma. Chlorenchyma layers, consisting of palisade M and BS cells, are located under the epiderm on the adaxial side; whereas, on the abaxial side the M cells adjacent to BS are small and rounded, and they are separated from the epiderm by two layers of spongy M cells (Fig. 4). Bundle sheath and M cells have ultrastructural features characteristic of NAD-ME biochemistry with granal BS chloroplasts and numerous mitochondria in BS cells; but, there is no difference in the granality between BS and M chloroplasts. This type of anatomy differs from all other known C_4 types. The concentric layer of Kranz tissue around veins has some features of Salsinoid type, but this type is unusual in having spongy parenchyma on the abaxial side of the leaf. The biochemical subtype of this species is NAD-ME (Voznesenskaya, Koteyeva and Edwards, unpublished).

B. Biochemical Diversity: C_4 Cycles and Energy Requirements for C_4 Subtypes

1. Chloroplasts and Mitochondria

Despite C_4 photosynthesis having evolved multiple times in different families and subfamilies with structural variations on Kranz anatomy, species belonging to each biochemical subtype tend to have features in common in terms of the structure of chloroplasts in M and BS cells, and the occurrence of mitochondria in BS cells. In NADP-ME type C_4 species, BS chloroplasts are deficient in grana compared to M chloroplasts, whereas in NAD-ME type species, the

M chloroplasts tend to be more deficient in grana development (Gamaley, 1985; Voznesenskaya and Gamaley, 1986; Fisher et al., 1997; Voznesenskaya et al., 1999). In PEP-CK type C_4 species, the granal development is similar for M and BS chloroplasts (Yoshimura et al., 2004; Voznesenskaya et al., 2006). This has been quantified in a number of studies on different photosynthetic types by determining the granal index (the length of all appressed thylakoid membranes as a percentage of the total length of all thylakoid membranes in a chloroplast). Early studies indicate that the degree of grana development in BS chloroplasts correlates to the capacity for photosystem II (PSII) activity, linear electron flow and capacity for generation of NADPH, with low grana-containing chloroplasts being richer in photosystem I (PSI)-mediated cyclic electron flow producing ATP (Edwards and Walker, 1983; Anderson, 1999). Thus, differences in grana development are associated with differences between M and BS cells in the need for NADPH relative to ATP to support C_4 photosynthesis.

Mitochondria are most abundant, and often larger, in BS cells of NAD-ME type species (where the decarboxylase is located in mitochondria), and least abundant in BS cells of NADP-ME type species (where the decarboxylase is located in chloroplasts). Bundle sheath mitochondria in PEP-CK species also function to provide ATP to support decarboxylation via PEP-CK in the cytosol (Burnell and Hatch, 1988), and they are generally intermediate in size and number compared to NAD-ME or NADP-ME type species (Yoshimura et al., 2004; Voznesenskaya et al., 2006).

The basal energy required per CO_2 assimilation in C_4 photosynthesis is the sum of energy to support the C_4 and C_3 cycles. Analyses to date on biochemical subtyping (by Western blots, enzyme assays) indicate each species has a major form of delivery of CO_2 to Rubisco through one type of C_4 acid decarboxylase. For each biochemical subtype, the amount of energy required to support the C_4 cycle for delivery of CO_2 and the C_3 cycle for fixation of CO_2 can be calculated. This is shown in Fig. 5, with illustrations of how the provision of energy can be met cooperatively by M and BS chloroplasts. This demonstrates how the photochemical demands for energy can be shared between the cell types, and the differences between the three types of C_4 cycles. However, the exact photochemical demands for energy within each subgroup may vary between species. This is most evident in NADP-ME type C_4 species, as discussed below.

2. Illustration of Energetics for NADP-ME Type Species

Summary

1. 5 ATP, 2 NADPH required per CO_2 assimilated (2 ATP for the C_4 cycle, 3 ATP, 2 NADPH for the C_3 cycle).
2. The C_4 cycle delivers primarily malate to BS cells (NADP-ME species are mainly malate formers).
3. In NADP-ME type C_4 species, BS chloroplasts have fewer grana than do M chloroplasts, but there is variation in the degree of deficiency of grana in the BS chloroplasts. The extremes range from the BS chloroplasts being agranal (in sugarcane, sorghum), to BS chloroplasts having granal indices about half that of M chloroplasts, observed in members of family Chenopodiaceae (see Voznesenskaya et al., 1999). The illustration in Fig. 5a is based on the granal index and linear electron flow to generate NADPH being two fold higher in M than in BS chloroplasts, as observed in some NADP-ME type chenopods (Voznesenskaya et al., 1999). The two fold higher use of reductive power in BS cells could vary due to the amount of 3-phosphoglyceric acid (PGA) shuttled from BS to M chloroplasts for reduction (in the current scheme, one sixth of the PGA). Alternatively, this balance in reductive power could be modified in NADP-ME dicots by a partial shuttle of aspartate from M to BS cells through NADP-ME, see Moore et al. (1984) and Meister et al. (1996). Also, the degree of grana development in BS chloroplasts of NADP-ME type species appears to correlate with the development of a secondary aspartate PEP-CK shuttle (Gutierrez et al., 1974; Wingler et al., 1999; Voznesenskaya et al., 2006).

The deficiency in PSII in BS chloroplasts in this subtype is thought to reduce production of O_2 in BS cells and help maintain a high CO_2/O_2 ratio, which is favorable for limiting ribulose 1,5-bisphosphate (RuBP) oxygenase activity and photorespiration. This results in an increased

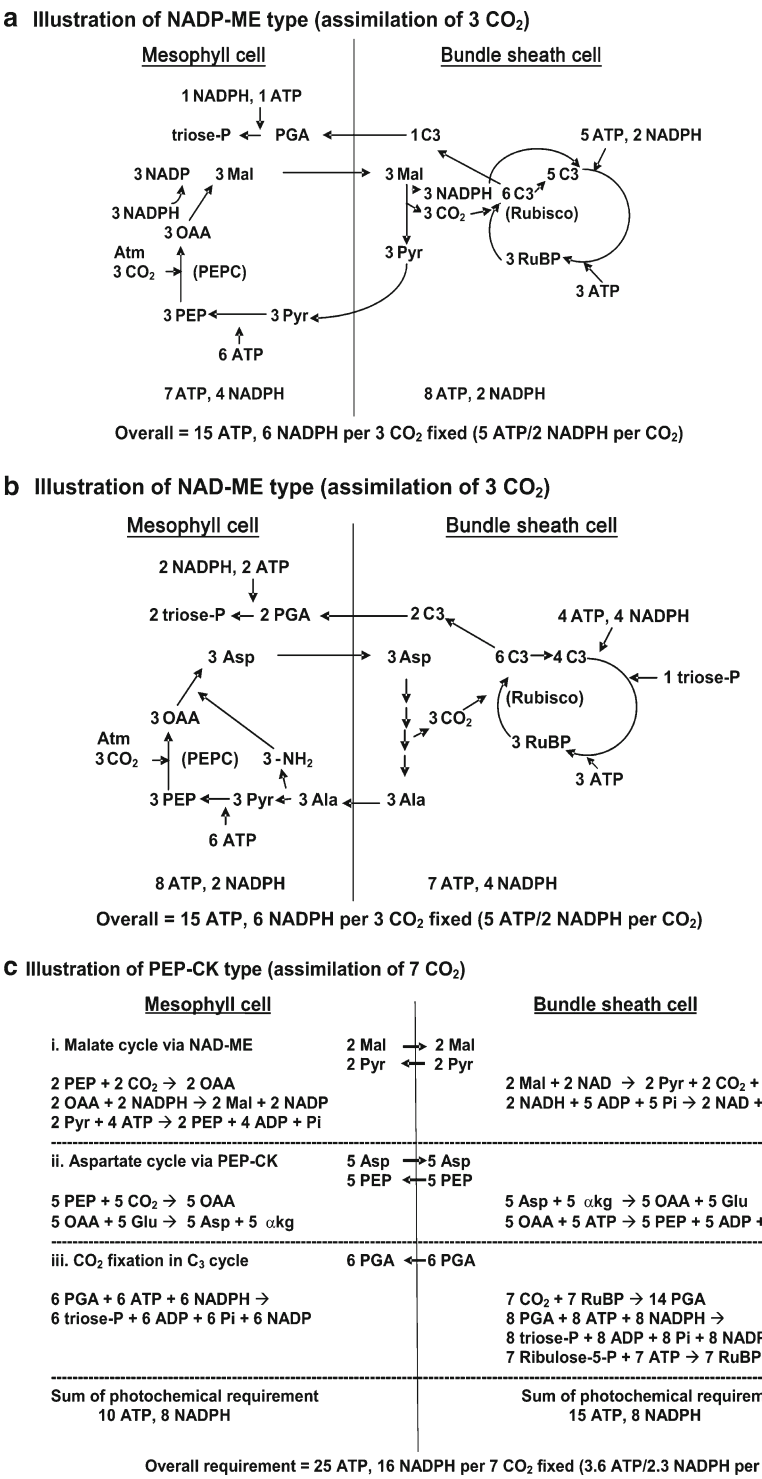


Fig. 5. Illustrations of the three types of C_4 cycles and their bioenergetics: **a**, NADP-ME, **b**, NAD-ME and **c**, PEP-CK subtypes. *αkg*, α-ketoglutarate; *Ala*, alanine; *Asp*, aspartate; *Atm*, atmospheric; *Glu*, glutamate; *Mal*, malate; *OAA*, oxaloacetate; *PEP*, phosphoenolpyruvate; *PEPC*, PEP carboxylase; *PGA*, 3-phosphoglyceric acid; *Pi*, inorganic phosphate; *pyr*, pyruvate; *RuBP*, ribulose 1,5-bisphosphate; *triose-P*, triose phosphate. Panels a and b, adapted from Voznesenskaya et al., 1999, Oxford University Press.

need for photochemically-produced NADPH in M chloroplasts, see (Edwards and Walker, 1983).

3. Illustration of Energetics for NAD-ME Type Species

Summary

1. 5 ATP, 2 NADPH required per CO_2 assimilated (2 ATP for the C_4 cycle, 3 ATP, 2 NADPH for the C_3 cycle).
2. The C_4 cycle delivers primarily aspartate to BS cells (NAD-ME species are primarily aspartate formers). The aspartate cycle requires only production of ATP (but not reductive power) to drive PEP regeneration from alanine by the M chloroplasts (Edwards and Walker, 1983; Voznesenskaya et al., 1999). This ATP may be provided via PSI cyclic electron flow in the M chloroplasts. Extensive studies of NAD-ME-type species in family Chenopodiaceae have shown that M chloroplasts are deficient in grana compared to BS chloroplasts (Gamaley, 1985; Gamaley and Voznesenskaya, 1986; Voznesenskaya and Gamaley, 1986; Glagoleva et al., 1991).
3. In the illustration of Fig. 5b, the use and generation of reductive power in BS chloroplasts is two-fold higher than in M chloroplasts. This occurs with the granal index and linear electron flow to generate NADPH being two fold higher in BS than M chloroplasts, as observed in some NAD-ME type chenopods (Voznesenskaya et al., 1999). Again, this partitioning of reductive power can be regulated by the amount of PGA shuttled from BS to M chloroplasts for reduction to triose-P (in the current scheme, one third of the PGA is shuttled to M cells).

4. Illustration of Energetics for PEP-CK Type Species

Summary

1. 3.6 ATP, 2.3 NADPH required per CO_2 assimilated (0.6 ATP and 0.3 NADPH per CO_2 for the C_4 cycles; 3 ATP, 2 NADPH for the C_3 cycle). The PEP-CK type requires less ATP, but more NADPH per CO_2 fixed than the malic enzyme type species.
2. There are two C_4 cycles, one shuttling aspartate, the other shuttling malate (Fig. 5c). Aspartate is utilized in the cytosol to generate oxaloacetate for the PEP-CK reaction. Malate is used by the mitochondria via NAD-ME, which generates CO_2 and NADH; the NADH is then utilized by the mitochondria to generate ATP to support the PEP-CK decarboxylase reaction (Burnell and Hatch, 1988; Walker and Chen, 2002; Voznesenskaya et al., 2006). In this scheme, the aspartate cycle generates about 70%, and the malate cycle about 30%, of the total CO_2 delivered to the BS cells.
3. In this illustration (Fig. 5c), an equal amount of reductive power is required by M and BS cells. This is consistent with M and BS chloroplasts of PEP-CK species having a similar granal index, suggestive of equivalent capacity for PSII activity for generating NADPH (Voznesenskaya et al., 2006). Again, this partitioning of reductive power can to some extent be regulated by the amount of PGA shuttled from BS to M chloroplasts for reduction (in the scheme, about 40%).

5. Additional Energy Requirements in C_4 Photosynthesis

Besides the basic requirements for energy to support the C_3 and C_4 cycles, additional energy will be consumed by over-cycling of the C_4 cycle (CO_2 leakage from BS cells, that is 2 ATP per CO_2 lost from BS cells), and due to the occurrence of limited photorespiration because of some O_2 reacting with RuBP, see Kanai and Edwards (1999). The cost of photorespiration is illustrated in Fig. 6. The requirement for reductive power per O_2 reacting with RuBP (eq. to 2 NADPH) is the same as for CO_2 reacting with RuBP, and the scheme illustrates how this can be shared equally between M and BS chloroplasts.

III. Single-Cell C_4 Photosynthesis in Terrestrial Plants

For decades following the discovery of C_4 photosynthesis in the 1960s, it was considered that the requirements for C_4, as summarized in the Introduction, could only be met in terrestrial plants by the presence of Kranz type anatomy. Thus, it was surprising to find species in family Chenopodiaceae that undergo traditional C_4 photosynthesis, but have a unique anatomy that does not consist of the Kranz dual-cell system. Instead, the single-cell C_4 system functions in individual

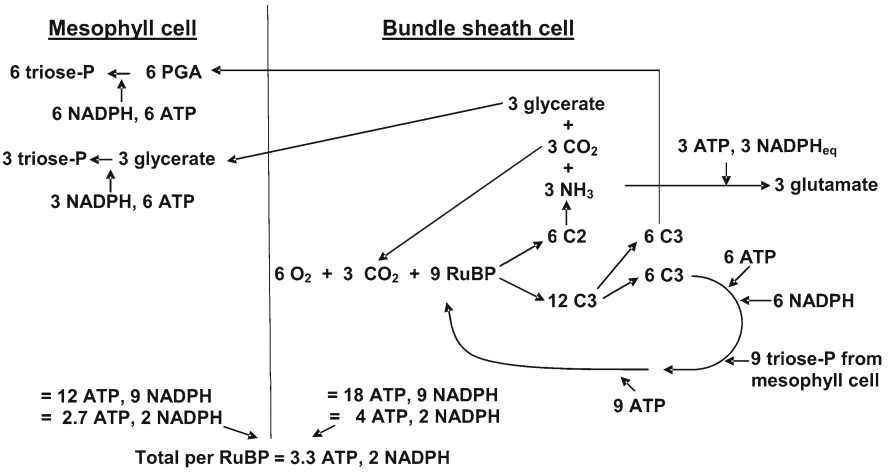


Fig. 6. Scheme of energetics of photorespiration in C_4 plants. *PGA*, 3-phosphoglyceric acid; *RuBP*, ribulose 1,5-bisphosphate; *triose-P*, triose phosphate.

chlorenchyma cells by means of intracellular biochemical and organelle compartmentation. Two very novel means of accomplishing this evolved in subfamily Suaedoideae. These systems function by spatial development of two cytoplasmic domains in chlorenchyma cells, which contain dimorphic chloroplasts. The arrangement of M and BS cells that so long has defined terrestrial forms of C_4 plants has now been joined by single-cell C_4 systems as functional anatomical alternatives (Voznesenskaya et al., 2001, 2002; Sage, 2002; Edwards et al., 2004; Akhani et al., 2005; Park et al., 2010).

A. Occurrence (Family and Phylogeny)

In family Chenopodiaceae, which has C_3 and C_4 species, all C_4 genera except for subfamily Chenopodioideae (including genus *Atriplex*) occur in a succulent clade made up of subfamilies Salicornioideae/Suaedoideae/Salsoloideae. The single-cell C_4 type species occur in subfamily Suaedoideae (Fig. 7), in two species in genus *Bienertia* (*B. cycloptera* Bunge ex Boiss., *B. sinuspersici* Akhani sp. nov.) and in one species in genus *Suaeda, S. aralocaspica* (Bunge) Freitag & Schütze (Kadereit et al., 2003; Schütze et al., 2003; Akhani et al., 2005; Kapralov et al., 2006). *Suaeda aralocaspica*, originally named *Borszczowia aralocaspica* Bunge, was subsequently classified in the monotypic *Suaeda* section Borszczowia, with a leaf type called Borszczowoid (Freitag and Stichler, 2000; Schütze et al., 2003; Kapralov et al., 2006).

There are four independent origins of C_4 photosynthesis in subfamily Suaedoideae: two parallel origins of Kranz C_4 anatomy (Salsinoid and Schoberioid in genus *Suaeda*) (see Section II A 3), and two independent origins of single-cell C_4. The single-cell C_4 plant *Suaeda aralocaspica* is in tribe Suaedeae. In this tribe, the veins are invariably located in one plane, with the primary vein in the center of the leaf and all deviating bundles in lateral positions. However, unlike other *Suaeda* species, *S. aralocaspica* has a primary vein in the center with peripheral veins adjacent to the chlorenchyma tissue (Freitag and Stichler, 2000; Schütze et al., 2003). This species is positioned between the C_3 section Schanginia and a C_3 shrubby section *Suaeda*, which suggests this type of photosynthesis evolved from C_3 ancestors rather than from a C_4 ancestor with Kranz anatomy (Kapralov et al., 2006). The two *Bienertia* species occur in an isolated tribe, Bienertieae, with a leaf type called Bienertioid, and the species have no known close relatives. We will use the common names Bienertia and Borszczowia to refer to these two types of single-cell C_4 taxa which are classified as C_4 structural forms called Bienertioid and Borszczowoid, respectively.

B. Biogeography of Single-Cell C_4 Species

The single-cell C_4 species grow in desert conditions. Borszczowia grows in central Asia from northeast of the Caspian lowlands east to Mongolia

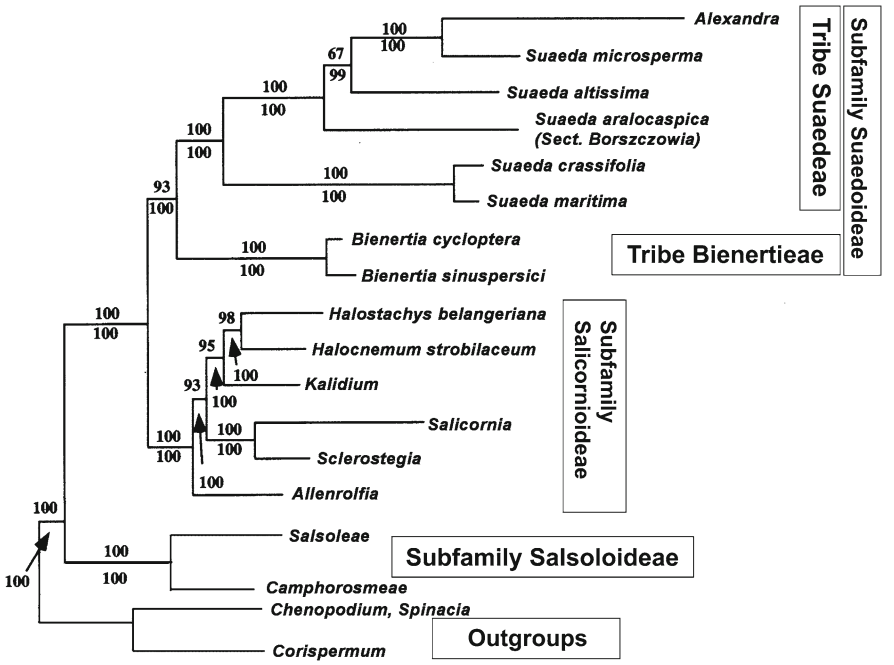


Fig. 7. Phylogenetic position of "single-cell" C_4 in Chenopodiaceae. The single maximum likelihood phylogram based on combined complete sequence information on nuclear ITS and five chloroplast DNA regions. Numbers above branches refer to bootstrap percentages and those below branches refer to Bayesian inference posterior probabilities (adapted from Kapralov et al., 2006).

and western China (Fig. 8). It is a hygro-halophyte that grows in temperate salt deserts with low night temperatures. The habitat consists of a high water table in salt marshes, which can support continuous leaf development and growth (Freitag and Stichler, 2000; Boyd et al., 2007).

Bienertia cycloptera grows from east Anatolia eastward to Turkmenistan and Pakistani Baluchestan. Its leaves are very succulent and sensitive to extended drought, which can cause wilting and leaf drop. It grows on dryer soils and is confronted with drought stress during the summer (Akhani et al., 2003).

Bienertia sinuspersici occurs in hot climates, and at lower latitudes and elevations than *B. cycloptera*. It is in a natural biogeographic range occurring from the westernmost coasts of Pakistan and extending westward along the coastal areas in southern Iran and countries surrounding the Persian Gulf. It shows an arc-like, latitudinal range that is separated from the range of *B. cycloptera* populations by the Zagros and Makran Mountains (Fig. 8). *Bienertia sinuspersici* differs anatomically by having mostly one to two layers of chlorenchyma cells, versus two to three layers in *B. cycloptera.* Furthermore, *B. sinuspersici* is distinguished from *B. cycloptera* in having longer cotyledon leaves and leaves proper, larger seeds, larger flowers, and larger chromosomes, together with a set of micro-morphological features (Akhani et al., 2005).

C. Overview of Two Types of Single-Cell C_4 Photosynthesis in Terrestrial Plants

Two means of partitioning the function of C_4 photosynthesis between two cytoplasmic compartments evolved in family Chenopodiaceae. Borszczowia produces elongated palisade chlorenchyma cells with dimorphic chloroplasts polarized towards opposite ends of the cell. This is somewhat analogous to having Kranz anatomy, with the M and BS arrangement, without the intervening cell walls. Surprisingly, a completely different solution to performing C_4 photosynthesis in a single cell is found in Bienertia. The chlorenchyma cells of the two Bienertia species have a peripheral, chloroplast-containing, thin layer of cytoplasm (peripheral compartment) and a very unusual

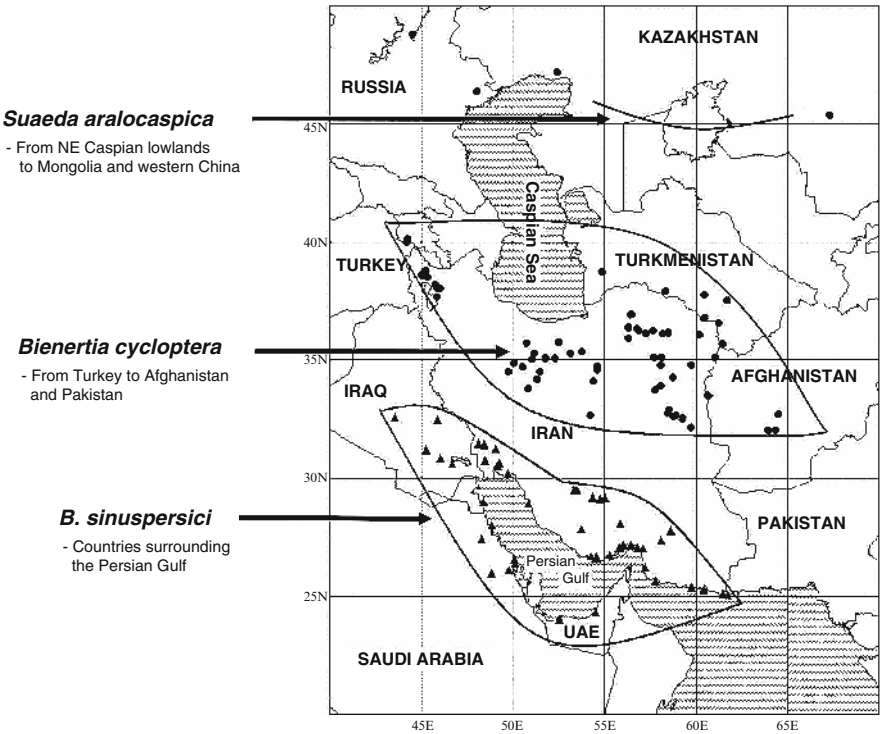


Fig. 8. Map showing the wide-spread distribution of single-cell C_4 species, *Suaeda* (=*Borszczowia*) *aralocaspica*, *Bienertia cycloptera*, and *B. sinuspersici.* The lines on the figure show the range for each species. Further research is needed to determine whether there is more diversity among these populations (subtypes or new species).

chloroplast-containing central cytoplasmic compartment, which are proposed to function like M and BS cells, respectively, in Kranz type C_4. In both systems, the partitioning of biochemically distinct organelles into discrete compartments is considered to result in concentration of CO_2 around the Rubisco-containing chloroplasts, causing inhibition of Rubisco oxygenase activity and photorespiration, as occurs in the typical Kranz system. Models of how these systems operate C_4 photosynthesis have been proposed (Fig. 9, Edwards et al., 2004).

D. Biochemical Evidence for Function of C_4 Photosynthesis in Single-Cell C_4 Plants

1. General Features Characteristic of C_4

Western Blots and Analysis of C_4 Enzymes

Analyses of photosynthetic enzymes by Western blots and enzyme assays show that the single-cell C_4 species have high levels of C_4 cycle enzymes PEPC and PPDK, similar to Kranz type *Suaeda*, and in contrast to very low levels in the C_3 type *Suaeda* species (Fig. 10, also see Voznesenskaya et al., 2002). Assays for C_4 acid decarboxylases show that these single-cell C_4 species are NAD-ME type (Fig. 10), as are all Kranz type C_4 species which have been examined in subfamily Suaedoideae, see Voznesenskaya et al., 2007. Also, the single-cell C_4 species have a C_4 type PEPC similar to that in Kranz type species in subfamily Suaedoideae (Lara et al., 2006). This includes having high specific activities, a serine residue near the amino-terminus which undergoes phosphorylation/dephosphorylation, and light/dark regulation by phosphorylation with differential sensitivity to malate (Lara et al., 2006).

C_4 Type Carbon Isotope Composition

Reports on carbon isotope values in Bienertia (Winter, 1981; Akhani et al., 1997, 2005; Freitag and Stichler, 2002; Voznesenskaya et al., 2002) and Borszczowia (Freitag and Stichler, 2000;

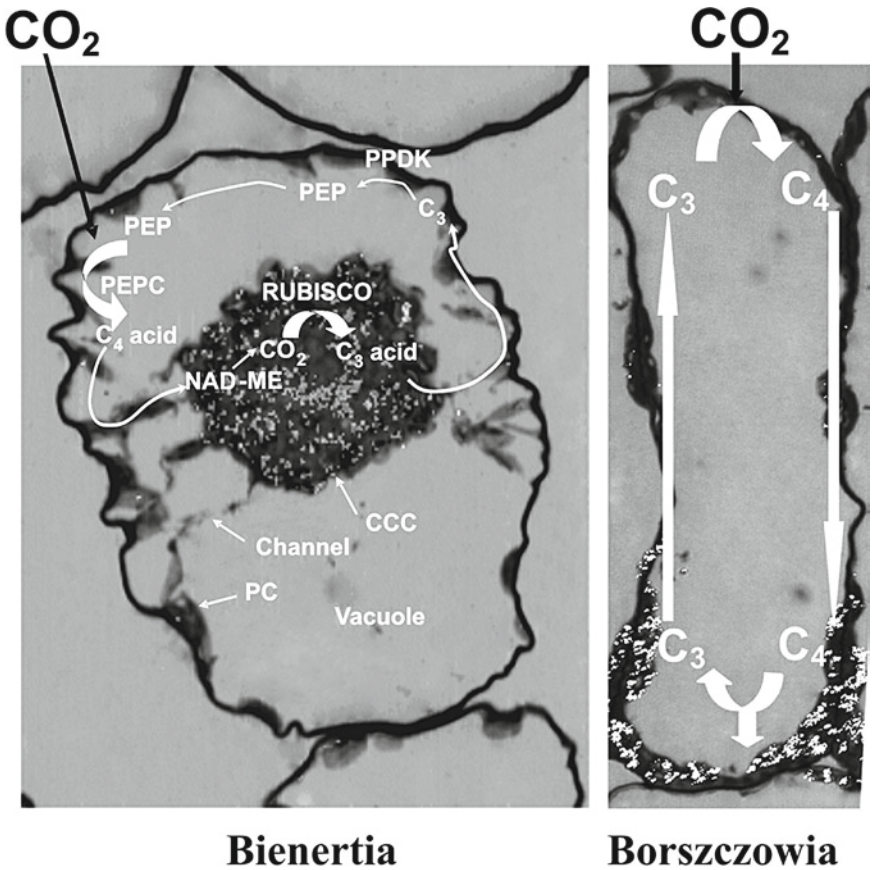


Fig. 9. General models of proposed function of C_4 photosynthesis in the two types of single-cell C_4 systems. The left panel for Bienertia illustrates atmospheric CO_2 entering the peripheral cytoplasm where it is fixed by PEPC, and a scheme which shows the path of carbon through the NAD-ME type C_4 cycle. The C_4 cycle delivers CO_2 to Rubisco (this immunogold-treated section for Rubisco shows label appearing as light deposits in the central cytoplasmic compartment). The right panel for Borszczowia illustrates atmospheric CO_2 entering the proximal end of the cell where it is fixed by PEPC; CO_2 is donated to Rubisco in the proximal end of the cell (immunolabeling for Rubisco shows light deposits in chloroplasts in the proximal end) via an NAD-ME C_4 cycle (as in Bienertia). *PC*, peripheral chloroplast; *CCC*, central cytoplasmic compartment; Channel, cytoplasmic channel connecting the PC and CCC; *PPDK*, pyruvate, Pi dikinase; *PEPC*, PEP carboxylase; *NAD-ME*, NAD-malic enzyme.

Voznesenskaya et al., 2001) indicated that they have C_4/CAM (Crassulacean acid metabolism) type carbon isotope composition. Although succulent, subsequent studies showed no evidence for performance of CAM (Voznesenskaya et al., 2001, 2002, 2003). Various collections of the two Bienertia species and Borszczowia, from natural habitats and from plants grown under controlled conditions in high light, show that they have C_4 type carbon isotope composition. Analysis of the carbon isotope composition during a growing season in Iran showed *B. cycloptera* performs C_4 photosynthesis during its life cycle in nature similar to Kranz type C_4 species (Akhani et al., 2009). In Bienertia species, more negative values (−16% to −19%) have been observed in young leaves and during growth under low light (100–200 photosynthetic photon flux density) (Freitag and Stichler, 2002; Voznesenskaya et al., 2002, 2005c). In young leaves, C_4 type chlorenchyma have not fully developed (Voznesenskaya et al., 2005c); growth under low light may limit the developmental transition from C_3 to a fully functional C_4 system (possibly due to incomplete development of dimorphic chloroplasts, or ability to concentrate CO_2 around Rubisco).

Physiological Response

The single-cell C_4 species and the Kranz type *Suaeda* species have low sensitivity of photosynthesis to O_2 under atmospheric levels of CO_2, and

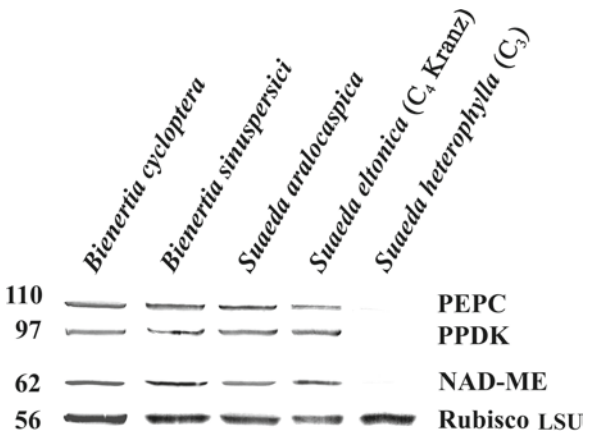


Fig. 10. Western blots of Rubisco and three C_4 cycle enzymes, PPDK, PEPC, and NAD-ME in the single-cell C_4 species *Suaeda* (=*Borszczowia*) *aralocaspica, Bienertia cycloptera* and *B. sinuspersici,* Kranz type *S. eltonica* and the C_3 *S. heterophylla* (see Chuong et al., 2006). Copyright American Society Plant Biologists, www.plantcell.org

low CO_2 compensation points, typical of C_4 plants (Voznesenskaya et al., 2001, 2002, 2007; Edwards et al., 2007). Also, the water use efficiency (μmol CO_2 $mmol^{-1}$ water) is about two fold higher in the single-cell C_4 species and the Kranz type C_4 species than in representative C_3 species in subfamily Suaedoideae (Edwards et al., 2007).

Resistance to CO_2 Loss

For C_4 photosynthesis to function, high efficiency in trapping of the CO_2 generated by the C_4 pump, and in refixation of photorespired CO_2, are required. This means that the diffusive resistance in C_4 plants for CO_2 from Rubisco to the intercellular air space must be substantially higher than that in C_3 plants. Analyses of the diffusive resistance in the single-cell C_4 species show it is about 50-fold higher than that in C_3 species, and that it is of the same order of magnitude of Kranz type C_4 plants (Edwards et al., 2007).

2. Spatial Compartmentation Enabling Function of NAD-ME Type C_4 Photosynthesis

Critical experiments showing how C_4 photosynthesis functions in these single-cell C_4 species were performed by studying the structural organization by microscopy, by immunolocalization of several photosynthetic enzymes, and by localization of starch. In each single-cell C_4 system, the mechanism of photosynthesis and spatial compartmentation of function are analogous to those of NAD-ME type Kranz species (Fig. 9, Voznesenskaya et al., 2001, 2002; Edwards et al., 2004). In the Bienertia species, the peripheral cytoplasm functions analogous to Kranz type C_4 M cells in fixation of atmospheric CO_2 into C_4 acids, while the central cytoplasmic compartment functions analogous to BS cells in donation of CO_2 from C_4 acids to Rubisco (Voznesenskaya et al., 2001, 2002; Edwards et al., 2004). As illustrated in the model in Fig. 9, CO_2 is fixed by PEPC in the peripheral cytoplasm leading to formation of C_4 acids (aspartate and malate) and their transport via cytoplasmic channels to the central compartment and decarboxylation in mitochondria via NAD-ME, with CO_2 donation to Rubisco. C_3 acids formed from decarboxylation are transported to the peripheral compartment and used for regeneration of PEP from pyruvate in the peripheral chloroplasts. In Borszczowia, the carboxylation phase of the C_4 pathway, with fixation of atmospheric CO_2, functions in the distal part of the cell (analogous to M cells in Kranz type), while donation of CO_2 to Rubisco from decarboxylation of C_4 acids occurs in the proximal part of the cell (analogous to BS cells in the Kranz type NAD-ME species).

Dimorphic Chloroplasts (Structure, Enzymes and Starch)

In each structural type of single-cell C_4, C_4 photosynthesis is accomplished in part by the partitioning of two biochemically and ultrastructurally distinct chloroplast types into separate compartments within the cell (Voznesenskaya et al., 2001, 2002, 2005c). These chloroplasts are dimorphic in biochemistry of photosynthesis, in ability to store starch, and in ultrastructure. In addition to immunolocalization studies by confocal microscopy, immunolocalization in Bienertia and Borszczowia by transmission electron microscopy shows rather strong selective labeling of PPDK in one chloroplast type, and Rubisco in the other chloroplast type (E. Voznesenskaya, N. Koteyeva, G. Edwards, unpublished results). The outer chloroplasts supporting the carboxylation phase of the C_4 cycle to fix atmospheric CO_2 have PPDK, which generates the substrate for PEPC by converting pyruvate to PEP, they store little or no starch, and they have a deficiency in grana development. The inner chloroplasts, which fix CO_2 generated from decarboxylation of C_4 acids, have Rubisco, they store starch

(and have ADPG pyrophosphorylase, the first committed step for starch biosynthesis), and they have well-developed grana. These features are the same as the respective M and BS chloroplasts in related Kranz type NAD-ME species. In C_4 plants, BS chloroplasts usually store larger amounts of starch than M chloroplasts. In NAD-ME type C_4 species, the grana-deficient chloroplasts are thought to be associated with a lower requirement for reductive power to support the C_4 carboxylation phase in this subgroup (see Section B2).

Mitochondria and Peroxisomes

In the single-cell C_4 species, the mitochondria are partitioned to the cytoplasmic compartment, where the Rubisco-containing chloroplasts are located (the proximal end of the cell in Borszczowia and in the central cytoplasmic compartment in Bienertia species). The mitochondria perform two important functions relative to C_4 photosynthesis: generation of CO_2 by decarboxylation of C_4 acids via NAD-ME and decarboxylation of glycine as a result of any photorespiration. Also, peroxisomes are predominantly located in the cytoplasmic compartment with Rubisco-containing chloroplasts (based on transmission electron microscopy and immunolocalization of catalase), which are presumably associated with metabolism of glycolate to glycerate in the glycolate pathway (Voznesenskaya et al., 2001, 2002; Chuong et al., 2006). While the C_4 cycle concentrates CO_2 around Rubisco and suppresses photorespiration, some photorespiration does occur. The selective localization of glycine decarboxylase in BS mitochondria makes photorespired CO_2 available for refixation by Rubisco.

E. Development of Spatial Compartmentation and Dimorphic Chloroplasts

An intriguing aspect of single-cell C_4 photosynthesis is the development of spatial compartmentation of functions and dimorphic chloroplasts. There is evidence that very young chlorenchyma cells have a single type of chloroplast (monomorphic) which is in a C_3 default mode, with all chloroplasts containing low levels of Rubisco without PPDK, and without spatial separation of organelles into two compartments (Voznesenskaya et al., 2005c). Since enzymes of the C_4 cycle like PPDK and the small subunit of Rubisco are nuclear encoded, there must be posttranscriptional regulation for selective expression of certain proteins in chloroplasts (see chapter 12 by Berry et al. for selective expression in Kranz type C_4). In mature chlorenchyma cells that have formed two cytoplasmic compartments and dimorphic chloroplasts, there is intricate development of the cytoskeleton, which consists of actin and microtubules. Cytoskeleton-disrupting drugs show that microtubules are important in maintaining the two cytoplasmic compartments (Chuong et al., 2006).

F. Form of Photosynthesis in Different Photosynthetic Organs in Single-Cell C_4 Species

Plants are usually characterized by photosynthetic type according to the mechanism of carbon assimilation in the leaf, which is generally the main photosynthetic organ. Among chenopods having C_4 photosynthesis in leaves, there is variation between species as to the type of photosynthesis in cotyledons (Butnik, 1979, 1984, 1991; Pyankov et al., 1999, 2000b, c; Voznesenskaya et al., 1999, 2004; Akhani and Ghasemkhani, 2007). There are C_4 chenopods, for example *Salsola richteri* (Salsoloid type), which have the same type of Kranz anatomy in leaves and cotyledons. There are also C_4 chenopods having C_4 photosynthesis in leaves and cotyledons, but different types of Kranz anatomy, for example *Salsola laricina*, which has Salsoloid anatomy in leaves and Atriplicoid type anatomy in cotyledons. Finally, there are dicots which have C_4 photosynthesis in leaves, but C_3 type anatomy and photosynthesis in cotyledons; for example *Salsola gemmascens* has Salsoloid type anatomy in leaves and C_3 type anatomy in cotyledons (Pyankov et al., 2000c).

Not all C_4 plants have leaves as the primary photosynthetic organ during vegetative growth. For example, in family Chenopodiaceae, C_4 species of *Anabasis*, *Haloxylon*, *Halosarcia, Hammada* and some species of *Halothamnus* have reduced leaves with stems as the primary

photosynthetic organ (Ocallaghan, 1992; Akhani et al., 1997; Pyankov et al., 2000b).

With respect to single-cell C_4 species, Borszczowia and both Bienertia species have green cotyledons, leaves, and flowers. Studies of the respective organs, including structure of chlorenchyma, immunolocalization of photosynthetic enzymes (Rubisco and PEPC), and Western blots of Rubisco and C_4 cycle enzymes, show they all have unique chlorenchyma cells which are structurally and biochemically developed to perform single-celled C_4 photosynthesis (Voznesenskaya et al., 2001, 2002, 2004; Edwards et al., 2004; Akhani et al., 2005; Boyd et al., 2007). Thus, these species perform single-cell C_4 photosynthesis throughout their life cycle. Leaves make up the majority of the green tissue during vegetative growth of these species, although younger branches have green stems. However, the flowers have green tepals which become a major photosynthetic organ during the latter reproductive phase of growth. Photosynthesis in flowers may have adaptive value for survival of these desert plants. In Bienertia, under increasing temperatures as flowers develop, lower leaves wither and senesce and are replaced by smaller floral leaves consisting of green tepals (Boyd et al., 2007). Also, the stems of *B. cycloptera* have single-cell C_4 type chlorenchyma cells beneath the epidermis. This, combined with the presence of stomata on the stems, starch in stem chlorenchyma, and light-dependent fixation of atmospheric CO_2 by the stems, suggests they contribute to carbon assimilation in Bienertia. In contrast, the stems of Borszczowia have C_3 type chlorenchyma cells scattered throughout the cortical tissue. They likely function to refix respired CO_2 in stems, since light dependent fixation of external CO_2 is only observed under high atmospheric levels (Boyd et al., 2007).

G. How Did Single-Cell C_4 Evolve?

In considering how single-cell C_4 plants might have evolved, an analogy can be made to the proposed evolutionary development of Kranz type C_4 plants from C_3 plants (see chapter 6 by Bauwe). In C_3 plants, M cells are the main photosynthetic tissue in the leaf, since the BS cells have few, or no, chloroplasts. It has been suggested that evolution of C_4 has occurred multiple times by a stepwise progression of structural and biochemical changes which were induced by CO_2-limiting conditions (Monson et al., 1984; Edwards and Ku, 1987; Monson and Moore, 1989; Rawsthorne and Bauwe, 1998; Sage, 2004). The occurrence of intermediates between C_3 and C_4 plants, particularly in the genus *Flaveria* (Asteraceae), has provided a basis for suggesting how C_4 may have evolved, from C_3, to intermediates which reduce photorespiration without a C_4 cycle, to intermediates having a partially-functioning C_4 cycle, to full development of C_4. In the first type of intermediate, normal C_3 type photosynthesis occurs in M cells; however, the release of CO_2 in photorespiration occurs in mitochondria in BS cells (by selective localization of glycine decarboxylase in BS mitochondria), where a degree of refixation by chloroplasts occurs. This increases the efficiency of photosynthesis under limiting CO_2 (von Caemmerer, 1989).

By analogy, under CO_2-limiting conditions a single-cell C_3-C_4 intermediate species may develop by spatial separation of the fixation of atmospheric CO_2 by the C_3 cycle and the refixation of photorespired CO_2 (see illustration in Fig. 11). This would generate a form of CO_2 concentrating mechanism and reduce loss of CO_2 by photorespiration. It could provide the initial spatial separation with chloroplasts in one cytoplasmic compartment fixing atmospheric CO_2, and chloroplasts and mitochondria in another cytoplasmic compartment functioning to minimize CO_2 loss by photorespiration. The spatial separation of chloroplasts and mitochondria in this hypothetical C_3-C_4 intermediate is like that in the single-cell C_4 plants. This would provide the initial spatial separation of organelles, with subsequent differentiation of chloroplasts and expression of C_4 cycle enzymes leading to development of a functional C_4 system.

IV. Future Perspectives

The discovery of C_4 plants among terrestrial species, approximately 40 years ago, and their association with Kranz anatomy, has led to numerous studies on the occurrence, photosynthetic mechanism, structural and biochemical diversity, molecular control of development of two photosynthetic cells, and evolution (Edwards and Walker, 1983; Hatch, 1987; Sage and Monson,

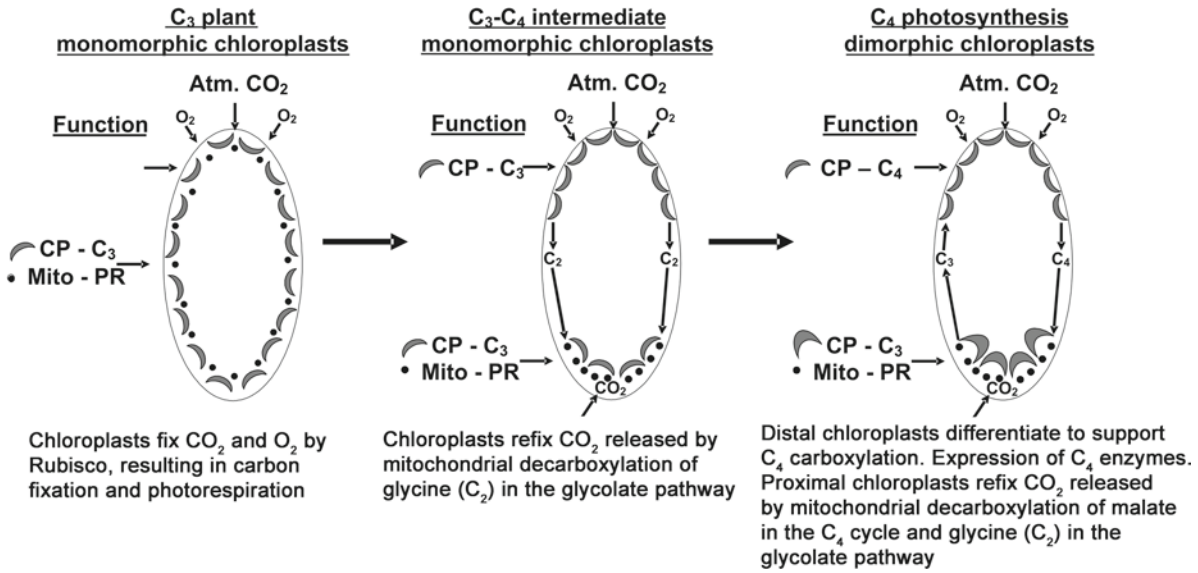


Fig. 11. Scheme illustrating a hypothetical intermediate stage in evolution of single-cell C_4 photosynthesis via a C_3-C_4 intermediate which reduces photorespiratory loss of CO_2 by refixation of CO_2 via Rubisco. *Atm*, atmospheric; *mito*, mitochondria; *PR*, photorespiration; *CP*, chloroplast.

1999). With the paradigm that this occurred in land plants via development of Kranz anatomy, the finding that terrestrial species can conduct C_4 photosynthesis within individual chlorenchyma cells provides a very different system to study C_4. This includes stages in evolution of C_4, the genetic control of development of the requisite spatial separation of functions, the mechanism of chloroplast differentiation, biochemical and biophysical requirements for the function of C_4 photosynthesis, and development of strategies for engineering C_4 photosynthesis into selected C_3 crops.

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