Photosynth Res (2015) 123:33–43
DOI 10.1007/s11120-014-0037-1

REGULAR PAPER

Salsola laricifolia, another C_3–C_4 intermediate species in tribe Salsoleae s.l. (Chenopodiaceae)

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Received: 8 July 2013 / Accepted: 14 August 2014 / Published online: 17 September 2014

Abstract This study identifies *Salsola laricifolia* as a C_3–C_4 intermediate in tribe Salsoleae s.l., Chenopodiaceae, and compares *S. laricifolia* with the previously described C_3–C_4 intermediates in Salsoleae. Photosynthetic pathway characteristics were studied in four species of this tribe including *S. laricifolia*, C_3 *Sympegma regelii*, C_3–C_4 *S. arbusculiformis*, and C_4 *S. arbuscula*, using the approaches of leaf anatomy and ultrastructure, activities of ribulose 1-5-bisphosphate carboxylase/oxygenase (Rubisco) and PEP carboxylase (PEPC), CO_2 compensation point, and immunolocalization of Rubisco, PEPC, and the P-subunit of glycine decarboxylase (GDC). *Salsola laricifolia* has intermediate features, with near continuous and distinctive Kranz-like cells (KLCs) compared with the C_3-Sympegmoid anatomical type and the C_3–C_4 intermediate *S. arbusculiformis*, a relatively low CO_2 compensation point (30.4 μmol mol^{-1}) and mesophyll (M)-to KLC tissue ratio, mitochondria in KLCs primarily occurring along the centripetal wall, and specific localization of P-protein GDC in the KLCs. The C_3-type isotope value (−22.4 ‰), the absence of the clear labeling for PEPC in M cells, and the low activity of the PEPC enzyme (61.5 μmol mg^{-1} chlorophyll^{-1} h^{-1}) support the identification of *S. laricifolia* as a type I C_3–C_4 intermediate. Although these C_3–C_4 intermediate species have different structural features, one with discontinuous KL cells and the other with continuous, they have similar characteristics in physiology and biochemistry.

Keywords C_3–C_4 intermediate species · Kranz anatomy · Chenopodiaceae · C_4 photosynthesis · Photosynthetic enzymes · *Salsola laricifolia*

Abbreviations

Rubisco	Ribulose 1-5-bisphosphate carboxylase/oxygenase
PEPC	Phosphoenolpyruvate carboxylase
GDC	Glycine decarboxylase
KLC	Kranz-like cell
M	Mesophyll
BS	Bundle sheath
KC	Kranz cell
Γ	CO_2 compensation point
A_{max}	The maximum rate of photosynthesis
$\delta^{13}C$	Carbon isotope value

Introduction

With ca. 550 species in ca. 10 independent C_4 lineages, the family Chenopodiaceae comprise the largest number of C_4 species and C_4 lineages among eudicot families (Kadereit et al. 2003; Pyankov et al. 2001b; Sage 2001). In Chenopodiaceae, there are eight mainly described C_4 leaf anatomical types (Carolin et al. 1975; Edwards and Voznesenskaya 2011), which are divided into 16 forms considering all differences in the structure and arrangement

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of M and bundle sheath (BS) cells, arrangement of vascular bundles and water storage, and presence or absence of hypodermis and sclerenchyma (Kadereit et al. 2003).

C_3–C_4 intermediates have been of interest because they may contribute to the understanding of C_4 evolution and mechanisms for reduction of photorespiration (Edwards and Ku 1987). C_3–C_4 intermediates have been identified in 17 genera of eudicots and monocots (Sage et al. 2011a), and include *Salsola* in Salsoleae and *Bassia* in Camphorosmeae in the family Chenopodiaceae (Freitag and Kadereit 2014; Kadereit and Freitag 2011; Voznesenskaya et al. 2001, 2013). Compared with C_3 species, C_3–C_4 intermediates have intermediate characters in leaf anatomy and photosynthesis, such as a relatively low M/BS volume ratio, increased organelle number in BS cells, concentration of numerous large mitochondria to the centripetal region of BS cells, a lower CO_2 compensation point, and the selective localization of GDC in BS mitochondria, which helps to recapture CO_2 released by the decarboxylation of glycine (Edwards and Ku 1987; Muhaidat et al. 2011; Rawsthorne 1992; Rawsthorne and Bauwe 1998; Sage et al. 2011b; Voznesenskaya et al. 2001, 2013).

The tribe Salsoleae s.l. is one of the largest in Chenopodiaceae, with the greatest diversity in photosynthesis and leaf anatomical structures among C_3, C_3–C_4 intermediate, and C_4 plants (Carolin et al. 1975; Edwards and Voznesenskaya 2011; Jacobs 2001; Kadereit et al. 2003; Voznesenskaya et al. 2001, 2013; Wen and Zhang 2011). In Salsoleae, the C_4 leaf anatomy type is called "Salsoloid" (Carolin et al. 1975), and is characterized by two layers of chlorenchyma, the outer of palisade M cells and the inner of specialized Kranz cells (KCs), the main vascular bundle in a central position, and small peripheral vascular bundles that contact KCs. In the C_4 Salsoloid type, the inner layer of chlorenchyma cells is defined as KCs rather BS cells, because the KCs form something of a sheath enclosing the veins and water-storage tissue, unlike an inner layer of BS cells, which forms a true sheath around individual peripheral veins in the most commonly used description of Kranz anatomy (Edwards and Voznesenskaya 2011; Voznesenskaya et al. 2013). The Salsoloid type can be divided into two subtypes, one with hypodermis, and the other without (Carolin et al. 1975). However, it is also known that the C_3 species in Salsoleae have a different leaf anatomy, named the Sympegmoid type, with two to three layers of M cells, and indistinctive BS cells arranged only adjacent to the peripheral veins (Carolin et al. 1975).

Salsola arbusculiformis and *S. divaricata* in Salsoleae have been identified as species with a C_3–C_4 intermediate type of photosynthesis (Voznesenskaya et al. 2001, 2013). Compared to species with the Sympegmoid type, *S. arbusculiformis* has a discontinuous layer of KLCs which surround the separate vascular bundles, but *S. divaricata* has a fully continuous layer of KLCs similar to that in C_4 *Salsola* species. Nevertheless, the two C_3–C_4 intermediates have similar physiological and structural features. Both have a rather low CO_2 compensation points compared with C_3 plants (36.7 μmol m^{-2} s^{-1} for *S. arbusculiformis* and 32 μmol m^{-2} s^{-1} for *S. divaricata*), a decreased outer M layer and M/KLC ratio, walls of KLCs that are thicker than those of M cells, numerous large mitochondria in KLCs, mitochondria positioned toward the inner KLC walls, and localization of GDC in KLC mitochondria (Voznesenskaya et al. 2001, 2013). These two species belong to the type I category of C_3–C_4 intermediates, which lack a functional C_4 cycle (Voznesenskaya et al. 2013).

Although *S. divaricata* and *S. arbusculiformis* have some similar C_3–C_4 intermediate characteristics, they are not closely related based on previous phylogenetic trees (Akhani et al. 2007; Wen et al. 2010). *Salsola divaricata*, in a single species branch, is related to a clade of C_4 species (Akhani et al. 2007; Voznesenskaya et al. 2013; Wen et al. 2010), while *S. arbusculiformis* + *S. laricifolia* form a sister pair, closely related to a C_3 branch containing *S. montana* + *S. masenderanica* based on analysis of one nuclear and one chloroplastic gene region (ITS and chloroplast *psb*B–*psb*H) (Wen et al. 2010). *Salsola laricifolia* has a close relationship with *S. arbusculiformis* not only in phylogenetic trees (Voznesenskaya et al. 2013; Wen et al. 2010, 2014) but also in morphological features (Freitag and Rilke 1997; Grubov 1999; Iljin 1936; Wen et al. 2014; Zhu et al. 2003). Based on our recent findings, leaves of *S. laricifolia* show a C_3-like carbon isotope value (−22.062 ‰), and leaf anatomy in this species is characterized by two to three layers of palisade M cells and a distinctive, Kranz-like innermost layer of chlorenchyma cells (Wen and Zhang 2011). More evidence is therefore needed to fully identify whether *S. laricifolia* is a new C_3–C_4 intermediate in Salsoleae.

To clarify the types of photosynthetic pathways in *S. laricifolia* and the C_3 species *Sympegma regelii*, a comparative study of leaf anatomy and ultrastructure, activity of Rubisco and PEPC, carbon isotope value, CO_2 compensation point, maximum rate of photosynthesis, and immunolocalization of Rubisco, PEPC, and P-protein of GDC were carried out. The aim of the study was to determine whether *S. laricifolia* is a C_3–C_4 intermediate plant and compare it with the confirmed C_3–C_4 intermediates *S. arbusculiformis* and *S. divaricata*, and C_4 species *S. arbuscula*.

Materials and methods

Plant material and growth conditions

Seeds of *Sympegma regelii* were collected on September 27, 2011 from plants growing in Toksun County (Turpan, Xinjiang, China). Seeds of *Salsola arbuscula* were collected

on September 24, 2011 from plants growing in Karamay City (Xinjiang, China). Seeds of *S. arbusculiformis* and *S. laricifolia* were collected on September 25, 2011 from plants growing in Tiechanggou Town (Toli County, Tacheng, Xinjiang, China). Seeds were stored at 4 °C before germination, and were germinated on moist paper at room temperature before being transplanted to soil.

All species were grown in greenhouse in pots with a mixture of 30 % topsoil, 60 % sand, and 10 % horticultural perlite. Plants were fertilized weekly with fertilizer made by ourselves (4 mM $(Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 1 mM NH_4NO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM $FeSO_4{\cdot}7H_2O$, 0.1 mM EDTA-Na_2, 0.005 mM KI, 0.1 mM H_3BO_3, 0.03 mM $ZnSO_4{\cdot}7H_2O$, 0.0008 mM $Na_2MoO_4{\cdot}7H_2O$, 0.0001 mM $CuSO_4{\cdot}5H_2O$, 0.0001 mM $CoCl_2{\cdot}6H_2O$). Plants for the study were grown in a greenhouse and supplemented with ~400 µmol photosynthetic quanta m^{-2} s^{-1} with a 14/10 h light/dark photoperiod. All measurements were done on expanded healthy leaves from 10- to 12-week-old plants. At the same time, leaves were sampled for analysis by light and electron microscopy, enzyme activity, in situ immunolocalization, carbohydrates staining, and carbon isotope value.

For studies on CO_2 compensation point and rates of photosynthesis under various conditions, plants were grown under field conditions in summer (from June to August, 2012) at the Xinjiang Institute of Geography and Ecology, Chinese academy of Sciences (Urumqi, Xinjiang, China). Plants were watered as necessary to avoid drought, and fertilized weekly with the above fertilizer.

Carbon isotope value

Carbon isotope values ($\delta^{13}C$) were measured using dried leaves, by means of standard procedures relative to PDB (Pee Dee Belemnite) limestone carbon isotope standard (Bender et al. 1973; Schulze et al. 1996). These measurements were done at the Chinese Academy of Forestry (Beijing, China) following Wen and Zhang (2011).

Leaf anatomy and ultrastructure

Leaf tissue for all microscopic observations was sampled from the middle of a mature leaf (3 sections per leaf, 1 leaf per plant, three plants per species), and prepared for light microscopy following Wen and Zhang (2011). Cross-sections for light microscopy were 8 µm thick. For electron microscopy, leaf tissues were fixed for 12 h at 4 °C in 0.2 M glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), washed by 0.1 M phosphate buffer (pH 7.2) for 2–3 h, and then post-fixed in 1 % osmium tetroxide (v/v) in 0.1 M phosphate buffer (pH 7.2) and washed by 0.1 M phosphate buffer (pH 7.2) for 1–2 h. Following standard dehydration in a graded ethanol–acetone series, samples were embedded in Spurr's resin. Cross-sections for electron microscopy were 100 nm thick.

Light microscopic images were used to measure %M cells, %BS cells/KLCs/KCs, M and BS/KLC/KC area, and the ratio of M-to-BS/KLC/KC tissue. Measurements of %M cells, %BS cells/KLCs/KCs followed Sage et al. (2011b). M and BS/KLC/KC areas were measured using Image-Pro plus software (Media Cybernetics, Silver Springs, MD, USA).

In situ immunolocalization

Leaf cross-sections for immunolocalization were prepared as described by Wen and Zhang (2011). Antibodies used were commercially available rabbit anti-spinach Rubisco (LSU) IgG (Agrisera, Sweden), rabbit anti-maize PEPC IgG (Agrisera, Sweden), and rabbit anti-spinach GDC (P-protein) IgG (Shanghai Sangon Biological Engineering Technology & Service, Shanghai, China). The cross-reactivity of each antibody was verified by running control labeling experiments on cross-sections of leaf tissues of *Salsola arbuscula* and *S. arbusculiformis* in which the enzymes were known to be expressed and accumulated in a specific manner (Voznesenskaya et al. 2001).

Cross-sections 8 µm thick, were de-waxed, then rehydrated through an ethanol series, covered by ddH_2O for 5 min, subsequently rinsed with citrate buffer (pH 6.0), and covered by ddH_2O for 5 min, and PBST for 5 min. Sections were blocked with 3 % BSA + PBST for 40 min, washed by PBST three times, and then incubated overnight at 4 °C with each antibody. The antibodies were diluted, to 1:500 for the anti-spinach Rubisco IgG and anti-maize PEPC IgG, and to 1:1000 for anti-spinach GDC IgG. After incubation, sections were covered by PBST, then incubated with supervision with anti-rabbit detection reagent (Shanghai Long Island Biological Technology Co., Ltd., China) at room temperature for 40 min, and then covered by PBST. Sections were stained with a DAB coloration kit (Fuzhou Maixin Biological Technology Co., Ltd., China), observed and viewed with an Olympus CX41 microscope (Olympus, Japan), and images were obtained using an Olympus DP70 imaging system (Olympus, Japan). Meanwhile, control slides lacking antibody (Rubisco, PEPC, and GDC) were also carried through each experiment.

Staining for carbohydrates

Cross-sections 8 µm thick, were dried onto gelatin-coated slides, followed by PAS (periodic acid + Schiff's) staining. Sections were incubated in periodic acid (1 % w/v) for 20 min, washed, and then incubated with Shiff's reagent (BASO, Zhuhai, China) for 25 min. After rinsing, sections were analyzed by light microscopy.

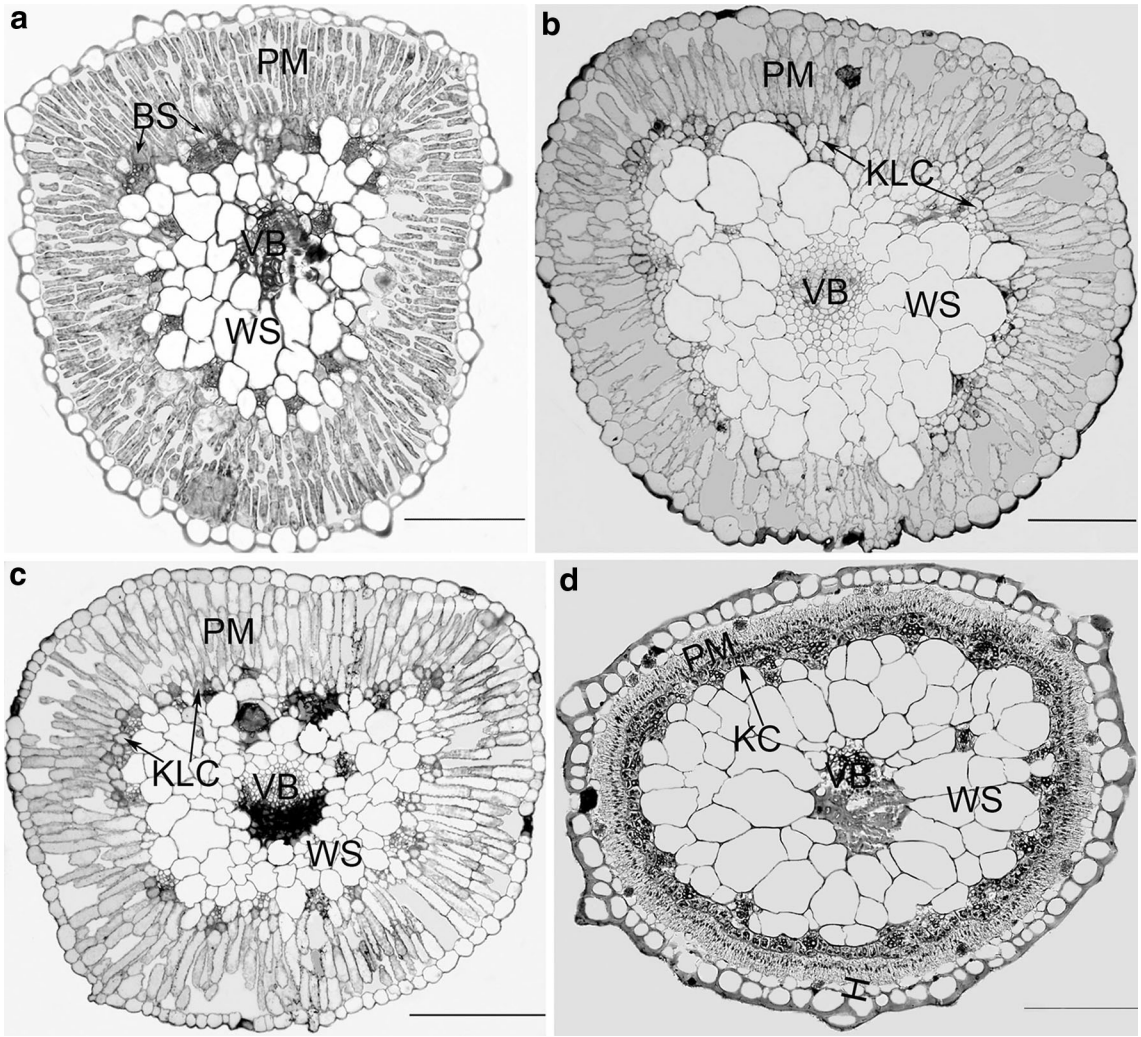

Fig. 1 Leaf anatomical structures in four species of Salsoleae. **a** *Sympegma regelii*; **b** *Salsola laricifolia*; **c** *Salsola arbusculiformis*; **d** *Salsola arbuscula*. *Scale bars* 100 μm. *BS* bundle sheath, *H* hypodermis, *KC* Kranz cell, *KLC* Kranz-like cell, *PM* palisade mesophyll, *VB* vascular bundle, *WS* water-storage tissue

Enzyme extraction and assay

For activities of PEPC, 0.3 g leaf tissue was ground on ice, using an extraction buffer containing 50 mM HEPES–KOH (pH 7.5), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM DTT, 0.2 mM Na_4-EDTA, 0.5 % BSA, and 2.5 % (w/v) insoluble PVP (Ueno 1992). The crude extract was centrifuged at 15000 rpm for 10 min at 4 °C and then assayed at 340 nm using a coupled enzyme assay. The assay medium contained 50 mM Tris–HCl (pH 8.0), 2 mM DTT, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.5 mM glucose-6-phosphate, 0.2 mM NADH, 5 mM PEP, 3 units mL^{-1} malate dehydrogenase, and 50 μL extract. The reaction was run at 25 °C and initiated by adding PEP.

For activities of Rubisco, 0.3 g leaf tissue was ground on ice, using an extraction buffer containing 100 mM HEPES–KOH (pH 8.0), 20 mM $MgCl_2$, 5 mM DTT, and 2 mM EDTA. The extracts were then centrifuged at 14000 for 5 min at 4 °C. The reaction was run at 25 °C and started by adding 50 μL of extract to 450 μL of grinding medium containing 1 mM RuBP and 10 mM $NaH_{14}CO_3$. The reaction was stopped after 30 s by addition of 0.5 mL 1 N HCl + 4 N formic acid, and the acid-stable ^{14}C counts were determined in a scintillation counter. To calculate the specific activity for CO_2 fixation, a separate reaction was run to completion with addition of 5 nmol RuBP, and the amount of ^{14}C fixed was determined (Voznesenskaya et al. 2001).

CO_2 compensation point and the maximum rate of photosynthesis

The CO_2 compensation point (Γ) and maximum rate of photosynthesis (A_{max}) were measured on expanded healthy

leaves from 10- to 12-week-old plants using a Li-6400 portable photosynthesis system with flat cuvette (Li-Cor, Inc., Lincoln, NE, USA) from the 20th to 22nd of August, 2012. Because of small size (about 0.4 cm^2), multiple leaves on a branch were placed in the chamber. The value of Γ was measured at 25 °C and a photon flux density of 1200 μmol m^{-2} s^{-1}. Γ was calculated as the *x*-intercept of a linear regression of the five lowest intercellular values and their net CO_2 assimilation (Sage et al. 2011b). To assess A_{max}, the CO_2 levels were set to 370, 500, 600, 700, and 800 μmol mol^{-1}.

Data analysis

All statistical tests were performed with SPSS version 13.0 using a one-way analysis of variance and Turkey's pairwise multiple comparisons. Sample sizes are indicated in the table.

Results

Leaf anatomy and ultrastructure

Sympegma regelii has a typical C_3-Sympegmoid anatomy, characterized by chlorenchyma consisting of two to three layers of palisade M cells without a defined layer of hypodermis, and an indistinct layer of BS cells, only adjacent to peripheral bundles (Fig. 1a). The leaf anatomy of *S. laricifolia* is similar to that of this species, but with more distinctive and continuous KLCs, and an outer layer of M cells that are much shorter than the inner M cells (Fig. 1b). The leaf anatomical structure of *S. arbusculiformis* is similar to that of *S. laricifolia*, but with a discontinuous layer of KLCs (Fig. 1c). *Salsola arbuscula* has a typical C_4 Salsoloid Kranz anatomy, consisting of hypodermal cells, one outer layer of palisade M cells, one inner layer of KCs associated with small peripheral vascular bundles, and with the main vascular bundle in the center surrounded by water-storage tissue (Fig. 1d).

Sympegma regelii has the highest M/BS tissue ratios and M: BS area ratios compared to *Salsola arbusculiformis*, *S. laricifolia*, and *S. arbuscula*. The M/KLC tissue ratios and M: KLC area ratios of *S. laricifolia* are less than those of *S. arbusculiformis*, but the differences are not significant (Table 1). Compared with the C_3 species *Sympegma regelii*, the mitochondria in the KLCs of *S. laricifolia*, *S. arbusculiformis*, and the KCs of *S. arbuscula* are positioned primarily along the centripetal wall (Fig. 2), and these mitochondria are in close association with, or completely enclosed by chloroplasts (Fig. 2b, c, d).

Carbon isotope value and enzyme activities

Only *Salsola arbuscula* has a typical C_4 $\delta^{13}C$ value of −12.14 ‰ (Table 1). *Sympegma regelii*, *S. arbusculiformis*, and *S. laricifolia* have C_3-like carbon isotope ratios, ranging from −22.4 to −25.03 ‰. Among these three species, *S. laricifolia* has the more positive carbon isotope value, −22.4 ‰.

Salsola arbuscula has a high level of PEPC activity, a typical value for C_4 photosynthesis (Table 1), and much greater than that of the other three species. *Salsola laricifolia* has a PEPC activity above that of *Sympegma regelii* and *S. arbusculiformis*, but it is only 7 % of the rate observed in *S. arbuscula*. *Sympegma regelii* has the highest Rubisco activity, followed by *S. arbusculiformis*, *S. laricifolia*, and *S. arbuscula*; the difference between Rubisco activities of *S. laricifolia* and *S. arbusculiformis* is not significant (Table 1; Fig. 3b, c).

CO_2 compensation point and the maximum rate of photosynthesis

Sympegma regelii exhibits a C_3-like Γ of 52.6 μmol mol^{-1} at 25 °C (Table 1). Those of *S. arbusculiformis* and *S. laricifolia* are more than half of that, 32.1 and 30.4 μmol mol^{-1}, respectively. The difference of Γ between *S. arbusculiformis* and *S. laricifolia* is not significant. In *S. arbuscula*, Γ is 6.1 μmol mol^{-1}, which is typical of C_4 plants. *Sympegma regelii*, *S. arbusculiformis*, and *S. laricifolia* have similar maximum rates of net CO_2 assimilation, in the range from 16.1 to 17.2 μmol m^{-2} s^{-1}. Compared with these three species, *S. arbuscula* has a higher A_{max}, 20.7 μmol m^{-2} s^{-1}.

Immunolocalization of Rubisco, PEPC, and P-protein GDC

The presence of the Rubisco is indicated as brown spots in all the chlorenchyma of the C_3 species *Sympegma regelii*, with a slight amount of staining in its water-storage tissues (Fig. 3a). Labeling for Rubisco in *S. arbusculiformis* and *S. laricifolia* is similar to that in *Sympegma regelii* (Fig. 3b, c). In leaves of C_4 *S. arbuscula*, labeling for Rubisco mostly occurs in the KCs, and only slightly in M cells, water-storage tissues, and hypodermal cells (Fig. 3d).

The photos of labeling for PEPC were not shown in this paper, because there was no specific staining for this enzyme in any of the species except for C_4 *S. arbuscula*. Labeling for PEPC as brown spots was seen in all the chlorenchyma cells of *Sympegma regelii*, *S. laricifolia*, and *S. arbusculiformis*. In leaves of C_4 *S. arbuscula*, strong labeling for PEPC was seen mostly in M cells and slightly in hypodermal and KCs.

Table 1 Summary on anatomical and physiological data for C_3 *Sympegma regelii*, C_3–C_4 *Salsola laricifolia*, *S. arbusculiformis*; and C_4 *S. arbuscula*

Parameter	Units	*n*	Species			
			Sympegma regelii	*Salsola laricifolia*	*Salsola arbusculiformis*	*Salsola arbuscula*
Anatomical data						
M/BS in C_3	Tissue ratio	9	8.13 ± 0.5[a]	6.44 ± 0.5[b]	6.8 ± 0.3[b]	2.4 ± 0.2[c]
M/KLC in C_3–C_4						
M/KC in C_4						
M:BS in C_3	Cell area ratio	9	8.43 ± 0.4[a]	6.26 ± 0.3[b]	6.69 ± 0.2[b]	2.25 ± 0.2[c]
M:KLC in C_3–C_4						
M:KC in C_4						
Physiological data						
A_{max}	μmol m^{-2} s^{-1}	3	16.1 ± 1.3[b]	17.2 ± 0.8[b]	16.9 ± 1.5[b]	20.7 ± 1.4[a]
Γ	μmol mol^{-1}	3	52.6 ± 1.2[a]	30.4 ± 2.6[b]	32.1 ± 1.5[b]	6.1 ± 1.0[c]
$\delta^{13}C$	‰	3	−25.03 ± 0.4[c]	−22.4 ± 0.7[b]	−23.9 ± 0.3[c]	−12.14 ± 0.5[a]
Rubisco activity	μmol mg^{-1} chlorophyll^{-1} h^{-1}	3	868.1 ± 10.2[a]	655.0 ± 5.1[b]	661.2 ± 7.9[b]	137.3 ± 6.4[c]
PEPC activity	μmol mg^{-1} chlorophyll^{-1} h^{-1}	3	18.3 ± 1.4[c]	61.5 ± 3.3[b]	50.6 ± 2.4[b]	902.7 ± 8.6[a]

A_{max} the maximum rate of photosynthesis, *BS* bundle sheath, *KC* Kranz cell, *KLC* Kranz-like cell, *M* mesophyll, Γ CO_2 compensation point, $\delta^{13}C$ carbon isotope value

Mean ± SE, letters indicate the statistical differences between species at $P < 0.05$

Labeling for P-protein GDC is seen as brown spots in all chlorenchyma cells of the C_3 species *Sympegma regelii* (Fig. 4a). The presence of P-protein GDC is indicated as brown spots exclusively in KLCs in *S. laricifolia*, *S. arbusculiformis*, and the KCs of *S. arbuscula* (Fig. 4b, c, d).

Starch localization

Only *S. arbusculiformis* and *S. laricifolia* were stained for carbohydrates; these two have similar features of starch localization (Fig. 5a, b). PAS staining for carbohydrates demonstrates that starch accumulation occurs in M cells and KLCs, but the density of PAS staining in KLCs near vasculature is higher than in M cells between bundles, and there are fewer small grains occurring in the water-storage tissue.

Discussion

Sympegma regelii was identified as a C_3 species based on leaf anatomy and its $\delta^{13}C$ carbon isotope fractionation value (Carolin et al. 1975; Wen and Zhang 2011). In this study, *S. laricifolia* is identified as a C_3–C_4 intermediate based on evidence of leaf anatomy, physiology, and biochemistry. Its leaf anatomy is characterized by two to three layers of M cells, but with more continuous and distinctive KLCs (Fig. 1b) than in the Sympegmoid type (Fig. 1a), or in the C_3–C_4 intermediate species *S. arbusculiformis* (Fig. 1c). Up to now, leaf anatomical types of C_3–C_4 intermediate plants in Salsoleae have been divided into two groups, one with discontinuous KLCs around vascular bundles, like *S. arbusculiformis*, and the other with a continuous layer of KLCs, like *S. divaricata* (Voznesenskaya et al. 2001, 2013). *Sympegma regelii*, *S. laricifolia*, and *S. arbusculiformis* all have two to three layers of M cells; but compared to the C_3 species *Sympegma regelii*, the other two have a reduced outer M layer in comparison with the inner M layer (Fig. 1a, b, c). *Salsola arbuscula* has leaves of a characteristic Salsoloid type with hypodermis, named the *Salsola soda* type (Kadereit et al. 2003; Wen and Zhang 2011) (Fig. 1d). The hypodermis may function in lowering H_2O loss, as a water-storage tissue, or as a site for deposition of crystals (Carolin et al. 1975; Gamaley 1985; Kadereit et al. 2003; Voznesenskaya and Gamaley 1986). Voznesenskaya et al. (2013) indicated that the reduced outer M layer in *S. divaricata* and *S. arbusculiformis* resembles the hypodermis layer in C_4 *Salsola* species.

The M-to-KLC tissue ratio for *S. laricifolia* is less than *S. arbusculiformis* and *Sympegma regelii*, but greater than that for *S. arbuscula* (Table 1). Decreases in the outer M size and M-to-BS/KLC tissue ratio, and increases in BS/KLC chloroplast numbers, as in *S. arbusculiformis* and *S. divaricata* (Voznesenskaya et al. 2013), are the initial anatomical preconditions leading to evolution of C_3–C_4 intermediacy, and to further possibly evolve a C_4-CO_2 concentration mechanism (Monson 1999; Sage 2004). Moreover, mitochondria in KLCs of *S. laricifolia* are positioned primarily along the centripetal wall, which is similar to the arrangement in *S. arbusculiformis* (Fig. 2b, c). These mitochondria

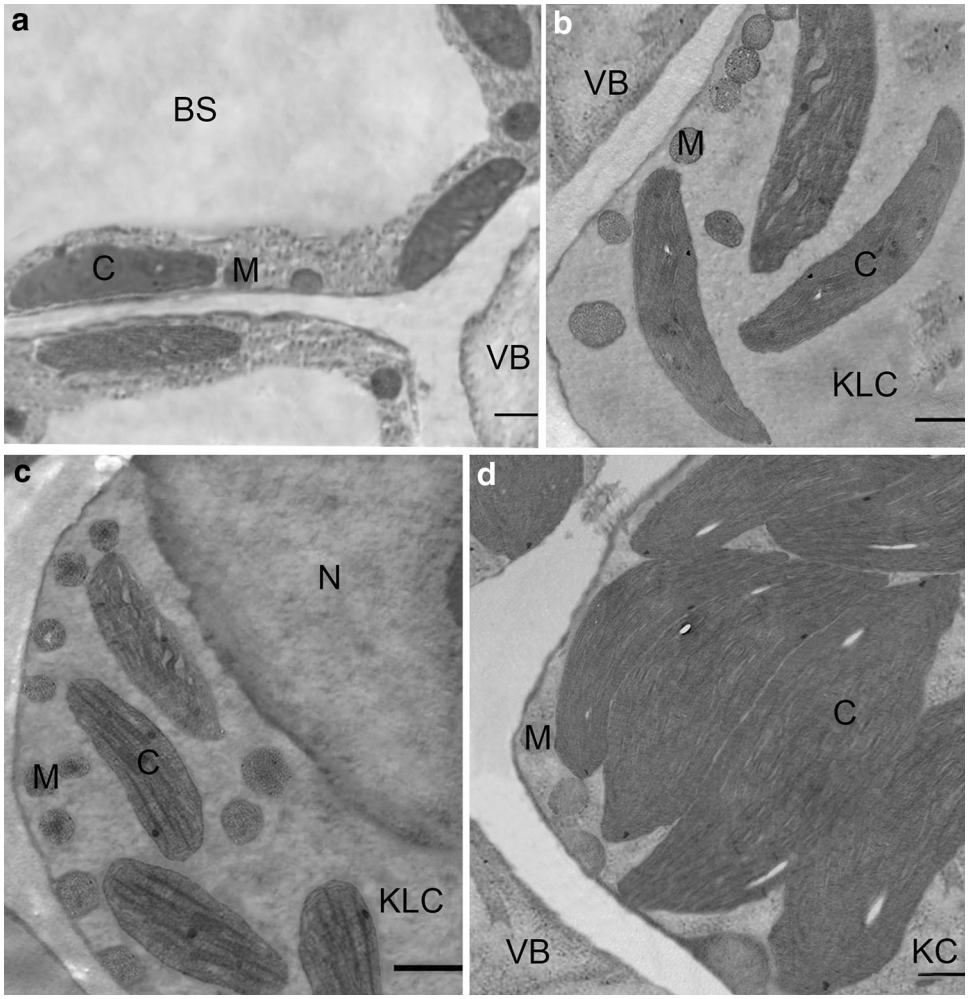

Fig. 2 Transmission electron micrographs of mitochondria and chloroplasts along the centripetal wall of bundle sheath cells in four species of Salsoleae. **a** *Sympegma regelii*; **b** *Salsola laricifolia*; **c** *Salsola arbusculiformis*; **d** *Salsola arbuscula*. *Scale bars* 0.5 μm. *BS* bundle sheath, *C* chloroplast, *KC* Kranz cell, *KLC* Kranz-like cell, *M* mitochondria, *N* nucleus, *VB* vascular bundle

in BS cells of C_3–C_4 intermediate plants are in close association with, or overlain and completely enclosed by chloroplasts, as seen in the three C_3–C_4 intermediates in *Panicum* (Brown et al. 1983b). This positioning of the mitochondria, which is the site of release of photorespired CO_2 by GDC (Rawsthorne et al. 1988), makes conditions favorable for refixation by chloroplasts, which lowers the CO_2 compensation point compared to C_3 plants.

An important feature for all identified C_3–C_4 intermediates is the compartmentalization of P-protein GDC in mitochondria of BS/KLC, as in *S. laricifolia* and *S. arbusculiformis* (Fig. 4b, c), such that glycine decarboxylase of the glycolate pathway is exclusively located in BS/KLC mitochondria. Thus, photorespiration as a consequence of RuBP Oxygenase activity in chloroplasts in M cells will occur through the glycolate pathway, with shuttle of glycine to BS/KLC, and generation of CO_2 by GDC; there it can be partially refixed by Rubisco in BS/KLC chloroplasts, which results in reduction in the CO_2 compensation point compared to C_3 plants (Bauwe 2011; Douce et al. 2001; Monson and Rawsthorne 2000; Morgan et al. 1993; Rawsthorne 1992). The confinement of GDC to BS cells could have been a primary event in the evolution of C_4 photosynthesis (Rawsthorne 1992). In C_3–C_4 intermediates, this mechanism is usually accompanied by increased organelle number in BS/KLC, concentration of mitochondria to the centripetal region of BS/KLC, and a significant enlargement of BS/KLC size in many eudicots with the exception of *S. arbusculiformis* and *S. divaricata* in Salsoleae (Brown et al. 1983a; Brown and Hattersley 1989; McKown and Dengler 2007; Monson and Rawsthorne 2000; Rawsthorne et al. 1988; Sage et al. 2011b; Voznesenskaya et al. 2013).

Γ is one of the most important physiological criterions for identification of whether a species is C_3–C_4 intermediate, and is also a qualitative measurement of apparent photorespiration (Edwards and Ku 1987). In the present study, *S. laricifolia* has a lower than expected Γ value (30.4 μmol mol^{-1}) suggestive of a C_3–C_4 intermediate, which is slightly below that in the confirmed C_3–C_4 intermediates *S. arbusculiformis* (32.1 μmol mol^{-1}) and *S. divaricata* (32 μmol mol^{-1}) (Voznesenskaya et al. 2013).

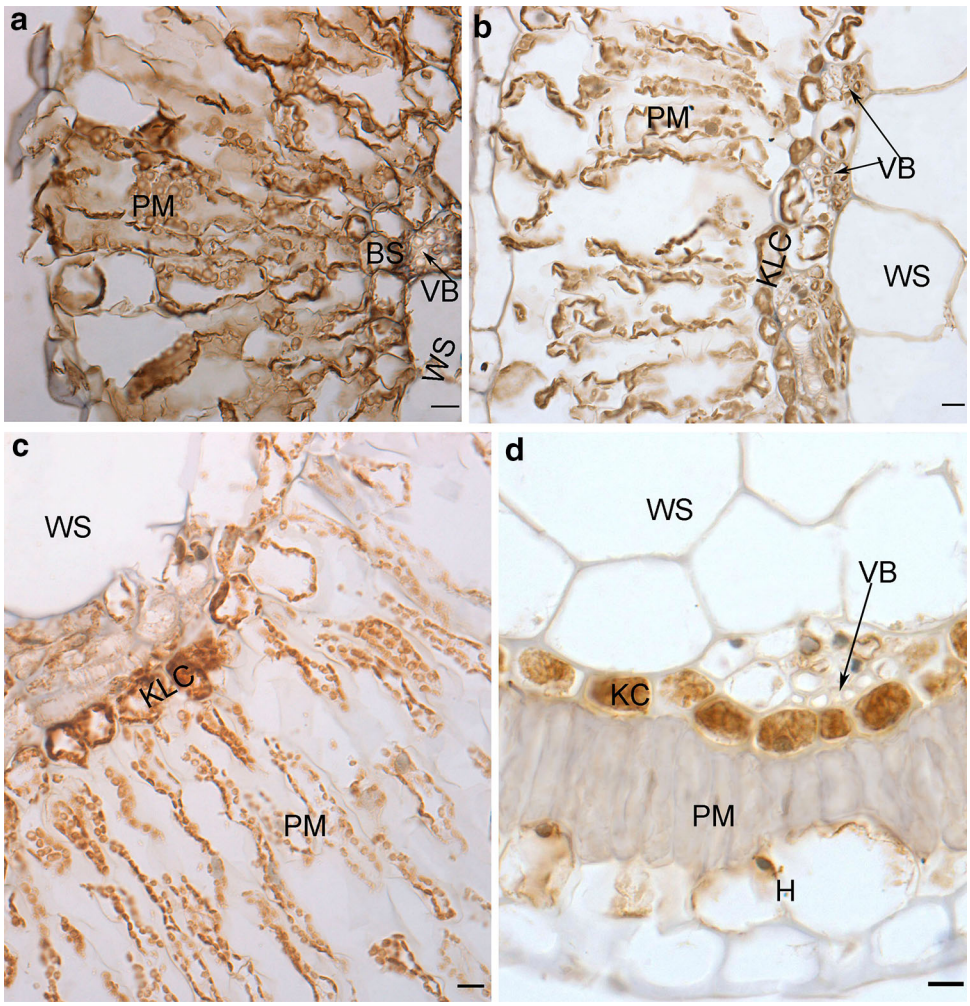

Fig. 3 Light micrographs illustrating in situ immunolocalization of Rubisco in leaves from four species of Salsoleae. **a** *Sympegma regelii*; **b** *Salsola laricifolia*; **c** *Salsola arbusculiformis*; **d** *Salsola arbuscula*. Brown precipitate indicates positive immunolabeling. *Scale bars* 20 µm. *BS* bundle sheath, *H* hypodermis, *KC* Kranz cell, *KLC* Kranz-like cell, *PM* palisade mesophyll, *VB* vascular bundle, *WS* water-storage issue

Results from this study on Γ (52.6 µmol mol^{-1}) and distribution of GDC between M and BS cells, together with the previous study on δ^{13}C carbon isotope fractionation value, suggest that *Sympegma regelii* is functioning like a C_3 species.

Carbon isotope value analysis can be employed to identify whether species are directly fixing atmospheric CO_2 via PEPC or via Rubisco in C_4 photosynthesis. In C_3 plants, Rubisco discriminates against atmospheric $^{13}CO_2$ (resulting in a more negative δ^{13}C values relative to C_4 plants), which is prevented or minimized in C_4 plants where atmospheric CO_2 is delivered to Rubisco in BS cells via the C_4 cycle (Edwards and Ku 1987; Voznesenskaya et al. 2013). Typically, δ^{13}C ratios in C_3 plants are usually between −21 and −30 ‰, and in C_4 plants range from −10 to −15 ‰ (Sage et al. 1999). In this study, *S. laricifolia* and *S. arbusculiformis* have carbon isotope values which are both within the expected range for C_3 plants (Table 1). This C_3-like isotope value shows that these two intermediates may fix atmospheric CO_2 via Rubisco in M cells, and reduce Γ by refixing photorespired CO_2 in KLCs, therefore functioning as type I intermediates; type II intermediates have a partially functional C_4 cycle, and the isotope composition is expected to have an intermediate value (Edwards and Ku 1987). The C_3-type isotope value (−22.4 ‰), the absence of clear labeling for PEPC in M cells, and the low activity of PEPC enzyme (61.5 µmol mg^{-1} chlorophyll^{-1} h^{-1}) support the identification of *S. laricifolia* as type I C_3–C_4 intermediate. Up to now, three confirmed intermediates *S. arbusculiformis*, *S. divaricata*, and *S. laricifolia* in Salsoleae have therefore been shown to be type I C_3–C_4 intermediates.

Salsola divaricata, *S. laricifolia*, and *S. arbusculiformis* belonged formerly to *Salsola* sect. *Coccosalsola* (Botschantzev 1976). However, recent phylogenetic studies do not support the existence of this section (Akhani et al. 2007). Section *Coccosalsola* is not monophyletic; its C_4 species fall into two clades, and these three C_3–C_4 intermediates also do not form a monophyletic group (Akhani et al. 2007; Wen et al. 2010). As mentioned, in the phylogenetic trees, *S. laricifolia* and *S. arbusculiformis* form a sister pair (Voznesenskaya et al. 2013; Wen et al. 2010, 2014), closely related to a C_3 branch (Wen et al. 2010).

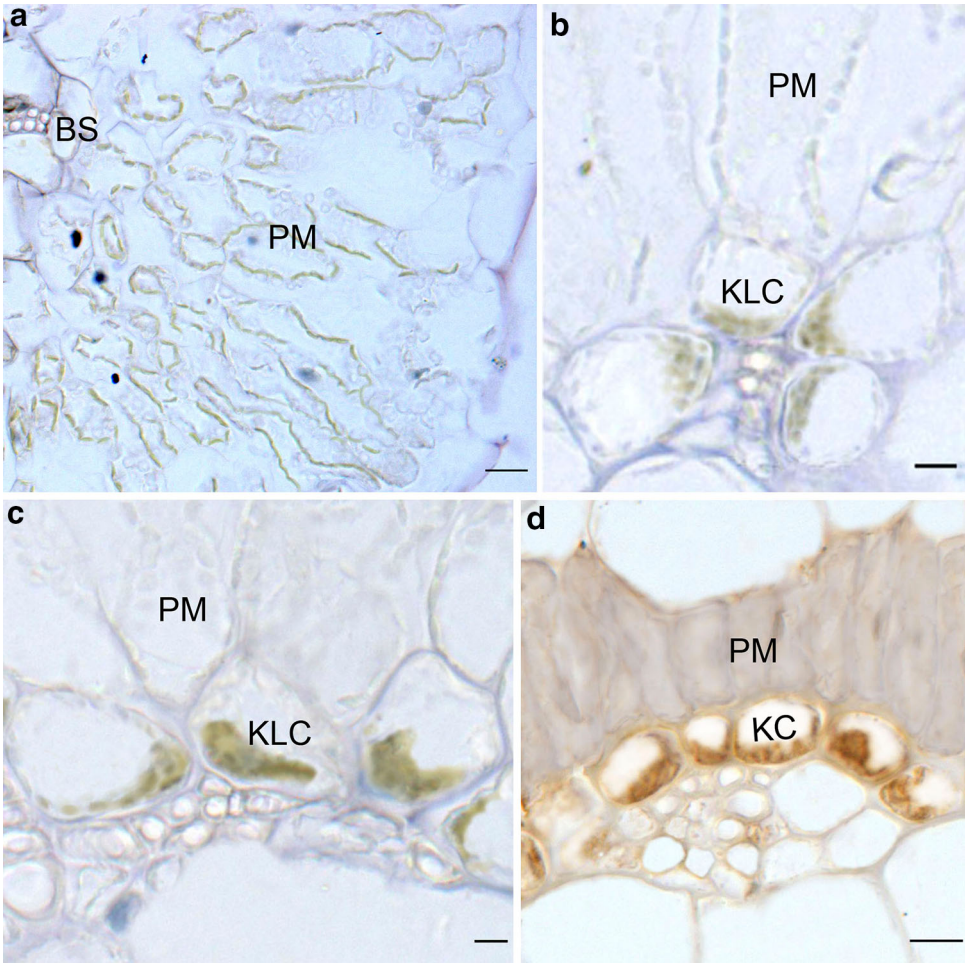

Fig. 4 Light micrographs illustrating in situ immunolocalization of P-protein of GDC in leaves from four species of Salsoleae. **a** *Sympegma regelii*; **b** *Salsola laricifolia*; **c** *Salsola arbusculiformis*; **d** *Salsola arbuscula*. Brown precipitate indicates positive immunolabeling. *Scale bars* 20 µm. *BS* bundle sheath, *KC* Kranz cell, *KLC* Kranz-like cell, *PM* palisade mesophyll

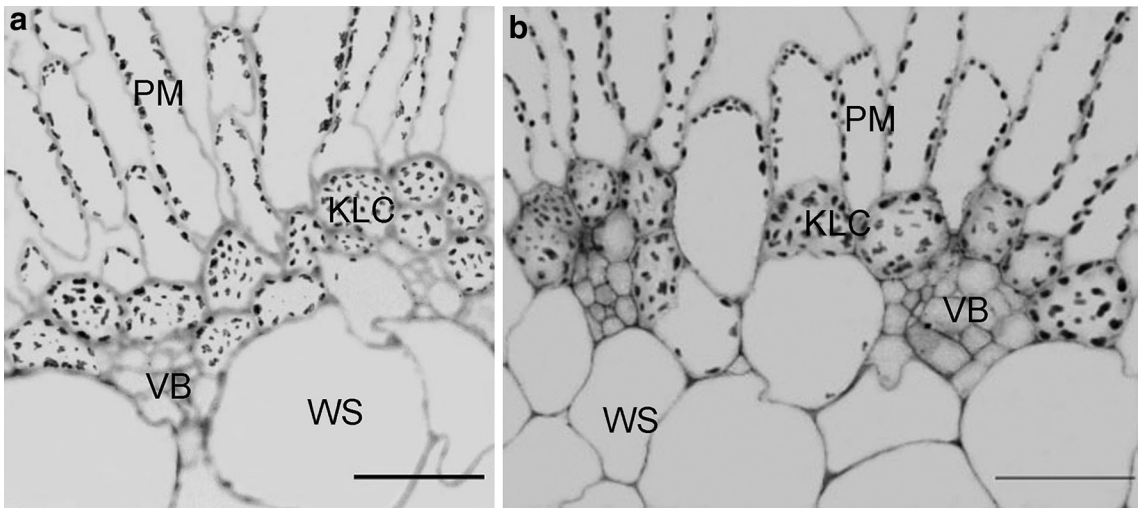

Fig. 5 Light micrographs illustrating starch localization in *Salsola laricifolia* (**a**) and *S. arbusculiformis* (**b**). Brown precipitate indicates starch localization. *Scale bars* 100 µm. *KLC* Kranz-like cell, *PM* palisade mesophyll, *VB* vascular bundle, *WS* water-storage issue

However, *S. divaricata* is not closely related to *S. laricifolia* and *S. arbusculiformis*, but is on a single species branch related to a clade of C_4 species (Akhani et al. 2007; Wen et al. 2010). Considering their distinct lineages, Akhani et al. (2007) proposed the genus '*Collinosalsola*' for *S. arbusculiformis* and *S. laricifolia*, and the genus

'*Canarosalsola*' for *S. divaricata*. *Salsola arbusculifomis* is a typical Irano-Turanian species mainly distributed from Iran and Turkmenistan to westernmost China, *S. laricifolia* is a central Asian floristic element mainly distributed from east Kazakhstan and Kyrgyzstan to northern China and Mongolia (Wen et al. 2014), and *S. divaricata* is an endemic species from the Canary Islands (Akhani et al. 2007). Although with different geographical distributions, they are mainly distributed in arid and semiarid ecosystems of temperate and subtropical regions (Delgado et al. 2006; Zhu et al. 2003), where C_4 plants in Salsoleae are also distributed (Akhani et al. 2007; Kadereit et al. 2003, 2005; Pyankov et al. 2001a). C_3–C_4 intermediates and C_4 plants are likely to evolve in habitats where photorespiration in C_3 plants is high (Sage et al. 2011b). Adaptation to dry habitats is beneficial to promotion of evolution from C_3 to C_4 in the Chenopodiaceae (Kadereit et al. 2012). A model for evolution from C_3 to C_4 in Salsoleae has been proposed based on structural and physiological analyses (Voznesenskaya et al. 2013). They proposed five conceptual phases, from performing C_3 photosynthesis with Sympegmoid anatomy, to development of proto-Kranz BS cells, functional C_3–C_4 intermediates with Kranz-like Sympegmoid type, and Kranz-like Salsoloid type, and performing C_4 with Salsoloid type anatomy. *Salsola arbusculiformis* belongs to the third phase with the Kranz-like Sympegmoid type, and *S. divaricata* belongs to the fourth phase with the Kranz-like Salsoloid type. Species from these two phases have similar trend in development of Kranz-like anatomy, like reduction in the outer M layer of cells to hypodermal-like cells, greater specialization of KLCs, and KLCs with increased cell wall thickness, organelle number, and selective expression of mitochondrial glycine decarboxylase (Voznesenskaya et al. 2013). Wider phylogenetic analysis and physiological and anatomical evaluations of Salsoleae species are needed to evaluate this model for evolution from C_3 to C_4.

Acknowledgments We are grateful to contributions made by Dr. Lin Wu and Dr. Xiaobin Zhou (measurement of CO_2 compensation points) from the Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences (CAS) (Urumqi, Xinjiang, China), Prof. Peixi Su (measurement of CO_2 compensation points) from the Cold and Arid Regions Environmental and Engineering Research Institute, CAS (Lanzhou, Gansu, China), Jianmin Luo (leaf ultrastructure) from Xinjiang University (Urumqi, Xinjiang, China), Prof. Elena V. Voznesenskaya (important comments) from Laboratory of Anatomy and Morphology, V. L. Komarov Botanical Institute of Russian Academy of Sciences (Saint-Petersburg, Russia), and Dr. Stewart C. Sanderson (English improvement) from the Shrub Sciences Laboratory (USDA, Utah, USA). This research was funded by the National Basic Research Program of China (2009CB825104), CAS West Light Foundation (No. XBBS201209), National Natural Science Foundation of China (31300217) and Xinjiang Institute of Ecology and Geography, CAS.

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