RESEARCH PAPER

Molecular phylogeny and forms of photosynthesis in tribe Salsoleae (Chenopodiaceae)

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Abstract

While many C3 lineages have Kranz anatomy around individual veins, Salsoleae have evolved the Salsoloid Kranz anatomy where a continuous dual layer of chlorenchyma cells encloses the vascular and water-storage tissue. With the aim of elucidating the evolution of C4 photosynthesis in Salsoleae, a broadly sampled molecular phylogeny and anatomical survey was conducted, together with biochemical, microscopic, and physiological analyses of selected photosynthetic types. From analyses of photosynthetic phenotypes, a model for evolution of this form of C4 was compared with models for evolution of Kranz anatomy around individual veins. A functionally C3 proto-Kranz phenotype (Proto-Kranz Sympegmoid) and intermediates with a photorespiratory pump (Kranz-like Sympegmoid and Kranz-like Salsoloid types) are considered crucial transitional steps towards C4 development. The molecular phylogeny provides evidence for C3 being the ancestral photosynthetic pathway but there is no phylogenetic evidence for the ancestry of C3–C4 intermediacy with respect to C4 in Salsoleae. Traits considered advantageous in arid conditions, such as annual life form, central sclerenchyma in leaves, and reduction of surface area, evolved repeatedly in Salsoleae. The recurrent evolution of a green stem cortex taking over photosynthesis in C4 clades of Salsoleae concurrent with leaf reduction was probably favoured by the higher productivity of the C4 cycle.

Key words: Ancestral character state reconstruction, C2 pathway, C3–C4 intermediates, CO2 compensation point, leaf anatomy, TEM, western blots.

Introduction

Reconstructing the evolution of C4 photosynthesis is challenging as it requires the complex coordination of anatomical, ultrastructural, biochemical, and gene regulatory changes from C3 ancestors (Hibberd and Covshoff, 2010; Gowik and Westhoff, 2011; Sage et al., 2012, 2014; Williams et al., 2012; Hancock and Edwards, 2014). In bringing together these
aspects, a model of C_4 evolution where Kranz anatomy is formed around individual veins has been developed over the last 30 years, which includes potential evolutionary precursors and a number of transitional, evolutionary-stable states (Monson et al., 1984; Edwards and Ku, 1987; Sage, 2004; Gowik and Westhoff, 2011; Sage et al., 2012, 2014). These theoretical states are based on distinct phenotypes observed in nature in close relatives of C_4 lineages and are characterized by a combination of C_3 and C_4 characteristics. From these, a stepwise progression from C_3 to proto-Kranz to photosynthetic intermediates, and finally to C_4 photosynthesis was proposed with a progressive reduction in photorespiration (Sage et al., 2014; hereafter named the ‘Flaveria model’ based on photosynthetic phenotypes studied in this genus).

In dicots, there are many anatomical forms of Kranz anatomy that differ in the arrangement of a dual layer of chlorenchyma cells performing the C_4 pathway. These includes forms where Kranz anatomy developments around individual veins; however, there are also nine forms where two concentric chlorenchyma layers surround all veins (Edwards and Voznesenskaya, 2011). According to Brown (1975), in C_4 plants we refer to cells of the inner chlorenchyma layer that become specialized for C_4 photosynthesis, irrespective of their position in the leaf, as Kranz cells (KC) and the outer layer as mesophyll (M) cells (Edwards and Voznesenskaya, 2011; Voznesenskaya et al., 2013). In C_3–C_4 intermediate phenotypes the inner layer of chlorenchyma, which has become specialized to support the C_3 cycle, is referred to as Kranz-like cells (KLC; Voznesenskaya et al., 2013). In C_3 species vascular bundles (VB) are surrounded by non-specialized parenchymatic bundle sheath (BS) cells.

Proto-Kranz phenotypes, first described in Heliotropium and Flaveria, are suggested to represent the initial phase of C_4 evolution where overall vein density is increased and BS cells have an increased number of organelles, with enlarged mitochondria located internally to chloroplasts in a centripetal position towards the VB (Muhaidat et al., 2011; Sage et al., 2012, 2013, 2014). C_3–C_4 intermediate phenotypes, which have been found in grasses and in a number of dicot families, have in common increased development of chloroplasts and mitochondria in the KLCs. Both M and KLC chloroplasts have Rubisco and the C_3 cycle. In the KLCs there is a distinctive layer of mitochondria that are located internally to the chloroplasts in a centripetal position. In C_3–C_4 intermediate phenotypes glycine decarboxylase (GDC) is selectively localized in the KLC mitochondria, which support a C_3 cycle by establishing a photorespiratory CO_2 pump. In the C_2 cycle photorespiratory glycine produced in the M cells is shuttled for decarboxylation by GDC to the KLCs where photorespired CO_2 is concentrated, enhancing its capture by KLC Rubisco (see Edwards and Ku, 1987; Sage et al., 2012, 2014; Voznesenskaya et al., 2013; Khoshravesh et al., 2016).

For the ‘Flaveria model’ C_1–C_4 intermediate phenotypes have been classified into two general groups: Type I and Type II C_3–C_4 species (Edwards and Ku, 1987; alternatively called Type 1 C_2 and Type 2 C_5, Sage et al., 2014). Type I C_3–C_4 species have developed little or no capacity for function of a C_4 cycle as activities/quantities of C_4 enzymes are low, similar to C_3 species. These intermediates mainly reduce losses of the CO_2 generated by photorespiration by its partial refixation in the KLCs. Type II intermediates have substantial expression of a C_4 cycle; e.g. the levels of the C_4 cycle enzymes phosphoenolpyruvate carboxylase (PEPC), pyruvate phosphate dikinase (PPDK), and NADP-malic enzyme (NADP-ME) are two- to five-fold higher in Type II C_3–C_4 species than in C_3 species (Ku et al., 1983, 1991; Edwards and Ku, 1987; Moore et al., 1987; Muhaidat et al., 2011; Sage et al., 2012). The values of CO_2 compensation points (I) in C_3–C_4 intermediate phenotypes are in between those of C_3 and C_4 species.

The fact that C_3–C_4 intermediate phenotypes thrive, persist, and occasionally have been found in lineages without any C_4 relatives, suggests that they represent an evolutionary-stable condition in their own right (Monson et al., 1984; Edwards and Ku, 1987). Their predominant occurrence close to C_4 groups may be strongly biased by the more intensive screening in these lineages. Thus, C_3–C_4 intermediate phenotypes do not necessarily represent transitional states that always lead to the establishment of C_4 photosynthesis. A C_4 cycle might already be favourable in conditions of high photorespiration, e.g. in hot, dry, and saline environments (Keerberg et al., 2014). The ‘Flaveria model’ is functionally plausible, and supported by phenotypes that actually exist in nature (Sage et al., 2014); however, phylogenetic evidence for the ancestry of the C_3–C_4 intermediate condition is scarce, and is hampered by the generally low number of species with intermediate phenotypes.

C_3–C_4 intermediate phenotypes have been recognized in 16 angiosperm genera (Sage et al., 2012, 2014; Khoshravesh et al., 2016). Often, the ancestry of the C_3–C_4 intermediate condition is inferred from a sister-group relationship of a C_4 lineage and a C_3–C_4 intermediate lineage (Sage et al., 2011, 2012) because the intermediate condition is a priori considered as less derived. However, in such cases it is impossible to distinguish between ancestry and a de novo evolution of the C_3–C_4 intermediate condition (compare with Hancock and Edwards, 2014). If those cases in which C_3–C_4 intermediate photosynthesis seems to precede C_4 photosynthesis, as suggested in Sage et al. (2011; 2012), are critically tested for unequivocal phylogenetic evidence, only Flaveria (Asteraceae) studied by McKown et al. (2005) holds up. In this case, a stepwise acquisition of C_4 photosynthesis in one lineage of Flaveria was shown (McKown et al., 2005; Lyu et al., 2015).

There are four other promising groups that are rich in C_3–C_4 intermediate phenotypes and therefore potentially informative lineages in terms of disentangling the steps of C_4 evolution and ancestral state reconstruction for C_3–C_4 intermediacy: Blepharis (Fisher et al., 2015), Anticharlis (Khoshravesh et al., 2012), Heliotropium (Sage et al., 2012), and Salsoleae sensu stricato (s.s.; Voznesenskaya et al., 2013). A better understanding of C_3–C_4 intermediate phenotypes in Salsoleae is particular important as these, in contrast to the other groups, seem to deviate from the ‘Flaveria model’ (see ‘Salsoleae model’, Voznesenskaya et al., 2013).

Salsoleae, especially the former section Cocosalsola, has long been known to contain C_3 and C_4 species (Winter, 1981; Akhani et al., 1997). In fact, Salsoleae has repeatedly been
suspected to contain species that represent reversions from C₄ back to C₃ photosynthesis (Carolin et al., 1975; P'yankov et al., 1997; Kadereit et al., 2014); however, this has been questioned by Kadereit et al. (2003). According to a survey by Voznesenskaya et al. (2013, see table 5) there are at least 21 species with δ¹³C values within the typical range of C₃ species in Salsoleae. So far, four of these have been shown to possess either proto-Kranz (Salsola montana), or a C₃–C₄ intermediate phenotype (S. arbustiformis, S. divaricata, and S. loricifolia (Voznesenskaya et al., 2013 and references therein; Wen and Zhang, 2015). In the ‘Flaveria model’ Kranz anatomy is formed around individual veins, requiring a series of anatomical changes in progression from C₃ to C₄. In Salsoleae, however, the photosynthetic tissue in leaves forms a continuous layer that surrounds all the vascular and water-storage tissue, i.e. in C₃ species by multiple layers of mesophyll tissue (Symplegma-type anatomy), and in C₄ species by a dual layer of chlorenchyma tissue forming a Kranz anatomy (Salsoloid-type anatomy). Voznesenskaya et al. (2013) proposed a model for transitions from C₃ to proto-Kranz to C₃–C₄ intermediates to C₄ in Salsoleae, based on limited photosynthetic phenotypes, which would require very different changes in leaf anatomy and regulation of development of the dual layer of chlorenchyma cells compared to that in the ‘Flaveria model’ for development of Kranz anatomy around individual veins.

Here, we conduct a large-scale analysis of Salsoleae, including species with C₃-type δ¹³C values. The results of a molecular phylogenetic study of 74 species and an anatomical survey of 77 species of Salsoleae s.s., and some outgroup species, are presented. Furthermore, in a search for additional C₃–C₄ intermediates in the tribe, anatomical, ultrastructural, enzyme content, and gas exchange analyses were performed on a number of species that have C₃-type δ¹³C values. Molecular clock and character optimization analyses were used to reconstruct the evolution of the C₄ pathway in Salsoleae. The following questions were addressed. (1) Is there evidence for additional C₃–C₄ intermediates in Salsoleae? (2) What is the current model for evolution of C₄ in Salsoleae based on analyses of photosynthetic phenotypes? (3) In what ways does this model differ from the ‘Flaveria model’ proposed in Sage et al. (2012, 2014)? (4) Where and when did C₄ photosynthesis originate in Salsoleae, and is there phylogenetic evidence for a reversion from C₄ back to C₃? (5) Does the C₃–C₄ intermediate condition represent an ancestral state to C₄ in Salsoleae?

Material and methods

Plant material and sampling

Species and samples included in the analyses with their respective voucher information are listed in Supplementary Table S1 at JXB online. We used herbarium samples and plants grown in the greenhouse at the Botanical Gardens of the University of Mainz, Germany, and at Washington State University (WSU), Pullman, WA, USA, and leaves of specimens that were fixed during various expeditions, mainly by H. Freitag. A few samples were kindly provided by other institutions. Species of the Kadi clade were mostly left out, because in the trees based on chloroplast sequence data they are separated from the Salsoleae s.s.

In WSU, plants were grown in 15-cm diameter pots with commercial potting soil in a growth chamber (model GC-16; Enconair Ecological Chambers Inc., Winnipeg, Canada) under a 14/10 h 25/18 °C day/night cycle under mid-day PPF of ~500 µmol quanta m⁻² s⁻¹, and 50% relative humidity for ~2 months. Plants were watered daily and fertilized once per week with Peter’s Professional fertilizer (20:20:20 Scotts Miracle-Gro, Marysville, OH, USA).

Our sampling of Salsoleae for the molecular phylogenetic analyses comprised 74 species representing all currently accepted genera (Supplementary Table S2) and included 15 species with C₃-like carbon isotope (δ¹³C) values (compare with table 5 in Voznesenskaya et al., 2013). Furthermore, representatives of other primary clades of Salsoleoideae (Supplementary Table S2) as well as representatives of Camphorosmoideae were included. For rooting and dating purposes Suaoideae and Salicordioideae were sampled as outgroups (Supplementary Table S1). For light microscopy 77 species were examined, mostly from the same material (Supplementary Table S1), including 34 species studied for the first time. Data for eight species were taken from the literature. Six species having different anatomical types (most not previously known in that respect) were chosen for study by electron microscopy, and δ¹³C, in situ immunolocalization, western blot, and gas exchange analyses.

With respect to nomenclature, apart from a few exceptions we follow previous accounts of the different subfamilies, in particular Botschantzev (1989), Akhani et al. (2007), and Kadereit and Freitag (2011), although we are aware that more nomenclatural adjustments are required.

Sequencing and phylogenetic inference

Total DNA was extracted from dried or fresh leaf material using the DNeasy Plant Mini Kit (QIAGEN, Germany) or innuPREP Plant DNA Kit (Analytik Jena, Germany) following the manufacturers’ protocols but increasing incubation times. PCRs for five markers (atpB-rbcL intergenic spacer, ndhF-rpL32 spacer, trnQ-rps16 spacer, rpl16 intron, ITS) were carried out in a T-Professional or T-Gradient Thermocycler (Biometra, Germany), or a PTC100 Thermocycler (MJ Research, USA). Primers sequences, PCR recipes, and cycler programs are documented in Supplementary Table S3. PCR products were checked on 0.8% agarose gels and purified using the NucleoSpin® Gel and PCR clean-up-Kit (Macherey-Nagel, Germany) or ExoSAP (Affymetrix, USA) following the manufacturers’ instructions. The Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) combined with the primers mentioned above was used for the sequencing reactions, followed by a purification step using IllustraTM SephadexTM G-50 Fine DNA Grade (GE Healthcare, UK). Sequencing was performed following the Sanger method on a 3130xI Genetic Analyzer (Applied Biosystems Inc., USA). The raw forward and reverse sequences were checked and automatically aligned in Sequencher 4.1.4 (Gene Codes Corporation, USA). The refined alignment was performed in Mesquite 2.75 (http://mesquiteproject.org) and carefully checked visually. The program SequenceMatrix (v. 1.7.8; Vaidya et al., 2011) was used to combine the four chloroplast (cp) marker data sets.

Phylogenetic analyses under the settings outlined below were initially conducted individually for the five selected DNA regions. Results of the individual analyses of the four cp markers revealed no topological conflict [i.e. incongruence with ML Bootstrap ≥65% and Posterior Probability (PP) ≥0.90] among individual markers and combination of the cp markers distinctly increased the resolution and support values. For the cp data further analyses were performed using two large and different data sets: (1) a data set with 106 taxa (106t data set) including a broad outgroup sampling; and (2) a data set with 75 taxa (75t data set) in which only Salsola genistoides served as the outgroup. The 106t data set was used to reveal primary clades in Salsoleoideae and for estimation of divergence time, whereas the 75t data set was used for character optimization (see below). Since the ITS tree and the cp tree showed supported conflict at basal branches, and the combination of cp data and ITS for the 75t data set led to a
significant decrease of resolution and support values in some parts of the tree, we concentrated on the cp tree for further analyses and only include the ITS tree for comparison. First, the best-fitting substitution model for the combined cp data sets was inferred using jModelTest (Posada, 2008). CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway V. 3.3. ML phylogenetic analyses were performed using RAxML (Stamatakis, 2006; Stamatakis et al., 2008), including bootstrapping that was halted automatically following the majority-rule ‘autoMRE’ criterion. Bayesian inference (BI) was conducted using BEAST (Bayesian Evolutionary Analysis by Sampling Trees v.1.8.2; Drummond and Rambaut, 2007) with GTR+G (general time-reversible; best-fitting according to jModeltest under AIC criterion) with a gamma-distribution in four categories as the substitution model. A birth-and-death demographic model was used as the tree prior. Markov Chain Monte Carlo (MCMC) analysis was performed with the following settings: randomly generated starting tree, 20,000,000 (106 data set) or 10,000,000 iterations (750 data set), discarded burn-in of 10%, and sampling every 1000 steps (750 data set). For the 106 data set a relaxed clock model was implemented in which rates for each branch are drawn independently from an exponential distribution (Drummond et al., 2006). The crown node of Salsoloideae and Camphoroideae was set to 47.0–25.5 Ma by on divergence-time estimates in the Chenopodiaceae/Amaranthaceae complex (Kadereit et al., 2012). We assumed a uniform distribution within the age bounds set. Other settings were left in default.

### Light and electron microscopy

For light microscopy, after routine checks by manual sectioning, the middle parts of well-developed leaves were selected for transections by a rotary microtome (Leitz 1515). The semi-thin sections of material fixed in FAA [2% (v/v) formaldehyde, 0.5% (v/v) acetic acid, 70% (v/v) ethanol] were studied under a Dialux 20 (Leitz, Wetzlar). Some were first examined and photographed in water to get a better contrast between lignified (blue) and non-lignified (purple) cell walls before embedding into Depex (Serva) for documentation. For detailed study, middle parts of fully developed leaves were fixed and processed in a similar way to that described in Voznesenskaya et al. (2013).

For screening purposes, leaf samples from herbarium specimens were first boiled for about 1–3 minutes and hand-cut sections were preserved in glycerol–gelatin. Selected samples for microtome transections were soaked in a 10% solution of NH4 for 10 d, dehydrated in ethanol, and embedded in Technovit 7100 ( Heraeus Kulzer). The samples were sectioned at 5–20 µm using a rotary microtome. Sections were stained in a 6:6:5:6 mixture of Azur II, Eosin Y, methylene blue, and distilled water and mounted in Eukitt (O. Kindler) after drying. Images of the sections were taken using a Leitz Diaplan light microscope combined with Leica Application Suite 2.8.1.

For ultrastructural characterization, ultra-thin sections were taken from the same samples prepared for the light microscopy study and embedded in Spurr’s resin as described in Voznesenskaya et al. (2013). The number and sizes of mitochondria in chloroplasts were estimated per cell section (about 10–15 cell images from 2–3 separate leaf samples) using an image analysis program (ImageJ 1.37v, https://imagej.nih.gov/ij/index.html).

### δ¹³C values

Carbon isotope composition of plant samples was determined at Washington State University using a standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard (Bender et al., 1973). Leaf samples (from plants growing in the WSU growth chamber) were dried at 60 °C for 24 h, and then 1–2 mg were placed in a tin capsule and combusted in a Eurovector elemental analyser. The resulting N2 and CO2 gases were separated by gas chromatography and admitted into the inlet of a Micromass Isoprobe isotope ratio mass spectrometer (IRMS) for determination of ¹³C/¹²C ratios (R). δ¹³C values were determined where δ = 1000 × (Rsample/Rstandard) − 1.

### In situ immunolocalization

Sample preparation and immunolocalization by transmission electron microscopy (TEM) were carried out according to Voznesenskaya et al. (2013). The antibody used (raised in rabbit) was against the P subunit of glycine decarboxylase (GDC) from Pisum sativum L. (courtesy of D. Oliver, Iowa State University). Pre-immune serum was used for controls. The density of labeling was determined by counting the gold particles on electron micrographs and calculating the number per unit area (µm2) using ImageJ 1.37v. For each cell type, replicate measurements were made on parts of cell sections (n = 10–15 cell images). Immunolabeling procedures were performed separately for different species; the difference in the labeling intensity reflects the difference between cell types but not between species. The level of background labeling was low in all cases.

### Western blot analysis

Extraction of total soluble proteins, protein separation, and blotting onto a nitrocellulose membrane were carried out according to Voznesenskaya et al. (2013). A loading control with protein samples (20 µg) separated by 10% (w/v) SDS-PAGE can be found in Supplementary Fig. S1. Western blots were performed using anti-Amaranthus hypochondriacus NAD-malic enzyme (NAD-ME) IgG, which was prepared against the 65-kDa α subunit (courtesy of J. Berry; Long and Berry, 1996) (1:5000), anti-Zea mays 62-kDa NADP-malic enzyme (NADP-ME) IgG (courtesy of C. Andreo; Maurino et al., 1996) (1:5000), anti-Zea mays PEPC IgG (1:10000), and anti-Zea mays pyruvate-Pi dikinase (PPDK) IgG (courtesy of T. Sugiyama) (1:5000). The intensities of bands in western blots were quantified using ImageJ 1.37v and expressed relative to the level in the C4 species Oryza sativa, which was set at 100%.

### CO₂ compensation point

Measurements of CO₂ compensation points (Γ) were made on an individual lateral branch using a Li-Cor lighted chamber (LI-6400-22L; Li-Cor Biosciences, Lincoln, NE, USA) designed for terete or semi-terete conifer leaves. For each species, a part of a branch of an intact plant was enclosed in the chamber and illuminated with a PPF of 1000 µmol quanta m⁻² s⁻¹ under 400 µmol mol⁻¹ CO₂ at 25 °C until a steady-state rate of CO₂ fixation was obtained (generally 45–60 min). For varying CO₂ experiments, the CO₂ level was first decreased, and then increased up to 400 µmol mol⁻¹ at 5 min intervals. Γ was determined by extrapolation of the initial slope of rate of CO₂ fixation (A) versus the intercellular CO₂ concentration in the leaf (C) through the x-axis where the net rate of CO₂ assimilation equals zero. The leaf area exposed to the incident light was calculated by taking a digital image of the part of the branch that was enclosed in the chamber, and then determining the exposed leaf area using ImageJ 1.37v.

### Statistical analysis

Where indicated, standard errors were determined, and ANOVA was performed using Statistica 7.0 software (StatSoft, Inc.). Tukey’s HSD (honest significant difference) test was used to analyze differences between amounts of gold particles in BS/KLC/KC versus M for each species, and δ¹³C and Γ values in different species. All analyses were performed at the 95% significance level.

### Character coding and analyses of character evolution

Analyses of character evolution were conducted for five traits: (1) type of photosynthesis; (2) KC/KLC function; (3) life form; (4) leaf selerenchyma; and (5) leaf reduction (Table 1). Traits were optimized over 1000 trees of 74 Salsoleae and Salsola genistoides as the outgroup obtained in a Bayesian analysis (see above) using the ML criterion in Mesquite (http://mesquiteproject.org). The fit of single-versus two-rate models was tested for traits with two character states using a likelihood ratio test. Table 1 gives information about the coding of the character states of the five traits.
Table 1. Traits of photosynthetic pathway, leaf anatomy and life form in Salsoleae s.s.

| Species of Salsoleae s.s. | Isolate no. for molecular analysis | Trait 1: Type of photosynthesis according to carbon isotope ratio | Leaf anatomy; type names according to Voznesenskaya et al. (2013) | Trait 2 | Trait 3 | Trait 4 | Trait 5 |
|---------------------------|-----------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|--------|--------|--------|--------|
| Anabasis aphylla L.       | chen 2743/2017                    | C4 (1, 2, 12)                                                | salsolid+H (1, 12, 6, this study)                              | 2      | 0      | 0      | 2      |
| Anabasis articulata (Forssk.) Moq. | chen 2360                   | C4 (7)                                                        | salsolid+H (6, 12, 8, this study)                              | 2      | 0      | 0      | 2      |
| Anabasis brevifolia C.A. Mey. | chen 2407                      | C4 (12)                                                       | salsolid+H (2, 11, this study)                                 | 2      | 0      | 0      | 1      |
| Anabasis calcarea (Charl. & Aellen) Bokhari & Wendelbo | chen 1841                      | C4 (1)                                                        | salsolid+H (1, this study)                                     | 2      | 0      | 0      | 2      |
| Anabasis ehrenbergii Schweinf. ex Boiss. | chen 2403/2741               | C4 (12)                                                       | salsolid+H (this study)                                        | 2      | 0      | 0      | 2      |
| Anabasis setifera Moq.    | chen 2373                        | C4 (7)                                                        | salsolid+H (1, 2, 6, 12, this study)                           | 2      | 0      | 0      | 1      |
| Arthrolytum betapakdalense Korov. | chen 0229                    | C4 **                                                         | salsolid+H (this study)                                        | 2      | 0      | 0      | 1      |
| Arthrolytum gracile Aellen | chen 2603                        | C4 (1)                                                        | salsolid+H (this study)                                        | 2      | 0      | 0      | 2      |
| Arthrolytum lehmaniannum Bunge | chen 2637                    | C4 (4)                                                        | salsolid+H (5, this study)                                     | 2      | 0      | 0      | 1      |
| Conulaca amblycantha Bunge | chen 0350                        | C4 **                                                         | salsolid+H+ (S, this study)                                    | 2      | 0      | 1      | 1      |
| Conulaca monacantha Delile | chen 0212                        | C4 (1, 12)                                                    | salsolid+H+ (S, 2, this study)                                 | 2      | 0      | 1      | 1      |
| Conulaca setifera (DC.) Moq. | chen 0304                     | C4 (12)                                                       | salsolid+H+ (S, 6, this study)                                 | 2      | 0      | 1      | 1      |
| Cytobasion fruticulosa (Bunge) Aellen | chen 0082                    | C4 (12)                                                       | salsolid+H+ (S, this study)                                    | 2      | 0      | 1      | 1      |
| Girgensohnia diplerta Bunge | chen 2639                        | C4 **                                                         | salsolid+H+ (S, this study)                                    | 2      | 1      | 1      | 2      |
| Girgensohnia minima E. Korov. | chen 2601                      | C4 **                                                         | salsolid+H+ (S, this study)                                    | 2      | 1      | 1      | 2      |
| Girgensohnia oppositiflora (Pall.) Fenzl | chen 0033                    | C4 (1, 12)                                                    | salsolid+H+ (S, 2, 6, this study)                              | 2      | 1      | 1      | 1/2    |
| Glycoryza giglietti Botsch. | chen 2819                        | C4 **                                                         | salsolid+H+ (this study)                                       | 2      | 0      | 0      | 0      |
| Halogelaton alpacaronoides (Delile) Moq. | chen 0300                    | C4 (1, 7, 12)                                                 | salsolid+H (6, 8, 12, this study)                              | 2      | 0      | 1      | 0      |
| Halogelaton archochnoides Moq. | chen 2605                        | C4 (1)                                                        | salsolid+H+ (this study)                                       | 2      | 1      | 0      | 0      |
| Halogelaton glomeratus (M. Bleb.,) C.A. Mey. | chen 0030                    | C4 (1)                                                        | salsolid+H (2)                                                 | 2      | 1      | 0      | 0      |
| Halogelaton sativus (L.) Moq. | chen 1229                        | C4 (1)                                                        | salsolid+H (8, this study)                                     | 2      | 1      | 0      | 0      |
| Halothamnus bottejaub. & Spach | chen 0351                        | C4 (12)                                                       | salsolid (7)                                                  | 2      | 0      | 0      | 2      |
| Halothamnus farganianus Botsch. | chen 0197                        | C4 **                                                         | salsolid (7)                                                  | 2      | 0      | 0      | 1      |
| Halothamnus ilinensis (Lipsky) Botsch. | chen 2668                    | C4 (1, 12)                                                    | salsolid (7)                                                  | 2      | 1      | 0      | 1      |
| Halothamnus somalensis (N.E. Br.) Botsch. | chen 2584                        | C4 **                                                         | salsolid (7)                                                  | 2      | 0      | 0      | 2      |
| Haloxylon ammodendron (C.A. Mey.) Bunge | chen 0035                     | C4 (1, 2, 12)                                                 | salsolid+H (11, this study)                                    | 2      | 0      | 0      | 2      |
| Haloxylon persicum Bunge ex Boiss. | chen 2815                     | C4 (7, 12)                                                    | salsolid+H (11)                                               | 2      | 0      | 0      | 2      |
| Hammada articulata (Moq.) O. Bolos & Vigo | chen 0196                        | C4 **                                                         | salsolid+H (2, 6, this study)                                  | 2      | 0      | 0      | 2      |
| Hammada eriantha Botsch. | chen 2813                        | C4 **                                                         | salsolid+H (this study)                                        | 2      | 0      | 0      | 2      |
| Hammada griffithii (Moq.) Iljin | chen 2635                        | C4 (1, 12)                                                    | salsolid+H+ (S, this study)                                    | 2      | 0      | 1      | 2      |
| Hammada negevensis Iljin & Zoh. | chen 2814                        | C4 (7, 12)                                                    | salsolid+H+ (S, this study)                                    | 2      | 0      | 0      | 2      |
| Hammada salicornica (Moq.) Iljin | chen 2752                        | C4 (1, 7)                                                     | salsolid+H (2, 8, 12, this study)                              | 2      | 0      | 0      | 2      |
| Hammada schmitthiania (Pomeli) Botsch. | chen 2629                        | C4 (1, 7, 12)                                                 | salsolid+H+ (this study)                                       | 2      | 0      | 0      | 2      |
| Hammada thomsonii (Bunge) Iljin | chen 0178                        | C4 **                                                         | salsolid+H+ (S, this study)                                    | 2      | 0      | 1      | 2      |
| Horaninowia capitata Sukhoro. | chen 0188                        | C4 **                                                         | salsolid+H+ (S, this study)                                    | 2      | 1      | 1      | 1      |
| Horaninowia platypetra Charl. & Aellen | chen 2602                        | C4 (1)                                                        | salsolid+H+ (S, this study)                                    | 2      | 1      | 1      | 1      |
| Horaninowia ulicina Fisch. & C.A. Mey. | chen 2589                        | C4 (1)                                                        | salsolid+H+ (S, 15, this study)                                | 2      | 1      | 1      | 1      |
| Horaninowia illiniae (Bunge) Korovin | chen 0182                        | C4 (4)                                                        | salsolid+H (11, this study)                                    | 2      | 0      | 0      | 0      |
| Lagernantha cycloptera (Stapf.) M.G. Gilbert & Fritsch | chen 2809                        | C4 **                                                         | salsolid+H (this study)                                        | 2      | 0      | 0      | 0      |
| Noaea minuta Boiss. & Bal. | chen 0079                        | C4 (12)                                                       | salsolid+H (S, this study)                                     | 2      | 1      | 1      | 0      |
| Noaea mucronata (Forssk.) Asch. & Schweinf. | chen 0019                        | C4 (1, 2, 7)                                                  | salsolid+H+ (S, 6, 9, this study)                              | 2      | 0      | 1      | 1/2    |
| Nuculania pennis Batt. | chen 2627                        | C4 **                                                         | salsolid+H (8, this study)                                     | 2      | 0      | 0      | 0      |
| Species of Salsoleae s.s. | Isolate no. for molecular analysis | Trait 1: Type of photosynthesis according to carbon isotope ratio | Leaf anatomy: type names according to Voznesenskaya et al. (2013) | Trait 2 | Trait 3 | Trait 4 | Trait 5 |
|--------------------------|-----------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|--------|--------|--------|--------|
| *Rhaphidophyton regelii* (Bunge) Ilin | chen 0075 | C₃ (4) | kranz-like salsoloid+S (this study) | 1 | 0 | 1 | 0 |
| *Salsola abrotanoides* Bunge | chen 2996 | C₃ (4, 6, 9) | symplegmoid (11, this study) | 0 | 0 | 0 | 0 |
| *Salsola acutifolia* (Bunge) Botsch. | chen 2640 | C₃ (1, 12) | salsoloid+H (this study) | 2 | 1 | 0 | 0 |
| *Salsola arbusculiformis* Drob. | chen 0176 | C₃ (1, 2, 6, 8, 11) | kranz-like symplegmoid (13, 16, this study) | 1 | 0 | 0 | 0 |
| *Salsola botschantzewii* Kurbanov | chen 2630 | C₃ (9) | proto-kranz symplegmoid (this study) | 0 | 0 | 0 | 0 |
| *Salsola cyrenaica* (Maire & Weiller) Brullo | chen 0354 | C₃ (9) | salsoloid+H (3, this study) | 2 | 0 | 0 | 0 |
| *Salsola deschaseauiaria* Littard. & Maire | chen 2758 (= 2641) | C₃ (9) | kranz-like salsoloid (this study) | 1 | 0 | 0 | 0 |
| *Salsola divaricana* Masson ex Link | chen 2779 | C₃ (6, 9) | kranz-like salsoloid (14, this study) | 1 | 0 | 0 | 0 |
| *Salsola drobowi* Botsch. | chen 0175 | C₃ (4, 6, 9) | proto-kranz symplegmoid (this study)** | 0 | 0 | 0 | 0 |
| *Salsola flordia* (M. Bleib.) Poir. | chen 2811 | C₃ (1, 12) | salsoloid+H (2, 6, this study) | 2 | 1 | 0 | 0 |
| *Salsola folosa* (L.) Schrad. | chen 0103 | C₃ (1) | salsoloid+H (11, this study) | 2 | 1 | 0 | 0 |
| *Salsola grandi Freitag, Vural & N. Adigüzel* | chen 0105 | C₃ ** | salsoloid+H (4, this study) | 2 | 1 | 0 | 0 |
| *Salsola gymnossachale* Maire | chen 0355 | C₃ (9) | kranz-like salsoloid (this study) | 1 | 0 | 0 | 0 |
| *Salsola juntatovii* Botsch. | not included | C₃ (9) | proto-kranz symplegmoid (this study) | 0 | 0 | 0 | 0 |
| *Salsola karneri* (Vol.) Botsch. | chen 2642 | C₃ (1, 6) | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Salsola larcifolia* Turcz. ex Litv. | chen 1355 | C₃ (6, 9, 10, 11) | kranz-like salsoloid (14, 15, 16, this study) | 1 | 0 | 0 | 0 |
| *Salsola lipschitzii* Botsch. | not included | C₃ (9) | proto-kranz symplegmoid (this study) | 0 | 0 | 0 | 0 |
| *Salsola melitensis* Botsch. | chen 2644 | C₃ (9) | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Salsola montana* Litv. | chen 2591 | C₃ (1, 2, 5, 6, 9) | proto-kranz symplegmoid (14) | 0 | 0 | 0 | 0 |
| *Salsola oppositifolia* Desf. | chen 0099 | C₃ (1, 6, 9, 12) | salsoloid+H (8, this study) | 2 | 0 | 0 | 0 |
| *Salsola oreophila* Botsch. | chen 2847 | C₃ (5, 9) | symplegmoid (10, this study) | 0 | 0 | 0 | 0 |
| *Salsola pachychylla* Botsch. | chen 2762 | C₃ (5, 6) | symplegmoid (11, this study)) | 0 | 0 | 0 | 0 |
| *Salsola rosmaninus* (Ehrenb. ex Boiss.) Akhani | chen 0303 | C₃ (1, 7) | salsoloid+H (2, 6, this study) | 2 | 0 | 0 | 0 |
| *Salsola schweinfurthii* Solms-Laub. | chen 2827 | C₃ (1, 6, 7, 12) | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Salsola soda* L. | chen 2834 | C₃ (1, 7) | salsoloid+H (8, this study) | 2 | 1 | 0 | 0 |
| *Salsola stocksi* Boiss. | chen 2646 | C₃ (1) | salsoloid+H (2) | 2 | 0 | 0 | 1 |
| *Salsola tianschanica* Botsch. | not included | C₃ (9) | symplegmoid (this study) | 0 | 0 | 0 | 0 |
| *Salsola tunetana* Brullo | chen 2647 | C₃ ** | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Salsola verticillata* Schousboe | chen 2648 | C₃ (this study)* | kranz-like salsoloid (this study) | 1 | 0 | 0 | 0 |
| *Salsola webbii* Moq. | chen 2828 | C₃ (1, 6, 9, 12) | symplegmoid (2, 8, 14, this study) | 0 | 0 | 0 | 0 |
| *Salsola zygophyilla* Batt. & Trab. | chen 2756 | C₃ (1, 6, 12) | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Salsola zygophyiloides* (Aellen & Townsend) Akhani | chen 2593 | C₃ ** | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Sevada schimperii* Moq. | chen 2590 | C₃ (3) | salsoloid+H (this study) | 2 | 0 | 0 | 0 |
| *Sympegma regiellii* Bunge | chen 383a/2766 | C₃ (4, 11) | symplegmoid (2, 9, 16, this study) | 0 | 0 | 0 | 0 |
| *Salsola genistoides* Juss. ex Poir. (outgroup) | chen 1155/1362 | C₃ (1, 9) | symplegmoid (11, this study) | 0 | 0 | 0 | 2 |

**References for C₃ versus C₄ type carbon isotope ratio:** 1 = Akhani et al. (1997), 2 = Akhani and Ghasemkhani (2007), 3 = Carolin et al. (1975), 4 = Freitag and Stichler (2000), 5 = P’yankov et al. (1997), 6 = P’yankov et al. (2001), 7 = Shomer-Ilan et al. (1981), 8 = Voznesenskaya et al. (2001), 9 = Voznesenskaya et al. (2013), 10 = Wen and Zhang (2011), 11 = Wen and Zhang (2015), 12 = Winter (1981).

**References for leaf anatomy:** 1 = Bokhari and Wendelbo (1978), 2 = Carolin et al. (1975), 3 = Freitag and Duman (2000), 4 = Freitag et al. (1999), 5 = Freitag and Stichler (2000), 6 = Khattib (1959), 7 = Kotte-Heinrich (1963), 8 = Mare (1982), 9 = Montiel (1998), 10 = P’yankov et al. (1997), 11 = P’yankov et al. (2001), 12 = Volkens (1887), 13 = Voznesenskaya et al. (2001), 14 = Voznesenskaya et al. (2013), 15 = Wen and Zhang (2011), 16 = Wen and Zhang (2015).

* In Voznesenskaya et al. (2013) this species was mentioned to have the C₃ pathway. However, samples for the carbon isotope value were taken from a wrongly identified specimen [D. Podlech 44954 (P)]. The correct identification for this specimen is *Salsola oppositifolia*, which indeed is a C₄ species.

**C₃ metabolism deduced from leaf anatomy, no carbon isotope values available.**

*** Classified as symplegmoid in Freitag and Duman (2000), P’yankov et al. (2001) and Khattib (1959)."
Results

Leaf anatomy

Light microscopy

Figure 1A shows that *S. abrotanoides* (*C₃*) mostly had two layers of palisade M cells. The peripheral vascular bundles (VBs) were surrounded by a layer of bundle sheath (BS) cells, which looked similar to the adjacent cells of the water-storage (WS) tissue but were smaller.

Four species (all *C₃–C₄*), *R. regelii*, *S. deschaseuxiana*, *S. gymnomaschala*, and *S. verticillata* (Fig. 1B–E) were similar in having two layers of chlorenchyma cells underneath the epidermis. While the inner layer consisted of elongated palisade cells (M₂), the cells of the outer layer (M₁) were 2–5 times shorter with a reduction to varying degrees in the number of chloroplasts depending on species and growth conditions. In *R. regelii* the M₁ cells were elongated (Figs 1B and 2), but in the other species the M₁ cells appeared almost globular to polygonal in shape; they were wider than the M₂ palisade cells and more similar to the typical hypodermis of *C₄* species (Fig. 1C–E). These species had a clearly defined continuous (or almost continuous in *R. regelii*) layer of chlorenchymatous Kranz-like cells (KLCs), which was situated above and between the peripheral VBs.

In *S. oppositifolia* (*C₄*) the palisade M₂ cells were rather short and M₁ cells were represented by a typical hypodermis consisting of large globular cells that were almost devoid of chloroplasts. The KCs had organelles in a centripetal position; they formed a continuous layer just beneath the palisade cells (Fig. 1F).

Among the *Salsola* species in this study, *C₃* *S. abrotanoides* had the lowest volume of WS tissue. In *R. regelii*, the inner part of the WS tissue was replaced by massive sclerenchyma tissue, which accounted for half of the leaf diameter and for

**Fig. 1.** General anatomy in leaves of five *Salsola* species (A, C–F) and *Rhaphidophyton regelii* (B). *Salsola abrotanoides* (A), *S. deschaseuxiana* (C), *S. gymnomaschala* (D), *S. verticillata* (E), and *S. oppositifolia* (F). The images show light microscopy on leaf cross-sections illustrating the position of the palisade mesophyll (M) and bundle sheath (BS)/Kranz-like cells (KLCs)/Kranz cells (KCs). Note the continuous layer of KLCs in *R. regelii*, *S. deschaseuxiana*, *S. gymnomaschala*, and *S. verticillata*, and the difference between the outer (M₁) and inner (M₂) layers of mesophyll. Sclerenchyma (S) and water-storage (WS) tissue are also indicated. Scale bars = 200 μm for (A); 100 μm for (B–F).

**Fig. 2.** Leaf cross-sections of *Rhaphidophyton regelii*, a *C₃–C₄* species with Kranz-like Salsoloid leaf anatomy. (A) Cross-section of entire leaf, and (B) close-up of the chlorenchyma. Abbreviations: E, epidermis; M, mesophyll; KLC, Kranz-like cells; WS, water storage tissue; S, sclerenchyma; CVB, central vascular bundle; PVB, peripheral vascular bundles; X, xylem; P, phloem; ST, stoma.
the stiff appearance of the leaves (Fig. 2). Crystal-bearing idioblasts were preferentially located in the hypodermis or hypodermis-like layer, and in the Kranz-like layer between the peripheral bundles, but they could occur scattered elsewhere. In *S. gymnomaschala* and in *S. verticillata* the epidermis was partially doubled. In all *Salsola* species the main vein was located more or less in the center of the leaf and surrounded by 2-4 layers of WS tissue.

**Transmission electron microscopy**

Figure 3 shows obvious differences in the quantity, position, size, and level of development of BS cell organelles in the C₃ species and in the corresponding KLCs in intermediates and the KCs of the C₄ species.

C₃ *Salsola abrotanoides* (Fig. 3A, B) had the lowest number of organelles in BS cells; a few chloroplasts and mitochondria were distributed more or less evenly along the cell wall, with

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**Fig. 3.** Electron microscopy of bundle sheath (BS)/Kranz-like cells (KLCs)/Kranz cells (KCs) and mesophyll (M) chlorenchyma cells in leaves of three *Salsola* species and *Rhaphidophyton regelii*: *S. abrotanoides* (A–D), *R. regelii* (E–H), *S. deschaseauxiana* (I–L), and *S. oppositifolia* (M–P). (A, E, I, M) Micrographs show M and BS/KLC/KC around vascular bundles. (B, F, J, N) Organelle distribution in BS/KLC/KC at a higher magnification. Note the difference in abundance of organelles in BS/KLC/KC between species, and the numerous mitochondria in KLCs of *R. regelii* (F) and *S. deschaseauxiana* (J), and in KCs in *S. oppositifolia* (N). (C, G, K, O) Chloroplast structure in BS/KLC/KC. (D, H, L, P) Structure of M chloroplasts. Scale bars = 20 μm for (A, I, M); 10 μm for (E, J, M); 5 μm for (N); 1 μm for (F); 0.5 μm for (B–D, G, H, K, L, O, P).
some mitochondria located in a centrifugal position. The structure of the thylakoid system was similar for BS and M chloroplasts (Fig. 3C, D).

In the KLCs of four species identified as C3–C4 intermediates, R. regelii (Fig. 3E, F), S. deschaseauxiana (Fig. 3I, J), S. gymnomaschala, and S. verticillata (not shown), the chloroplasts were at least twice as numerous (per cell section) than those of the BS in S. abrotanoides; they were distributed along the cell wall but tended to be enriched in the centripetal position. The mitochondria were also twice as numerous (per cell section) and 1.5–2 times larger than in BS cells of S. abrotanoides; and most of them were located in the centripetal position, close to the inner periclinal or radial cell walls (Fig. 3F, J). KLC chloroplasts (Fig. 3G, K) and M chloroplasts (Fig. 3H, L) in R. regelii and S. deschaseauxiana (and the other two Salsola intermediates, not shown) had a similar structure with a well-developed system of medium-sized grana consisting of 7–11 thylakoids.

The KCs in C4 S. oppositifolia contained numerous organelles in the centripetal position (Fig. 3M, N). The chloroplast structure differed remarkably among M cells and KCs: while the M chloroplasts had small to medium-sized grana of 2–5 thylakoids in stacks (Fig. 3P), the KC chloroplasts had numerous single thylakoids that interconnect small grana of paired thylakoids, or a few grana consisting mostly of 3–5 thylakoids (Fig. 3O).

Mitochondria in BS and M cells of S. abrotanoides had a similar size and structure (~0.4 µm), whereas in the KLCs of S. deschaseauxiana, S. gymnomaschala, S. verticillata, and R. regelii they were about 1.3–1.5 times larger compared to the M cells. In KCs and M cells of S. oppositifolia the mitochondria were almost identical in size (~0.5 µm).

Carbon isotope composition (δ13C) and CO2 compensation point (Γ)

Of the species studied biochemically and physiologically here, S. oppositifolia had C4 δ13C values (~13.7‰) while the other species had δ13C values ranging from −28.8 to −31.5‰, typical for C3 plants (Table 2). Γ was measured at 25 °C, 1000 PPFD, and 20% O2 in mature leaves of six Salsola species and R. regelii (Table 2). Γ values were characteristic of C4 species for S. oppositifolia (3.7 µmol mol⁻¹) and characteristic of C3 species for S. abrotanoides (61.2 µmol mol⁻¹). The Γ values in the other five species (R. regelii, S. deschaseauxiana, S. gymnomaschala, S. verticillata, and S. divaricata) were intermediate between C3 and C4, being about 32 µbar in the four Salsola species and 36.2 µmol mol⁻¹ in R. regelii (Table 2).

Immunolocalization of GDC

In situ immunogold labeling for GDC using the antibody to the P protein was examined by electron microscopy, and a quantitative analysis was made based on the density of gold particles, in C3 S. abrotanoides, C3–C4 R. regelii, S. deschaseauxiana, S. gymnomaschala, and S. verticillata, along with the C3–C4 species S. divaricata and C4 S. oppositifolia. Analysis of the immunolabeling distribution showed that there was no significant difference in density of the gold particles between the mitochondria of M and BS cells in C3 S. abrotanoides (Fig. 4, Supplementary Fig. S2). In contrast, in the C4 species S. oppositifolia gold particles were selectively localized in KC mitochondria with low labeling in M mitochondria, with a 10-fold difference in their number. In the intermediates R. regelii, S. deschaseauxiana, S. gymnomaschala, and S. verticillata, as well as in S. divaricata, the number of gold particles was also ~5.8–10 times higher in KLCs compared to M mitochondria (Fig. 4).

Western blot analysis of key C4 enzymes

Immunoblots for the key C4 cycle enzymes PEPC, PPDK, NAD-ME, and NADP-ME from total soluble proteins extracted from leaves of the studied species are presented in Fig. 5. The C4 species S. oppositifolia had very high labelling for the C4 pathway enzymes, PEPC and PPDK, and the two decarboxylases, NADP-ME and NAD-ME. Compared to the C4 species, the C3 species S. abrotanoides and the C3–C4 intermediates R. regelii, S. deschaseauxiana, S. gymnomaschala, S. verticillata, and S. divaricata had very low labelling for the C4 cycle enzyme PPDK and, to varying degrees, less labelling for PEPC, NAD-ME, and NADP-ME.

Molecular phylogeny of Salsoleae and mapping of key traits

The molecular phylogenetic analysis of the chloroplast genome revealed two unambiguous C4 lineages in Salsoleae s.s., (1) Halothamnus, and (2) Anabasis clade + Noaea clade +

Table 2. Carbon isotope discrimination (δ13C) and CO2 compensation point (Γ) for a subset of Salsoleae s.s. Values with different letters are significantly different according to one-way ANOVA with a post hoc Tukey HSD.

| Species                   | Carbon isotope discrimination δ13C, ‰<sub>oo</sub> | CO2 compensation point, Γ, µmol mol⁻¹ |
|---------------------------|-----------------------------------------------|--------------------------------------|
| S. abrotanoides, C3       | -31.2 ± 0.6 (n = 4) a                         | 61.2 ± 0.7 (n = 2) a                 |
| R. regelii, C3–C4         | -31.5 ± 0.3 (n = 8) a                         | 36.1 ± 2.2 (n = 4) b                 |
| S. deschaseauxiana, C3–C4 | -29.9 ± 0.3 (n = 6) ab                        | 31.9 ± 1.8 (n = 4) b                 |
| S. gymnomaschala, C3–C4  | -28.8 ± 0.3 (n = 12) b                        | 31.2 ± 1.0 (n = 3) b                 |
| S. divaricata, C3–C4      | -29.9 ± 0.3 (n = 16) ab                       | 33.3 ± 2.5 (n = 3) b                 |
| S. verticillata, C3–C4    | -29.1 ± 0.4 (n = 14) b                        | 32.2 ± 2.0 (n = 6) b                 |
| S. oppositifolia, C4      | -13.0 ± 0.3 (n = 6) c                         | 3.7 ± 0.9 (n = 4) c                  |
outgroup (only *Salsola genistoides*) the two C₃–C₄ intermediate clades merged into a monophyletic group that still formed a polytomy with the *Anabasis* clade, *Noaea* clade, and *Haloxylon* clade (Fig. 6). From the crown group age of *Halothamnus* (7.9–1.3 mya), *Anabasis* (10.2–2.6 mya), and *Noaea* (10.3–2.8 mya) it can be assumed that in these lineages C₄ photosynthesis has been present since the Late Miocene/Early Pliocene. Only for the *Haloxylon* clade can a distinctly older minimum age for the origin of C₄ photosynthesis of 16.9–6.6 mya be inferred from the molecular dating. In the case of common ancestry of the *Anabasis* clade + *Noaea* clade + *Haloxylon* clade, C₄ photosynthesis might date back to 19.2–7.6 mya (Supplementary Fig. S3).

The ML character optimization inferred a perennial life form and fully developed leaves without massive central sclerenchyma as the ancestral condition in Salsoleae s.s. (Fig. 6). An annual life form evolved at least six times independently in the tribe. A massive central sclerenchyma also evolved repeatedly (Fig. 6). In some cases, this feature was characteristic at the generic level, as in *Girgensohnia, Horaninovia, Cornulaca, Raphidophyton*, and *Noaea*. The reduction or complete loss of a true leaf lamina and a shift of photosynthetic function to the young stems was a common feature in Salsoleae and evolved multiple times in C₄ lineages of Salsoleae s.s., but also in *S. genistoides*. The occurrence of leafless species was clustered in certain genera, such as *Anabasis, Haloxylon*, and *Hammada*, the latter of which seemed to be highly polyphyletic.

Furthermore, the ML character optimization inferred a C₃ metabolism and C₃-type BS cells as ancestral in Salsoleae s.s. According to the ancestral character state reconstruction, a switch towards C₄ seems to have already occurred along the branch leading to the large sister group of the C₃ species *Salsola pachyphylla*, which contains three C₄ subclades but also one clade of C₁ and C₁–C₄ intermediates (highlighted green in Fig. 6). This clade of C₁ and C₁–C₄ intermediate species did not contain any C₄ species, and the clade was part of a polytomy of C₄ clades; thus, there is no indication in the cp tree that the C₁–C₄ intermediates represent ancestral states leading towards full C₄ photosynthesis.

Resolution in the ITS tree was weak in many parts of the tree (Supplementary Fig. S4). Combining cp and ITS data resulted in very low resolution (tree not shown) due to conflicting topologies. Branches that were in conflict between the two data sets (with bootstrap >75) are marked on the ITS tree (Supplementary Fig. S4).

**Discussion**

Evidence for newly identified C₃–C₄ species in Salsoleae

Results from gas exchange (I), compartmentation of GDC between M cells and KLCs, analyses of carbon isotope composition, and analyses of levels of C₄ enzymes, along with the structure of the respective cells, indicated that four species, *S. deschaseauxiana*, *S. gymnomaschala*, *S. verticillata*, and *R. regelii*, are C₅–C₄ intermediates, while...
The carbon isotope composition of these four intermediates as well as the C_3–C_4 intermediate S. divaricata showed they all have values in the range of those of C_3 species compared to the C_4-type value in S. oppositifolia (Table 2). However, values for plants grown in growth chambers are more negative (i.e. up to 4–7‰) than samples from natural habitats (Voznesenskaya et al., 2013). The more positive δ^{13}C values in the natural habitat may be due to growth under arid conditions limiting CO_2 diffusion into leaves (Cerling, 1999), or to induction of a partially functional C_4 cycle. According to our study, among Salsoleae there are at least 19 species with C_3 isotope values, seven of which are C_3–C_4 species (Table 1).
Structural, biochemical, and functional analyses are needed in order to determine whether species having C₄-type δ¹³C values are C₄, proto-Kranz, or C₁-C₄. An important test is measurement of δ, since values are lower in C₁-C₄ than in C₄ plants, which is indicative of a reduction in photorespiration (Edwards and Ku, 1987). Gas exchange analyses of S. deschaseaustiana, S. gymnomaschala, S. verticillata, and R. regelii showed that all species have δ values that are intermediate between C₄ S. oppositifolia and C₁ S. abrotanoides (Table 2). Additionally, C₃–C₄ intermediates, like C₄ species, have selective compartmentation of GDC in KLC mitochondria (Rawsthorne et al., 1988; Voznesenskaya et al., 2001, 2013; Sage et al., 2012, 2014), supporting refixation of photorespired CO₂. Analysis of GDC levels by immunolocalization in these four intermediates indicated selective localization in mitochondria of Kranz-like cells (KLCs), while in the C₁ species S. abrotanoides the density of immunolabeling for GDC was similar in M and BS mitochondria.

Western blot analysis of C₄ enzymes showed that levels in the C₁ species S. abrotanoides and the C₁–C₄ intermediates S. deschaseaustiana, S. gymnomaschala, S. verticillata, and R. regelii were very low compared to the C₄ species S. oppositifolia. The levels of PEPC in the four C₃–C₄ intermediate species were higher than in the C₁ species S. abrotanoides. However, except for R. regelii, levels of PPDK were low and barely detectable in both the C₃ and intermediate species. R. regelii had higher levels of PPDK, but low levels of C₄ decarboxylases similar to the C₁ species.

Currently, the results suggest that all seven known C₁–C₄ species of Salsoleae, R. regelii, S. arbuculiformis, S. deschaseaustiana, S. divaricata, S. gymnomaschala, S. laricifolia, and S. verticillata (Voznesenskaya et al., 2001, 2013; Wen and Zhang, 2015; this study, Table 1), are Type I, where the reduction of δ comes from refixation of photorespired CO₂ in KLCs with little or no function of a C₄ cycle (Edwards and Ku, 1987). Whether there is a contribution from a limited C₄ cycle to photosynthesis in these intermediates could be more directly analyzed by the method of Alonso-Cantabrana and von Caemmerer (2016) via online measurements of photosynthesis and carbon isotope discrimination.

### A model for evolution of C₄ photosynthesis in Salsoleae based on identified photosynthetic phenotypes

Of the 77 species of Salsoleae analyzed, without those of the Kali clade (Table 1), 24 species were studied for the first time. Our sampling was comprehensive and surpasses Carolin et al. (1975) with 43 species and Pyankov et al. (2001) with 38 species. Of the 77 species, 19 had C₃-type carbon isotope composition (consisting of seven C₃ species, five proposed proto-Kranz species, and seven C₁-C₄ intermediates) while 58 were C₄ species with C₄-type carbon isotope composition and Salsoloid-type leaf anatomy (Table 1). In Fig. 7 five photosynthetic phenotypes in Salsoleae are described based on the anatomical, ultrastructural, and biochemical analyses of species in the current study together with a few species described by Voznesenskaya et al. (2013). In this model, C₄ is proposed to have evolved structurally and functionally from C₃ Sympegmoid to Proto-Kranz Sympegmoid to C₃–C₄ Kranz-like Sympegmoid to C₁–C₄ Kranz-like Salsoloid to C₄ Salsoloid-type anatomy. There are two subtypes of Kranz-like Salsoloid C₃–C₄ intermediates (with or without sclerenchyma) and five anatomical subtypes with Salsoloid type.

### Non-Kranz anatomy, functionally C₃

The Sympegmoid leaf type is anatomically and functionally C₃. It is characterized by usually two well-developed layers of palisade M cells (M1 and M2) and indistinct C₁-M cells around peripheral VBs containing only a few organelles. Species of this type have C₃ δ¹³C values, C₁-type δ¹³C values, and structural features of M and BS cells characteristic of C₃ plants (including the occurrence of GDC in both M and BS mitochondria). It is found in S. abrotanoides (this study, Figs. 1A, 3, 4), S. genistoides, S. oreophila, S. pachyphylla, S. webbii (Carolin et al., 1975; Voznesenskaya, 1976; Pyankov et al., 1997; Pyankov et al., 2001; Voznesenskaya et al., 2013), and Sympegma regelii (Wen and Zhang, 2011). Based on anatomical evidence alone, we conclude that Salsola tianschanica belongs to this group, which would then comprise seven species in total (Table 1). An additional trait observed in this group is the comparatively low volume of water storage (WS) tissue and the position of peripheral VBs embedded in the WS tissue rather than at its periphery (Fig. 1A). From known data and the taxonomic literature, in particular the pertinent revisions of section Coccosalosa by Botschantzev (1976, 1989), the occurrence of this leaf type in other species is unlikely.

### Proto-Kranz anatomy, functionally C₃

Proto-Kranz species have anatomical changes in BS cells that may be the earliest phase of C₄ evolution, preceding development of the C₄ cycle (Sage et al., 2014). In Salsoleae, the Proto-Kranz Sympegmoid type only differs from the Sympegmoid type by having distinct cells with chloroplasts and mitochondria arranged preferentially along the inner and the radial walls between peripheral VBs and the chlorenchyma (Fig. 7). Currently this type is only documented in S. montana (Voznesenskaya et al., 2013). It has C₃-like δ¹³C and δ¹⁸O values, and immunolabelling for GDC is similar for M and BS mitochondria. However, based on analysis of leaf anatomy (by light microscopy of fresh leaf or herbarium samples fixed in FAA) there are additional probable candidates for proto-Kranz anatomy among the Central Asian Salsola species that have C₃ δ¹³C values, namely S. botschantzevii, S. drobovii, S. junatovii, and S. lipschitzii (Table 1).

### Kranz-like anatomy, functionally C₃–C₄ intermediate

Anatomically there are two types of intermediates in Salsoleae, the Kranz-like Sympegmoid type, and the Kranz-like Salsoloid type (Fig. 7). They resemble C₄ Salsola species in having KLCs with numerous organelles in the centripetal position. Both have C₄-type δ¹³C values, selective localization of GDC in KLC mitochondria, and intermediate δ¹³C values indicating functionally C₃-type species.

The Kranz-like Sympegmoid type is very similar to the aforementioned Sympegmoid forms; but the outer M cells
photosynthesis evolution in Salsoleae

(M1) are distinctly shorter and smaller than the inner M cells (M2) and the KLCs are restricted to the peripheral VBs. This type of structure has so far only been found in S. arbusculiformis (Voznesenskaya et al., 2001), and it is suggested to represent the first functional step towards C₄-type anatomy.

In the Kranz-like Salsoloid type of intermediacy, the M₁ appear more like the hypodermal cells in C₄ species, while still containing more or less numerous chloroplasts. M₁ cell size and M/KLC ratio is reduced in comparison with the Sympegmoid types, and the KLCs form a more or less continuous layer (interrupted by crystal-containing idioblasts) around the leaf, as in C₄ Salsola species. The KLCs contain chloroplasts and numerous large mitochondria positioned towards the inner cell wall, characteristic of other C₃–C₄ intermediate species (Edwards and Ku, 1987; Rawsthorne and Bauwe, 1998; Voznesenskaya et al., 2007, 2010; Muaidat et al., 2011; Sage et al., 2012, 2014). This type is currently found in six species (Table 1): S. laricifolia (Wen and Zhang, 2011, 2015), S. divaricata (Voznesenskaya et al., 2013), and S. deschaseauxiana, S. gymnomaschala, S. verticillata, and R. regelii (this study, Figs 1 and 7). However, R. regelii represents a different subtype by its very strong central sclerenchyma (Fig. 2), which, according to our knowledge, is unique among the C₃–C₄ intermediates identified in Salsoloideae.

Of note in the Kranz-like Salsoloid type, considerable variation occurs, mainly in the size, shape and the number of
organelles in the M1 cell layer and the arrangement of the KLCs. Sometimes multiple sections within species revealed a certain degree of variation, showing phenotypes more similar to the Kranz-like Symplegmarkoid type or phenotypes approaching C4 plants with typical Salsoloid leaf anatomy. Therefore, more detailed studies are needed to assess the phenotypic plasticity of the functionally intermediate types.

Kranz-type anatomy, functionally C4

The Salsoloid leaf anatomy in C4 lineages of Salsoleae differs substantially from C4 eudicots having Atriplicoid-type leaf anatomy with Kranz anatomy around individual veins in flat leaves. Species with Salsoloid-type anatomy are functionally C4, with a continuous layer of Kranz cells (KC) around WS tissue and VBs. If the M1 layer of cells is present it occurs as a hypodermis with few or no organelles. There is a further reduction in the M/KC ratio, with organelles in the KCS in a centripetal (or, rarely, in centrifugal) position. In other lineages, as in *Halothamnus, Noaea, Kali, Nanophyton, and Climacoptera*, the hypodermis is lacking (*Kadereit et al., 2003; Wen and Zhang, 2011*). Our data on *S. oppositifolia*, and on several other C4 species that had not previously been studied, do not add substantially to the well-known Salsoloid-type anatomy. Together with 40 other species, *S. oppositifolia* displayed the most common Salsoloid type that has a hypodermis and lacks central sclerenchyma; on the other hand only 12 species account for the variant with central sclerenchyma. The Salsoloid type without a hypodermis and without central sclerenchyma was represented in our sampling by four species of *Halothamnus* only. This form is present in many other species of that genus (*Kothe-Henrich, 1993*), and in almost all species of *Kali* (*Rilke, 1999*), while the variant with a central sclerenchyma was seen only in the two *Noaea* species.

Comparison with the ‘Flaveria model’ for C4 evolution

The various photosynthetic phenotypes in Salsoleae fit the general model of evolution from C3 to proto-Kranz, to intermediate, to Kranz anatomy with a progressive reduction in functional losses due to photorespiration. However, in the ‘Flaveria model’ where Kranz anatomy forms around individual veins (*Edwards and Ku, 1987; Sage et al., 2012*) the structural modifications are very different from the modifications from a Symplegmarkoid type to a Salsoloid Kranz type where the Kranz anatomy is formed around all veins and WS tissue. In evolution from C3 to C4 with Kranz anatomy around individual veins, there is increased vein density and size of BS cells around veins as they develop KC features. In contrast, in the Salsoleae the vein density in C4 species does not appear to be higher than in the C3 species. In addition, the size of the KCS is not significantly increased in the C4 species in comparison with their forerunners in C3 species (*Voznesenskaya et al., 2013*). Furthermore, in the Salsoleae model (see fig. 9 in *Voznesenskaya et al., 2013*) a decrease in the M/KC ratio might also be a precondition, as in grasses (*Christin et al., 2013*) and dicots (*Sage et al., 2012*); however, it happens by development of a continuous layer of KCS and reduction in the M1 layer rather than by an increase of veins and BS size.

Where and when did C4 photosynthesis originate in Salsoleae?

The chloroplast gene tree of Salsoloideae resolves five primary clades in the subfamily, namely the *Nanophyton* clade, the *Caroxylon* clade, the *Salsola* *genistoides* clade, the *Kali* clade, and the Salsoleae s.s. clade (Supplementary Fig. S3; *Kadereit and Freitag, 2011*), with the first four forming the sister group to Salsoleae s.s. (Supplementary Fig. S3). Molecular trees based on the nrDNA marker ITS (Supplementary Fig. S4) also reveal these clades but they are contradictory in their positions (for a short discussion on this matter see Supplementary Fig. S3). According to our results in both data sets only two clearly independent C4 lineages in Salsoleae seem likely. The first lineage is *Halothamnus*, with a crown age of 7.9–1.3 mya, and probably plus the *Kali* clade in the ITS data set. Most species of both clades have Salsoloid leaf anatomy and lack a hypodermis (e.g. as in *Traganum*). In the cp tree the monospecific C4 genus *Sympegma* is sister to *Halothamnus* while in the ITS tree it is sister to all C3–C4 intermediates and C4 clades in the Salsoleae sensu lato (s.l., Supplementary Fig. S4). The *Halothamnus* and *Kali* clades seem to have no close relatives with a C3–C4 intermediate phenotype. The second C4 lineage in Salsoleae s.s. probably consists of all other C4 species. Since the C4 clades of *Anabasis*, *Noaea*, and *Haloxylon* form a polytomy with the C3–C4 intermediates clade in the cp tree (Fig. 6), one large monophyletic C4 clade and a sister-group relationship to the C3–C4 intermediate remains possible. Unfortunately, the weak resolution of this particular part of the ITS tree within Salsoleae s.s. does not support this (Supplementary Fig. S4). The overall similar Salsoloid leaf anatomy with hypodermis (except for the two species of *Noaea*) and NADP-ME biochemistry is in favor of common ancestry of the C4 syndrome in this lineage. The predicted age of this large C4 lineage in Salsoleae s.s. is 19.2–7.6 mya (Supplementary Fig. S3), which is in accordance with the origin of many other C4 lineages during the Middle to Late Miocene (*Christin et al., 2011*).

**Does the C3–C4 intermediate condition represent an ancestral state to C4 in Salsoleae? Is there phylogenetic evidence for a reversion from C4 back to C3?**

The ML optimization suggests that early Salsoleae were shrubs or subshrubs that performed C3 photosynthesis in well-developed leaves with a Symplegmarkoid leaf type (Fig. 6). Along with one C3 species (*S. oreophila*), 10 species (consisting of proto-Kranz and C3–C4 intermediates) in Salsoleae form one (Fig. 6) or two clades (Supplementary Fig. S3) in the cp trees. Lack of resolution in the phylogenetic trees just at the node where these phenotypes and their closest C4 relatives arise hampers a reconstruction of these proto-Kranz and C3–C4 intermediates as ancestral (see also Supplementary Fig. S4). The ML optimization even suggests that the node from which these phenotypes arose was most likely C4 (pL=0.98, Fig. 6), which would imply the origin of these C3–C4 species from C4. However, in general a reversion from C4 back to C3 or intermediate phenotypes seems to be exceedingly rare, if
not improbable. Although a few cases have been reported in which C₃ species or C₃-C₄ intermediates are nested in a C₄ clade, there has also been plausible evidence for a scenario of multiple C₄ origins (e.g. Ocampo et al., 2013; Bohley et al. 2015). In the case of Salsoleae s.s., we assume that a convincing reconstruction is severely hampered by the low number of C₃-C₄ intermediate lineages (not by the low number of intermediate species) since all C₃-C₄ intermediate species seem to belong to just one lineage.

This C₃-C₄ intermediate-rich lineage might, however, be of major interest for future studies. Studying the ecology, physiology, and biochemistry of closely related proto-Kranz and C₃-C₄ intermediate species with and without selective localization of GDC might provide further insights into the selective advantage of proto-Kranz anatomy and the C₂ pathway. So far the selective advantage of displacement of BS organelles towards the centripetal position in proto-Kranz compared to C₃ species is not clear. Possibly the proto-Kranz state leads to a slight increase in fixation of the CO₂ generated by GDC in BS cells; however, this could be difficult to detect experimentally (e.g. by measurements of Γ). A C₂ cycle is indeed able to generate distinctly higher CO₂ levels in leaves (Keerberg et al., 2014) and therefore has an ecophysiological advantage. Since a reversion from intermediate to C₃ clade and hence from C₃-C₄ intermediate species cannot be determined as ancestral; although they display logical stepwise, ‘model-conforming’ phenotypes from C₃ to C₄ photosynthesis. From a phylogenetic point of view, they may represent an evolutionarily independent solution, enabling the respective species to survive in harsh environments, even in competition with distantly related species of the same tribe possessing full C₄ photosynthesis.

Conclusions

In the model for evolution of C₄ in Salsoleae, putative C₃ ancestors have M tissue surrounding the entity of veins with a limited volume of WS tissue; differentiation occurs with development of KLCs next to minor veins, reduction in size of M cells, and ultimately development of an internal layer of KLCs that surrounds all the vascular and WS tissue. Compared to the ‘Flaveria model’ for C₄ development around individual veins, in Salsoleae the proposed biochemical and functional transitions suggest convergence; however, there is obvious divergence in how structural changes were made in C₃ ancestors to develop Kranz anatomy. In Salsoleae, a number of structural changes that are important in the evolution of C₄ flat-leaved species are missing: individual KC size often does not increase, but KC volume increases due to the formation of the continuous layer, a decrease of the M/KC ratio occurs mainly due to the reduction of the M1 layer, and the density of venation does not change. These differences might be related to the succulent nature of Salsoleae, with an increase of the volume of WS tissue during the transition from C₃ to C₃-C₄ intermediates and to C₄ species.

The Salsoleae phylogenies unambiguously reveal all C₃ species, except for S. oerophila, in basal positions, and both the C₃ species and the C₃-C₄ intermediates as derived, but occurring in different clades. Intermediates, proto-Kranz, and one C₃ species are clustered in one (cp tree) or four (ITS tree) monophyletic groups that might either be sister to a large C₄ clade or nested within it. In the absence of closely related C₄ species, their intermediacy cannot be determined as ancestral; although they display logical stepwise, ‘model-conforming’ phenotypes from C₃ to C₄ photosynthesis. From a phylogenetic point of view, they may represent an evolutionarily independent solution, enabling the respective species to survive in harsh environments, even in competition with distantly related species of the same tribe possessing full C₄ photosynthesis.

Supplementary data

Supplementary data are available at JXB online

Table S1. Details of the specimens of Salsoleae and outgroups included in the molecular analyses.

Table S2. Sampling of primary clades of Salsoloideae (Salsoleae, Caroxyloneae, Nanophyton clade, Salsola kali clade and Salsola genistoides clade).

Table S3. Primers, PCR recipes and cycler programs.

Fig. S1. Representative membrane stained with Ponceau S after transfer of proteins to a nitrocellulose membrane and before immunoblotting.

Fig. S2. Electron microscopy of in situ immunolocalization of GDC in chlorenchyma cells of Salsola abrotanoides (C₃), Rhaphidophyton regelii (C₃-C₄), S. verticillata (C₃-C₄), and S. oppositifolia (C₄).

Fig. S3. Dated molecular phylogenetic tree of Salsoloideae (Chenopodiaceae) based on four cp markers (atpB-rbcL spacer, ndhF-rps32 spacer, trnQ-rps16 spacer, rpl16 intron).

Fig. S4: ML tree based on ITS sequences of Salsoleae and Caroxyloneae with representatives of Salicornioideae and Suaedoeideae as the outgroup.

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