Kranz and single-cell forms of $\text{C}_4$ plants in the subfamily Suaedoideae show kinetic $\text{C}_4$ convergence for PEPC and Rubisco with divergent amino acid substitutions

Josh J. Rosnow$^1$, Marc A. Evans$^2$, Maxim V. Kapralov$^3$, Asaph B. Cousins$^1$, Gerald E. Edwards$^{1,*}$
Eric H. Roalson$^{1,*}$

$^1$ School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA
$^2$ Department of Mathematics, Washington State University, Pullman, WA 99164-3113, USA
$^3$ School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool L3 3AF, UK

* To whom correspondence should be addressed. E-mail: eric_roalson@wsu.edu; edwardsg@wsu.edu

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Abstract

The two carboxylation reactions performed by phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are vital in the fixation of inorganic carbon for $\text{C}_4$ plants. The abundance of PEPC is substantially elevated in $\text{C}_4$ leaves, while the location of Rubisco is restricted to one of two chloroplast types. These differences compared with $\text{C}_3$ leaves have been shown to result in convergent enzyme optimization in some $\text{C}_4$ species. Investigation into the kinetic properties of PEPC and Rubisco from Kranz $\text{C}_4$, single cell $\text{C}_4$, and $\text{C}_3$ species in Chenopodiaceae s. s. subfamily Suaedoideae showed that these major carboxylases in $\text{C}_4$ Suaedoideae species lack the same mutations found in other $\text{C}_4$ systems which have been examined; but still have similar convergent kinetic properties. Positive selection analysis on the N-terminus of PEPC identified residues 364 and 368 to be under positive selection with a posterior probability >0.99 using Bayes empirical Bayes. Compared with previous analyses on other $\text{C}_4$ species, PEPC from $\text{C}_4$ Suaedoideae species have different convergent amino acids that result in a higher $K_m$ for PEP and malate tolerance compared with $\text{C}_3$ species. Kinetic analysis of Rubisco showed that $\text{C}_4$ species have a higher catalytic efficiency of Rubisco ($k_{\text{cat}}$ in mol CO$_2$ mol$^{-1}$ Rubisco active sites s$^{-1}$), despite lacking convergent substitutions in the $rbcL$ gene. The importance of kinetic changes to the two-carboxylation reactions in $\text{C}_4$ leaves related to amino acid selection is discussed.

Key words: Bienertia, $\text{C}_4$ photosynthesis, PAML, phosphoenolpyruvate carboxylase, positive selection analysis, Rubisco, Suaedoideae.

Introduction

When organisms develop the same solution to an abiotic or biotic stress resulting in a similar character state, it is referred to as convergent evolution or phenotypic convergence. One of the most documented convergent phenotypes in plants is the repeated development of $\text{C}_4$ photosynthesis, an adaptation that uses four carbon acids to increase photosynthesis under conditions where carbon assimilation can be limited by high photorespiration (Sage et al., 2012). The number of
times that C₄ independently developed (at least 66) makes it an extremely useful phenotype for analysing the genetics of adaptations (Christin et al., 2010; Sage et al., 2011).

The genetic mechanisms responsible for C₄ photosynthesis remain largely unknown, but they are thought to involve co-ordinated changes to genes that affect leaf anatomy, cell ultrastructure, energetics, metabolite transport, and the location, content, and regulation of many metabolic enzymes (Hibberd and Covshoff, 2010). One approach to gain further insight into the underlying genetic regulation of C₄ photosynthesis is to analyse how enzymes are optimized for C₄ biochemistry.

In C₄ plants there is spatial separation between the capture of atmospheric CO₂ with synthesis of C₄ acids, and the donation of CO₂ to Rubisco by decarboxylation of C₄ acids, which, in most species, occurs in mesophyll and bundle sheath (BS) cells, respectively. In the mesophyll cells, atmospheric CO₂ is initially converted into bicarbonate (HCO₃⁻) by carbonic anhydrase and the chloroplasts generate phosphoenolpyruvate (PEP) from pyruvate by pyruvate, Pi dikinase. Then, in the cytosol, the HCO₃⁻ and PEP are utilized as substrates for phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) for the synthesis of oxaloacetate (Chollet et al., 1996). The oxaloacetate is subsequently reduced in the chloroplast to malate (MA) by NADP-malate dehydrogenase or transaminated to aspartate (Asp) by Asp aminotransferase. The MA and Asp are transported to BS cells where CO₂ is donated to Rubisco via C₄ acid decarboxylases. How C₄ photosynthesis is regulated, by the level of enzymes and their kinetic properties, their state of activation, and their control by allosteric effectors, is important for understanding the mechanism and how they accomplish high rates of photosynthesis under CO₂ limiting conditions.

The kinetic properties of PEPC and Rubisco from C₄ plants are different from those in C₃ plants, which are considered to have optimized their function in the C₄ system (Ghannoum et al., 2005; Gowik and Westhoff, 2011; Whitney et al., 2011b). These differences have led to questions about how these changes occurred during the evolution of C₄ from C₃ by positive selection on certain amino acid residues. PEPC in C₄ plants have high enzymatic activities, as much as 20-40-fold higher than C₃ plants (per mg of chlorophyll), and Km values for PEP are several fold higher than C₃ plants (Ting and Osmond, 1973; Kanai and Edwards, 1999; Englemann et al., 2003; Lara et al., 2006). The C₄ PEPC can have cooperativity with PEP as substrate (reflected in higher Hill coefficients), be less sensitive to the inhibition of catalysis by Asp and MA, and react to the positive allosteric effectors glucose, 6-phosphate (G6P), glyceraldehyde-3P, and glycine (Gowik and Westhoff, 2011). G6P decreases the Km for PEP (Engelmann et al., 2003; Gowik et al., 2006), and lowers the inhibition by MA (Gupta et al., 1994; Chollet et al., 1996; Englemann et al., 2003). Positive selection analysis to identify amino acid residues under selection, that may account for the observed kinetic properties of the C₄ PEPC, have been made in family Asteraceae (in C₃, intermediate, and C₄ species in the genus Flaveria), Cyperaceae, and Poaceae (Christin et al., 2007; Besnard et al., 2009; Gowik and Westhoff, 2011). This includes the identification of amino acid substitution at residue 780 to a serine in the C₄ species which has been considered a key substitution in PEPC for C₄ kinetics.

Rubisco in C₄ plants functions where the ratio of CO₂ to O₂ is elevated, resulting in a decrease of the oxygenase reaction with RuBP and photorespiration. The high CO₂ concentration provides selective pressure for a faster turnover of the enzyme under saturating CO₂, resulting in higher kcat and Km(CO₂) values (Yeoh et al., 1981; Seemann et al., 1984; Sage, 2002; Kubien et al., 2003, 2008; Ghannoum et al., 2005). These kinetic changes to Rubisco in C₄ plants allow for a reduced investment in the enzyme, as much as half as in C₃ leaves, while achieving higher rates of photosynthesis under warm temperatures and current ambient levels of CO₂ due to their CO₂-concentrating mechanism (Long, 1999; von Caemmerer, 2013). Rubisco is a heterooctomer composed of multiple small and large subunits which are encoded by nuclear RbcS and chloroplast rbcL genes, respectively (Whitney et al., 2011a). Analyses for rbcL amino acid residues under positive selection in C₄ lineages have been made mainly in families Poaceae, Cyperaceae, and Amaranthaceae s.l. (Kapralov and Filatov, 2007; Christin et al., 2008, 2009; Kapralov et al., 2011, 2012).

Among eudicot families, Chenopodiaceae s.s. has the largest number of eudicot C₄ species and the most diversity in forms of C₄, yet there is no information comparing the kinetic properties of the carboxylases and positive selection of amino acid residues in C₄ lineages. The focus of the current study was on subfamily Suaedoideae which has diverse forms of C₄ along with C₃ species (Edwards and Voznesenskaya, 2011; Kadereit et al., 2012). There are four independent origins of C₄ in the subfamily, including two distinct Kranz anatomies in Suaeda sections Salsina s.l. and Schoberia, and two independent origins of single-cell C₄ anatomy, in Suaeda aralocaspica and in genus Bienertia (Kapralov et al., 2006; Rosnow et al., 2014). A recent positive selection analysis on C₄ PEPC in Suaedoideae showed that there was divergence in where positive selection was occurring compared with previous studies in grasses and sedges (Rosnow et al., 2014). In the current study, the kinetic properties of PEPC across C₃ and C₄ Suaedoideae species, including the affinity for PEP, the kinetic response to allosteric effectors (G6P and MA), and the degree of cooperativity with varying PEP as substrate, were investigated together with additional PEPC sequence information.

With respect to Rubisco, positive selection analysis for rbcL in Amaranthaceae s.l. showed evidence for selection of residues at positions 281 and 309 among C₄ species, which has also been observed in C₄ monocots (Kapralov et al., 2012). Also a functional analysis with hybrids of Rubiscos utilizing rbcL genes from C₃ versus C₄ Flaveria species indicated that a substitution in the rbcL gene at position 309 from a methionine to an isoleucine results in a higher Rubisco kcat in a methionine to an isoleucine results in a higher Rubisco kcat (Whitney et al., 2011b). However, the three Suaedoideae C₄ species which were previously analysed (Suaeda altissima, S. microphylla, and Bienertia cycloptera) lacked substitutions at 281 and 309 (Kapralov et al., 2012). This raises questions about Rubisco kinetics (kcat) and rbcL sequences in Suaedoideae C₄ lineages.
In this study, kinetic properties and sequence information for PEPC and Rubisco from the subfamily Suaedoideae were analysed. The results show that the C₄ species have divergent amino acid positive selection resulting in convergent C₄-type kinetic properties for PEPC and Rubisco.

Materials and methods

Plant material

All plants used in this study were started from seed and grown in controlled environmental chambers (Econair GC-16; Bio Chambers). Seedlings were started under low light [100 photosynthetic photon flux density (PPFD; pmol quanta m⁻² s⁻¹)] and temperature conditions with a day/night temperature of 25/22 °C and a photoperiod of 14/10h. The plants were moved to high light and temperature conditions (1,000 PPFD, with a day/night temperature of 35/25 °C and a photoperiod of 14/10h) once well established. A few leaves, for each replication, were sampled from 2–6-month-old plants and used for kinetic analysis.

Enzyme extraction

Chlorophyll content, the quantity of Rubisco binding sites for RuBP, and Rubisco and PEPC activities, were measured on flash-frozen leaves from plants exposed to at least 5h of light in the chambers, using a liquid-nitrogen-chilled mortar and pestle (the extraction included 250mg leaf tissue plus 1 ml extraction buffer). For Rubisco assays the extraction buffer consisted of [100 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS, pH 8.0), 1 mM EDTA, and 10 mM dithiothreitol (DTT)]; preliminary tests showed no difference in activity with or without the protease inhibitor (Sigma Protease Inhibitor Cocktail,P9599). For PEPC assays, the extraction buffer consisted of [100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.6), 1 mM EDTA, 1 mM sodium fluoride, and 10 mM dithiothreitol (DTT)]. The PEPC extraction included 1 mM sodium fluoride to prevent the possible action of phosphatases on the PEPC carboxylase protein. The frozen leaf powder was homogenized in the extraction buffer and, prior to centrifugation, a portion of the extract was placed in 80% acetone for chlorophyll determination (Porra et al., 1989). The extract was centrifuged at 10,000 g relative centrifugal force for 1 min at room temperature; the supernatant was collected and placed on ice. In the case of extracts for analysis of PEPC, the supernatant was desalted in a cold Sephadex G-50 column pre-equilibrated with the extraction buffer (to remove low-molecular-weight metabolites including the allosteric effectors malate, aspartate, and G6P, as well as cations, which may affect the assay).

PEPC kinetic assays

Assays were performed immediately following desalting, and there was no apparent loss in activity during the assay period. The activity was coupled to the MA dehydrogenase reduction of OAA and measured as a decrease in absorbance at 340 nm resulting from the oxidation of NADH. The standard assay mixture contained 100 mM HEPES–KOH (pH 7.6), 10 mM MgCl₂, 10 mM NaHCO₃, 0.2 mM NADH, 12 U NADH-MA dehydrogenase (MP Biomedicals), and 10 μl of enzyme extract in a total volume of 1 ml. The reaction was started by the addition of PEP (with or without G6P as indicated). In order to determine the Kₘₐₐ, Vₘₐₓ, and Hill coefficient for PEPC, the Hill equation was fitted to the experimental data by non-linear regression analysis with the software package KaleidaGraph 4.5 (Synergy Software):
using the manufacturer’s protocol (Promega, USA). Single colonies were grown overnight and plasmid DNA was purified using alkaline lysis with SDS (Sambrook and Russell, 2001). Plasmid inserts were PCR amplified using GOTaq (Promega, USA), Sp6 and T7 primers, and were visualized on a gel. Prior to sequencing, the PCR product was mixed with 2.5 U of Antarctic Phosphatase and 4 U of Exo-Sap Nuclease in Antarctic Phosphatase buffer (New England BioSciences, USA) to degrade primers and nucleotides, and subsequently diluted 1:10. Sequencing reactions were performed using the Big Dye terminator master mix v3.1 (Applied BioSciences, USA), using sequence specific internal primers along with Sp6 and T7 (see Supplementary Table S1 at JXB online). Sequencing was carried out at Washington State University genomics core. Sequence data was assembled using Sequencher software (USA). Nucleotide sequences were translated, aligned, and visualized using Se-Al and MacVector (USA). All sequences were deposited in GenBank (see Supplementary Table S2 at JXB online). Positive selection analysis on additional N-terminus PEPC residues was performed using the methodologies of Rosnow et al. (2014). The same phylogenetic tree from the previous study was used for selection analysis, but was pruned to exclude S. heterophylla, Salsola genistoides, and Salsola divaricata as these species were not sequenced in this study. Throughout this paper, the numbering of PEPC residues is based on Salsola divaricata as these species were not sequenced in this study.

Results

PEPC sequence analysis

To complement previous sequence and phylogenetic information on PEPC in Suaedoidae (Rosnow et al., 2014), N-terminal PEPC sequence was obtained for 19 species (S. heterophylla, Salsola genistoides, and Salsola divaricata were not included) using homologous upstream primers that overlapped with known C-terminal ppc-1 sequence. The region of coverage included part of exon 2 through exon 8, stopping where previous C-terminal sequence analysis had been performed (Rosnow et al., 2014). The sequenced region resulted in an additional 370 N-terminal amino acids of the ppc-1 coding sequence. Based on gene homology to previously sequenced Alternanthera species PEPCs (Gowik et al., 2006), this is approximately 87 N-terminal amino acids short of complete ppc-1 gene coverage.

Positive selection analysis, using phylogenetic relationships, models amino acid change identifying significant non-synonymous amino acid changes; for model descriptions see Rosnow et al. (2014) (Yang, 2007). There were no codons identified as being under positive selection with a posterior probability >0.95 by BEB in the M2A model or M8 model (see Supplementary Table S4 at JXB online) (P value=0.82 and 0.0071, respectively). There were seven codons (99, 171, 324, 333, 364, 365, 368) that were shown to be under positive selection with a posterior probability >0.95 by BEB, when only branches leading to C4 clades were labelled as foreground branches (P value <0.0001). Positions 364 and 368 were the only two residues identified to have a posterior probability >0.99 by BEB in Model A, when only branches leading to C4 clades were labelled as foreground branches (see Supplementary Table S4 at JXB online). Residues 364 and 368 are in the N-terminal region which is shown to be involved in the allosteric regulation of activators like G6P (Blasing et al., 2002; Engelmann et al., 2002; Takahashi-Terada et al., 2005). Residue 364 had four alternative amino acids present in this dataset, Arg present in C3 species, and either Lys, Gln, or Pro in C4 species (in order of prevalence). Residue 368 has Asn present in C3 species and Ser in C4 species. Both residues had a substitution in all C4 species (see Supplementary Fig. S1 at JXB online). There were no codons shown to be under positive selection with a posterior probability >0.95 by BEB, when foreground branches leading to Kranz C4 clades or branches leading to single-cell C4 clades alone were labelled (see Supplementary Table S4 at JXB online) (P values=0.65 and 1, respectively). By labelling all C4 branches as foreground branches, 10 codons were identified as being under positive selection (157, 159, 171, 198, 314, 318, 324, 353, 364, 368) with a posterior probability >0.95 by BEB (see Supplementary Table S4 at JXB online) although the results are not significant (P value=0.22). Four of these residues (171, 324, 364, 368) were identified as being on branches leading to C4 clades.

PEPC kinetics

The $K_m$ value for a given substrate in Michaelis–Menten kinetics is the concentration at which the rate of reaction...
is at half the maximal rate, which generally has an inverse relationship to affinity of enzyme for substrate. Since some forms of PEPC show cooperativity with PEP as substrate, the Hill equation was used to determine the $K_m$, the Hill coefficient ($h$) for PEP, and $V_{max}$. The analyses of PEPC kinetics in members of subfamily Suaedoideae representing different photosynthetic types, are shown in Tables 1–4 and Fig. 1. (see Supplementary Table S3 at JXB online for $\delta^{13}$C values of C$_4$ and C$_3$ species in the study)

In Table 1, results are shown with PEPC for three forms of C$_4$, Kranz-type Schoberia C$_4$ (two species) Kranz-type Salsina C$_4$ (two species), the single cell C$_4$ S. aralocaspica, along with C$_3$ type Suaeda (three species), and the C$_4$ monocot Z. mays. Substitutions on amino acid residues at positions 733 and 780 are candidates for affecting the $K_m$ for PEPC (Rosnow et al., 2014). The four combinations among the Suaeda species for 733 and 780, respectively, are MS Schoberia, LA Salsina, LA single-cell C$_4$, and FA C$_3$ species. The results are shown from kinetic analyses for $K_m$, PEP, the Hill coefficient, and the effect of the allosteric effector G6P. In Table 2, statistical analyses are shown for significant differences in $K_m$ for PEP, the Hill coefficients, and the $V_{max}$ of PEPC. Contrasts 1–3 in Table 2 show where there are differences in these parameters based on the three combinations of residues in Suaeda species at positions 733 and 780 (MS, LA, and FA). Contrasts 4–9 show where there are differences in these parameters based on the four photosynthetic types of Suaeda.

**Absence of G6P**

In kinetic analyses, in the absence of the allosteric effector G6P, the $K_m$ values for PEP were higher in the three types of C$_4$ species than in the C$_3$ species (Table 1; Table 2, contrasts 7–9, $P < 0.05$). Unlike the C$_4$ species, the C$_3$ species have a Phe (F) residue at position 733 (Table 1). Among the C$_4$, the $K_m$ PEP was significantly higher in Kranz Schoberia species (with a Met at 733 and a Ser at residue 780) compared with the Kranz Salsina species (with a Leu at 733 and an Ala at 780; Table 1; Table 2, contrast 4). In addition, the $K_m$ PEP for MS Schoberia was significantly higher than the $K_m$ PEP for the species having LA residues (Salsina C$_4$ and single-cell C$_4$; Table 2, contrast 1). There was no significant difference in the contrast between the single-cell C$_4$ species (SC C$_4$-LA) with either of the Kranz-type C$_4$ species (Table 2, contrasts 5 and 6).

The cooperativity of PEPC for the binding of PEP was investigated by determining the Hill coefficient, from the curve fitting of the Hill equation to the data set. In the absence of G6P, the Hill coefficients were higher in C$_4$ species (from 1.45–2.71) than in the C$_3$ species (0.94–1.19) indicating cooperativity in binding of PEP in C$_4$ species and no cooperativity in the C$_3$ species (Table 1; Fig. 1; Table 2, contrasts 7–9). There was the same pattern of significant differences among species in the Hill coefficients as in the $K_m$ for PEP. The C$_4$ species had higher Hill coefficients than C$_3$ species, the Schoberia C$_4$ had higher Hill coefficients than the Salsina C$_4$, while there was no significant difference in the coefficients between the single-cell C$_4$ species S. aralocaspica and either Kranz C$_4$ type (Table 2, contrasts 4–9).

**Presence of G6P**

In the presence of G6P there was a large decrease in the $K_m$ for PEP in both C$_4$ and C$_3$ species (Table 1) which was significantly different in the absence of G6P (see Supplementary Table S5 at JXB online). This difference is highlighted by the fold increase in rate at 0.3 mM PEP, where all species had at least a 2-fold increase in rate in the presence of G6P, with the highest increase in rate being found in C$_4$ species (Table 1). The $K_m$ values for PEP in the presence of G6P were higher in the C$_4$ than in the C$_3$ species (Table 1; Table 2, contrasts 2, 3, and 7–9). Among the C$_4$ species there were no significant differences in $K_m$ for PEP (Table 2, contrasts 1, and 4–6).

In the presence of G6P there was a large decrease in the Hill coefficient in the C$_4$ species, whereas there was no significant difference in the C$_3$ species with and without G6P (Table 1; see Supplementary Table S5 at JXB online). With G6P, in both C$_3$ and C$_4$ species the Hill coefficients were low (0.9–1.29) indicating little or no cooperativity in binding of PEP, and there was no significant difference in the coefficients between the photosynthetic groups (Table 2).

The change in $K_m$ values for PEP in Z. mays with or without G6P was similar to that in the C$_4$ Suaeda species. With the addition of G6P, the $K_m$ PEP decreased from 1.23 to 0.15 mM

Table 1. PEPC $K_m$-PEP values (pH 7.6) in representative species in subfamily Suaedoideae

Values were determined by curve-fitting the Hill equation to the data. Values represent the average of two biological and two technical replicates. The amino acid residues at positions 733 and 780, M (methionine), S (serine), L (leucine), A (alanine), F (phenylalanine), and V (valine) for the species are from Rosnow et al. (2014) and Besnard et al. (2003) for Z. mays.

| Species        | Photosynthetic mode | Amino acid at residue 733 | Amino acid at residue 780 | $K_m$ PEP (mM) | $V_{max}$ PEP (mM) |
|----------------|---------------------|---------------------------|---------------------------|----------------|-------------------|
| S. accuminata  | Schoberia Kranz C$_4$ | M                         | S                         | 0.83±0.12       | 0.10±0.01         |
| S. eltonica    | Schoberia Kranz C$_4$ | M                         | S                         | 1.04±0.14       | 0.14±0.02         |
| S. moquinii    | Salsina Kranz C$_4$  | L                         | A                         | 0.46±0.12       | 0.14±0.01         |
| S. fruticosa   | Salsina Kranz C$_4$  | L                         | A                         | 0.67±0.11       | 0.14±0.04         |
| S. aralocaspica| Single-Cell C$_4$    | L                         | A                         | 0.74±0.08       | 0.14±0.02         |
| S. linearis    | C$_3$                | F                         | A                         | 0.21±0.01       | 0.03±0.01         |
| S. physophora  | C$_3$                | F                         | A                         | 0.27±0.02       | 0.05±0.01         |
| S. linifolia   | C$_3$                | F                         | A                         | 0.35±0.03       | 0.04±0.01         |
| Zea mays       | C$_4$                | V                         | S                         | 1.23±0.07       | 0.15±0.08         |
in Z. *mays*, with values in the presence of G6P similar to the C$_4$ *Suaeda* species. Unlike the C$_4$ *Suaeda* species, the Hill coefficients in *Z. mays* were low with and without G6P (~1.2) indicating no change in cooperativity with the allosteric effector (Table 1; see Supplementary Table S5 at *JXB* online).

$V_{\text{max}}$

The maximum velocity ($V_{\text{max}}$ on a chlorophyll basis) of the PEPC reaction was determined from the curve-fitting of the Hill equation; as expected, C$_4$ species had much higher PEPC rates than C$_3$ species (Table 3; Table 2, contrasts 7, 8, and 9). There was a significant increase in $V_{\text{max}}$ for each C$_4$ species in the presence of G6P, where the mean fold increase was 1.8 (Table 3; see Supplementary Table S5 at *JXB* online). In the C$_3$ species, G6P had no significant effect on $V_{\text{max}}$ in two of the C$_3$ species; in C$_3$ *S. linearis*, which had very low activity, there was some increase with G6P (Table 3; see Supplementary Table S5 at *JXB* online). Among the C$_4$ contrasts, the $V_{\text{max}}$ in the *Salsina* C$_4$-LA is higher than *Schoberia* C$_4$-MS, and SC C$_4$-LA is higher than *Schoberia* C$_4$-MS, while there is not a significant difference between *Salsina* C$_4$-LA and SC C$_4$-LA (Table 2, contrasts 4, 5, and 6).

**Figure 1** shows the differences in activity in response to varying PEP for the different types of PEPC according to amino acid residues (LA type in *Salina* and SC-C$_4$, MS type in *Schoberia*, and FA type for C$_3$ species), with and without G6P. On a chlorophyll basis at high PEP, the C$_4$ LA type has higher activity than the C$_4$ MS type, while the C$_3$ species have very low activity. Both the LA type and the MS type respond in a similar way. At 5 mM PEP, the addition of G6P results in about a 2-fold increase in activity. In both types, at low levels of PEP, there is a large increase in activity with the addition of G6P as a consequence of lowering the $K_m$ for PEP. This increase in activity by G6P at low PEP (e.g. at ~0.5 mM PEP) is more dramatic in the MS type *Schoberia* (Fig. 1), which has a higher $K_m$ for PEP and a higher Hill coefficient in the absence of G6P than the LA type (Table 1).

**Malate inhibition**

The concentration of a metabolic inhibitor that reduces the rate of an enzyme by 50% ($IC_{50}$) is a useful determination in considering how *in vivo* metabolites might regulate enzyme activity. Table 4 shows the $IC_{50}$ values for MA with species representing different photosynthetic types in Suaedoideae. Amino acid differences are shown for residues 868, 879, and 890, along with residue 780 which are candidates for affecting the $IC_{50}$ for MA (Kai et al., 2003; Paulus et al., 2013b). There was a significant increase in the $IC_{50}$ values in the presence of G6P in all species except in *Salsina*. The two *Salsina* C$_4$ species had $IC_{50}$ values that were significantly higher, with and without G6P, than any other species tested; the *Salsina* species also had different amino acid residues at position 868, 879, and 890. PEPC in the C$_4$ species were the most sensitive to MA, both in the presence and absence of G6P. The two *Schoberia* C$_4$ species that have Ser at residue 780 and Arg at residue 868, had $IC_{50}$ values which were higher than the C$_3$ species, but lower than *Salsina* C$_4$ species (Table 4). The C$_3$ species, which had the lowest $IC_{50}$ values, were different from other species in having Lys at residue 868. The $IC_{50}$ values for the single cell C$_4$ *S. aralocaspica* were similar to the *Schoberia* type, and was different from other types in having a Gln residue at 868, and a Glu residue at 879. There was no significant difference in PEPC $IC_{50}$ values based on the presence of a Ser versus an Ala residue at 780.

**Rubisco rbcL sequence information**

A full-length rbcL sequence was generated for 20 Suaedoideae species, including at least two species from each Suaedoideae clade. There were 19 polymorphic Rubisco large subunit residues across the Suaedoideae species analysed, but none of the amino acid substitutions was invariantly fixed across C$_4$ species (see Supplementary Table S6 at *JXB* online). Two C$_3$ (*S. linifolia, S. vera*) and two C$_4$ (*S. accuminata, S. aralocaspica*) species, representing four different sections, had identical amino acid sequences (see Supplementary Table S6 at *JXB* online). The PAML branch-site test for positive selection did not show significant evidence for selection along C$_4$ branches (data not shown).
Convergent carboxylase kinetics via divergent amino acids in Suaedoideae

Table 2. Contrasts of Suaedoideae PEPC kinetic parameters (Kₘₐₜ for PEP, Hill coefficient, and Vₘₐₓₜ under saturating Mg²⁺ and HCO₃⁻)

Contrasts were done based on amino acid residues 733 (M, L, or F) and 780 (S or A) or photosynthetic mode; see Table 1. In contrasts 1–3, amino acids are compared, MS (C₄) occurs in Schoberia species, LA (C₃) occurs in Salsina species and single-cell S. aralocaspica, and FA (C₃) represents C₃ species. In contrasts 4–9, the four photosynthetic modes are being compared, Schoberia type C₄ (S. eltonica and S. accuminata), Salsina type C₄ (S. fruticosa and S. moquinii), single-cell C₄ (S. aralocaspica), and C₃ species (S. linearis, S. linifolia, and S. physophora). *, Significant at the P < 0.05 level of significance. (+) Indicates whether the first component of the contrast is larger than the second component and (–) indicates that the first component of the contrast is smaller than the second component. Maize data were excluded from analysis.

| Contrast                        | Kₘₐₜ No G6P | 5 mM G6P | Hill coefficient No G6P | 5 mM G6P | PEPC Vₘₐₓₜ No G6P | 5 mM G6P |
|---------------------------------|-------------|----------|-------------------------|----------|-------------------|----------|
| 1. MS (C₄)×LA (C₃)              | (+)         | (–)      | (+)                     | (+)      | (–)               | (–)      |
| 2. MS (C₄)×FA (C₃)              | (+)         | (+)      | (–)                     | (+)      | (+)               | (+)      |
| 3. LA (C₃) × FA (C₃)             | (+)         | (+)      | (–)                     | (–)      | (+)               | (+)      |
| 4. Schoberia C₄ (MS)×Salsina C₃ | (+)         | (–)      | (–)                     | (-)      | (–)               | (–)      |
| 5. Schoberia C₄ (MS)×SC C₄ (LA)  | (–)         | (–)      | (–)                     | (-)      | (+)               | (+)      |
| 6. Salsina C₃ (LA)×SC C₃ (LA)    | (+)         | (+)      | (–)                     | (–)      | (–)               | (–)      |
| 7. Schoberia C₄ (MS)×C₃ (FA)      | (–)         | (–)      | (–)                     | (-)      | (+)               | (+)      |
| 8. Salsina C₃ (LA)×C₃ (FA)        | (+)         | (+)      | (+)                     | (+)      | (+)               | (+)      |
| 9. SC C₃ (LA)×C₃ (FA)            | (+)         | (+)      | (+)                     | (+)      | (+)               | (+)      |

Table 3. PEPC Vₘₐₓₜ values with saturating Mg²⁺ and HCO₃⁻ for representative species in subfamily Suaedoideae at pH 7.6

| Species          | Photosynthetic mode | Vₘₐₓₜ (μmol min⁻¹ mg⁻¹ chl) | Fold increase in activity in presence of G6P |
|------------------|---------------------|----------------------------|---------------------------------------------|
|                  |                     | No G6P 5 mM G6P |                                            |
| S. accuminata    | Schoberia Kranz C₄  | 4.6  10.0       | 2.2                                         |
| S. eltonica      | Schoberia Kranz C₄  | 6.5  12.9       | 2.0                                         |
| S. moquinii      | Salsina Kranz C₃    | 9.9  15.6       | 1.6                                         |
| S. fruticosa     | Salsina Kranz C₃    | 11.5 17.6       | 1.5                                         |
| S. aralocaspica  | Single-Cell C₄      | 16.5 24.1       | 1.5                                         |
| S. linearis      | C₃                   | 0.1  0.4        | 3.5                                         |
| S. physophora    | C₃                   | 1.0  0.8        | 0.8                                         |
| S. linifolia     | C₃                   | 0.4  0.4        | 1.1                                         |
| Zea mays         | C₄                   | 15.1 21.1       | 1.4                                         |

Table 4. Estimates of malate IC₅₀ values for half-maximum inhibition of PEPC activity at pH 7.6 (PEP concentration, 2× the Kₘₐₜ) in representative photosynthetic types in subfamily Suaedoideae

The amino acid residues potentially involved in malate tolerance are presented. For species comparisons, different letters indicate a significant difference within a category of G6P (+ or –) while comparison of G6P levels within a species is indicated by an asterisk (*) for significance at the P < 0.05 level.

| Species          | Photosynthetic mode | Residue at 780 | Residue at 868 | Residue at 879 | Residue at 890 | IC₅₀ (mM) No G6P | IC₅₀ (mM) 5 mM G6P | Significant effect of G6P |
|------------------|---------------------|---------------|---------------|---------------|---------------|-----------------|-------------------|-------------------|
| S. accuminata    | Schoberia Kranz C₄  | S             | R             | D             | R             | 0.6 c           | 1.4 c             | *                 |
| S. eltonica      | Schoberia Kranz C₄  | S             | R             | D             | R             | 1.0 b           | 1.9 b             | *                 |
| S. moquinii      | Salsina Kranz C₃    | A             | L             | N             | M             | 4.5 a           | 5.9 a             | –                 |
| S. fruticosa     | Salsina Kranz C₃    | A             | L             | N             | M             | 4.5 a           | 5.2 a             | –                 |
| S. aralocaspica  | Single Cell-C₄      | A             | Q             | E             | R             | 0.9 b           | 1.6 bc            | *                 |
| S. physophora    | C₃                   | A             | K             | D             | R             | 0.3 d           | 0.9 d             | *                 |
| S. linifolia     | C₃                   | A             | K             | D             | R             | 0.3 d           | 0.8 d             | *                 |

Rubisco kₜₐₜ

Measurement of Rubisco kₜₐₜ using the coupled enzyme assay showed that C₄ species had significantly higher values than C₃ species (Table 5). The average kₜₐₜ value for C₄ was 2-fold higher than that of C₃ species (3.6 versus 1.8 mol CO₂ mol⁻¹ binding sites s⁻¹). There was no significant difference in Rubisco kₜₐₜ between the single-cell C₄ and Kranz species.
Table 5. Rubisco kcatc (mol CO₂ mol⁻¹ binding sites s⁻¹) values for representative photosynthetic types in subfamily Suaedoideae

| Species               | Photosynthetic mode | Rubisco kcatc | SD  |
|-----------------------|---------------------|---------------|-----|
| S. accuminata         | Schobienia Kranz C₄ | 2.95          | 0.33|
| S. eltochina          | Schobienia Kranz C₄ | 3.34          | 1.18|
| S. moquinii           | Salatina Kranz C₄   | 3.76          | 0.30|
| S. fruticosa          | Salatina Kranz C₄   | 4.23          | 0.46|
| S. altissima          | Salatina Kranz C₄   | 3.19          | 0.17|
| Mean Kranz C₄         |                     | 3.49 b        |     |
| S. aralocaspica       | Single-Cell C₄      | 3.77          | 0.39|
| Bienertia cycloptera  | Single-Cell C₄      | 3.90          | 0.27|
| Bienertia sinuspersci | Single-Cell C₄      | 3.46          | 0.34|
| Mean Single-Cell C₄   |                     | 3.71 b        |     |
| Zea mays              | Kranz C₄            | 3.58          | 0.00|
| S. linearis           | Cₛ                | 1.77          | 0.28|
| S. physophora         | Cₛ                | 1.52          | 0.09|
| S. linifolia          | Cₛ                | 2.08          | 0.17|
| S. vera               | Cₛ                | 1.82          | 0.49|
| Mean Cₛ              |                     | 1.80 a        |     |

Discussion

PEPC kinetic features in Suaedoideae: Vₘₐₓ, affinity for PEP, regulation by G6P and MA

The maximum activities of PEPC (Vₘₐₓ, μmol mg⁻¹ chlorophyll) from leaves of the C₄ Suaeda species were much higher than the C₃ species, which is characteristic of C₄ plants (Kanai and Edwards, 1999). In addition, compared with the C₃ species, all of the C₄ species analysed had a significantly higher Kₘ for PEP, both in the absence and presence of G6P (Tables 1, 2), which is the same general trend that has been reported throughout the literature (Svensson et al., 1997; Gowik et al., 2006; Lara et al., 2006; Jacobs et al., 2008). From studies in the genus Flaveria with ppc-2, the location of amino acids responsible for an increase in PEPC Kₘ was shown through reciprocal domain swapping to be in region 2 (amino acids 302–442) and region 5 (amino acids 651–966). In region 5, the single amino acid change to a Ser at residue 780 was suggested to be an important substitution resulting in the increase in Kₘ in C₄ PEPC (Blasing et al., 2000; Engelmann et al., 2002). Subsequently, this substitution has been considered to be a key substitution for increasing the Kₘ in PEPC analyses of various C₄ species (Christin et al., 2007; Besnard et al., 2009; Gowik and Westhoff, 2011). However, the results of the current study, and from analysis of Hydrella verticillata (a facultative aquatic C₄ species) PEPC (Rao et al., 2008), suggest that alternative substitutions can change the affinity for PEP. In Suaedoideae C₄ species, a substitution at residue 733 in region 5 is a candidate for raising the Kₘ, and the cooperativity in PEP binding (higher Hill coefficients).

Previous investigations on PEPC in C₄ plants showed that the addition of phosphorylated sugars (e.g. G6P and triose-P) reduced the sigmoid nature of Michaelis–Menten kinetics plots, reducing the Hill coefficient to near one, demonstrating that allosteric effectors can reduce the cooperative binding of PEP (Coombs and Baldry, 1975; Huber and Edwards, 1975; Nakamoto et al., 1983; Bauwe and Chollet, 1986; Doncaster and Leegood, 1987; Tovar-Mendez et al., 2000; Engelmann et al., 2003; Gowik et al., 2006). In addition, G6P has been shown to crystallize in the active site of the Flaveria trinervia ppc-2 gene, demonstrating that it can also act as a competitive inhibitor (Schlieper et al., 2014). In the present study, inclusion of the allosteric effector G6P in the assay of PEPC (pH 7.6) lowered the Kₘ for PEPC and increased enzyme activity in both the C₃ and C₄ species of Suaeda. However, in the absence of G6P, the C₄ species showed cooperativity with PEP (the mean Hill coefficient for five species is 2.1) while the PEPC in C₃ species showed no cooperativity (the mean Hill coefficient for three species is 1.0). This suggests certain substitutions in the C₄ PEPC result in both an increase in Kₘ for PEP and an increase in the cooperativity of PEP binding.

Region 2 in the N-terminus was previously identified as the G6P regulatory site in C₄ PEPC in Z. mays and it has also been suggested to influence the affinity of the enzyme for PEP (Kai et al., 2003). In a study of representative photosynthetic types in Flaveria, residue 352 in region 2 of ppc-2 (aka ppcA) was the only amino acid that showed differences between the C₄ and C₃-like Flaveria species which have a Lys residue, while the C₃ and C₄-C₃ intermediate Flaveria have an Arg residue at this position. The C₄ PEPC in Z. mays also has a Lys at residue 352 (Engelmann et al., 2003). By contrast, current analysis of the N-terminus in Suaeda species showed position 352 is either a Thr or Ser residue (see Supplementary Fig. S1 at JXB online), and this residue is also an invariant Thr across Alternanthera PEPCs (Gowik et al., 2006). In the Suaeda species, positive selection was found in region 2 at residues 364 (for Gln) and 368 (for Ser; see Supplementary Table S5 and Supplementary Fig. S1 at JXB online). The Alternanthera ppc-1 gene has positive selection for Ser at residue 368, while residue 364 is invariant. Interestingly, the ppc-1 gene in Z. mays and the ppc-2 gene of F. trinervia (C₄) has Asn at residue 368 (the same residue observed in all Suaedoideae C₃ PEPC), while F. pringeli (C₃) has Ser (the same amino acid observed in all Suaedoideae C₄ PEPC). These results suggest that paralogous genes (ppc-1 versus ppc-2) have undergone different selection processes. In C₄ Suaedoideae and C₄ Alternanthera ppc-1, substitution at residue 368 is a candidate for affecting the cooperativity with PEP as substrate, and regulation by binding G6P as an allosteric effector.

From species surveyed in Suaedoideae, the IC₅₀ values for MA indicate that it is an effective inhibitor of PEPC at mM levels (Table 4). PEPC from C₃ Suaeda species was more sensitive to inhibition by MA (assayed either with or without G6P) which is consistent with other studies where C₃ PEPCs are generally reported to be more tolerant to MA compared with C₃ orthologous genes or paralogous genes (Svensson et al., 1997; Dong et al., 1998; Blasing et al., 2002; Paulus et al., 2013a). Among the C₄ Suaeda, the two Schoberia Kranz species, had significantly higher IC₅₀ values indicating higher tolerance to MA (when assayed in the presence or absence of G6P), compared with the Schoberia Kranz species and the single-cell C₄ species S. aralocaspica. Also, with the addition
of G6P, the IC\textsubscript{50} for MA increased in the C\textsubscript{4} Schoberia and C\textsubscript{3} species, but not in the C\textsubscript{4} Salsina. Studies on Z. mays show C\textsubscript{4} PEPC has an allosteric site that binds MA and Asp, which is so close to the catalytic site that these metabolites act competitively with the substrate PEP, resulting in a less active enzyme (Izui et al., 2004). In Suaedoideae, an amino acid substitution at residue 868 (Leu) is observed in all C\textsubscript{4} species studied in the subfamily except Bienertia (see Supplementary Fig. S1 at JXB online) which may explain the difference in IC\textsubscript{50} values between C\textsubscript{3} and C\textsubscript{4} species. The high IC\textsubscript{50} values for MA in the Salsina species may be linked to their PEPC having, in addition to substitution at 868, substitutions at 879 (Asp), and 890 (Met) which is different from the other Suaeda species based on previous C-terminal PEPC sequence information (Rosnow et al., 2014). Amino acid substitution at 868 is also observed in Z. mays and other Amaranthaceae C\textsubscript{4} ppc-1 genes, but not Flaveria C\textsubscript{4} ppc-2. In other studies on MA inhibition of PEPC, a substitution at residue 884 from an Arg to a Gly in Flaveria was recently shown to increase tolerance to MA (Paulus et al., 2013b). This substitution is also observed in some, but not all, C\textsubscript{4} grass species (Paulus et al., 2013a). However, this substitution is not observed in any C\textsubscript{4} Suaedoideae species (Rosnow et al., 2014). Using heterologously expressed chimeric ppc-2 enzymes from Flaveria, the replacement of Ala 780 by Ser caused a slight increase in MA tolerance (observed in the presence, but not in the absence of G6P), which was not considered as the main determinant for higher MA tolerance in C\textsubscript{4} PEPC (Jacobs et al., 2008). In the present study, the highest tolerance to MA was in the Salsina C\textsubscript{4} species, which have an Ala 780 and which also indicates other residues are the main determinants of tolerance.

The lack of strong convergence for a substitution near the MA/Asp allosteric pocket, suggests that there is less selective pressure on increasing MA tolerance than on increasing the $K_m$ for PEP (decreased affinity), and G6P activation. Tolerance to MA may increase with alternative substitutions at different residues, without convergent amino acids, together with G6P activation and phosphorylation of PEPC in the light reducing sensitivity to MA. In the light, C\textsubscript{4} PEPC is regulated by phosphorylation at a conserved N-terminus Ser residue, which leads to activation of the enzyme by reducing its sensitivity towards the allosteric inhibitors MA and Asp (Jiao and Chollet, 1991; Vidal and Chollet, 1997; Nimmo, 2003).

The current results raise questions about the molecular route for a C\textsubscript{4} PEPC to acquire modified kinetic properties; i.e. modification to the allosteric activator site (residue 364/368) before or after increasing PEP $K_m$ near the reaction site (733/780), and the impact of the order of mutations on selective pressure. Further analyses are needed to address the influence of amino acid substitutions on PEPC tolerance to MA in C\textsubscript{4} Suaedoideae and other C\textsubscript{4} species, versus the impact of in vivo phosphorylation of PEPC in the light (in this study extractions were made in the light).

Overall, the results indicate that phylogenetically distant C\textsubscript{4} origins can optimize PEPC with divergent amino acid substitutions. The kinetic properties of C\textsubscript{4} PEPC are considered to be optimized for function in C\textsubscript{4} photosynthesis without interference with other metabolic processes. During the day, the positive allosteric effectors triose-P, G6P, and glycine are produced during photosynthesis in C\textsubscript{4} plants (Leegood and von Caemmerer, 1988, 1989; De Veau and Burris, 1989; Zelitch et al., 2009). These positive allosteric effectors increase the affinity of PEPC for PEP and its effective use in the C\textsubscript{4} cycle while the IC\textsubscript{50} values for the C\textsubscript{4} acids MA and Asp increases, which minimizes inhibition by products of C\textsubscript{4} photosynthesis. Activity of PEPC at night can be controlled by the enzyme having a high $K_m$ for PEP, due to relatively low levels of positive allosteric effectors (G6P, triose-P, and glycine) and by the non-phosphorylated form of the enzyme at night having a low IC\textsubscript{50} for C\textsubscript{4} acids (Doncaster and Leegood, 1987).

**Convergent evolution of Rubisco kinetics in C\textsubscript{4} Suaedoideae achieved via non-parallel amino acid substitutions**

In the current study of Suaedoideae, the determination of Rubisco $k_{cat}$ showed that the enzyme in C\textsubscript{4} species representing four lineages has, on average, approximately 2-fold higher catalytic rates than the C\textsubscript{3} species. The mean $k_{cat}$ value for these C\textsubscript{4} species (all NAD-ME type), are similar to those of NAD-ME type C\textsubscript{4} grasses (Ghannoun et al., 2005). Although the $k_{cat}$ values in Suaedoideae are higher for the C\textsubscript{4} than the C\textsubscript{3} species, sequence analysis of rbcL did not show any evidence for positive selection across lineages which could account for this adaptation.

The C\textsubscript{4} species S. aralocaspica (section Borschzowia) and S. accuminata (section Schoberia) and the C\textsubscript{3} species S. linifolia (section Schanginia) and S. vera (section Suaeda) have Rubisco large-subunit sequences that are identical (see Supplementary Table S6 at JXB online). This suggests that, in some C\textsubscript{4} species, Rubisco with higher specific activity evolved via amino acid changes in the Rubisco small subunits. Positive selection on the small subunit encoding RbcS gene has previously been demonstrated for C\textsubscript{4} Flaveria, which was strongly correlated with higher $k_{cat}$ values and weakly correlated with higher $K_m$(CO$_2$) values (Kraprov et al., 2011). Although Rubisco catalytic sites are located within the large subunits, significant changes in kinetics were shown when small subunits from C\textsubscript{3} rice were replaced with those from C\textsubscript{4} Sorghum, suggesting a differential role of S-subunits in Rubisco kinetics (Ishikawa et al., 2011). Further work is necessary to determine if there are amino acids encoded by certain RbcS genes that are under positive selection, and candidates for determinant of the higher $k_{cat}$ values in some Suaedoideae C\textsubscript{4} lineages.

In C\textsubscript{4} species representing sections Salsina and Bienertia, analysis of the Rubisco large-subunit residue polymorphism indicates that there are differences in amino acid residues compared with C\textsubscript{3} species which may be associated with increased Rubisco $k_{cat}$ values. The two single-cell C\textsubscript{4} species from the genus Bienertia have three amino acid replacements putatively associated with increased Rubisco $k_{cat}$. These are Ile 225, reported among submerged aquatic macrophytes (Iida et al., 2009) which may be linked to kinetic properties of Rubisco associated with CO$_2$-concentrating mechanisms.
Conclusions

The likelihood of a gene being repeatedly and independently recruited and changed to develop a convergent phenotype is most likely linked to the tissue in which it is expressed and its optimization for catalytic regulation. At the molecular level, when gene families are either recurrently recruited or when there are identical amino acid replacements in distant lineages, it suggests that there is limited genetic material suitable for new functions or that there is a restricted number of substitutions which can confer specific enzymatic properties (Christin et al., 2010). For PEPC, the differences in substrate affinity and the reaction towards allosteric effectors, suggest that C₄ PEPC’s harbour specific C₄ determinants that were acquired during the evolution of C₄ photosynthesis (Gowik and Westhoff, 2011). The results presented here suggest that the development of C₄ photosynthesis can occur with divergent amino acid substitutions that alter enzyme kinetics to converge on the same function. To our knowledge this is the first report to demonstrate that PEPC from C₄ terrestrial plants without Ser at position 780 have C₄-like PEPC kinetics (and to identify candidates for positive selection at positions 364, 368, and 733). Similarly, this is the first case which shows that there are C₄ species which have C₄-type Rubisco $k_{cat}$ values while lacking amino acid substitutions in the large subunit of Rubisco. This demonstrates that there are multiple molecular routes to the same C₄ carboxylase phenotype.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Table S1. Name and sequence of primers used.

Supplementary Table S2. Species origin, voucher, and sequence accession numbers.

Supplementary Table S3. Carbon isotope fraction values for leaf biomass.

Supplementary Table S4. ppc-1 positive selection results.

Supplementary Table S5. Statistical analysis for PEPC $K_m$ for PEP, Hill coefficient, and $V_{max}$.

Supplementary Table S6. Rubisco large subunit residue polymorphisms.

Supplementary Fig. S1. Phylogeny of Suaedoidae taxa used for ppc-1 positive selection analysis showing key amino acid changes.

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