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Rubisco Evolution in C₄ Eudicots: An Analysis of Amaranthaceae *Sensu Lato*

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Abstract

Background: Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) catalyses the key reaction in the photosynthetic assimilation of CO_2. In C₄ plants CO_2 is supplied to Rubisco by an auxiliary CO_2-concentrating pathway that helps to maximize the carboxylase activity of the enzyme while suppressing its oxygenase activity. As a consequence, C₄ Rubisco exhibits a higher maximum velocity but lower substrate specificity compared with the C₃ enzyme. Specific amino-acids in Rubisco are associated with C₄ photosynthesis in monocots, but it is not known whether selection has acted on Rubisco in a similar way in eudicots.

Methodology/Principal Findings: We investigated Rubisco evolution in Amaranthaceae *sensu lato* (including Chenopodiaceae), the third-largest family of C₄ plants, using phylogeny-based maximum likelihood and Bayesian methods to detect Darwinian selection on the chloroplast *rbcL* gene in a sample of 179 species. Two Rubisco residues, 281 and 309, were found to be under positive selection in C₄ Amaranthaceae with multiple parallel replacements of alanine by serine at position 281 and methionine by isoleucine at position 309. Remarkably, both amino-acids have been detected in other C₄ plant groups, such as C₄ monocots, illustrating a striking parallelism in molecular evolution.

Conclusions/Significance: Our findings illustrate how simple genetic changes can contribute to the evolution of photosynthesis and strengthen the hypothesis that parallel amino-acid replacements are associated with adaptive changes in Rubisco.

Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) serves as the main gateway for inorganic carbon to enter metabolic pathways in most ecosystems and hence is unique in its importance to support life. Observations of significant variation in Rubisco kinetics between plant species [1,2,3], the correlation of Rubisco kinetics with temperature [4] and CO_2 availability [5], and positive selection on Rubisco at the molecular level in all principal lineages of land plants [6] support the hypothesis that all Rubiscos may be well adapted to their subcellular environment [7]. However, the molecular mechanisms responsible for optimizing the relationship between Rubisco specificity and its maximum rate of catalytic turnover in particular conditions are still open to debate [8]. Here we use a phylogeny-based approach to investigate how the occurrence of C₄ photosynthesis has influenced Rubisco evolution at the molecular level in eudicots as represented by the family Amaranthaceae *sensu lato*.

Rubisco discriminates imperfectly between CO_2 and O_2 as substrates, and under present-day atmospheric conditions (385 p.p.m. CO_2), the carboxylase activity of Rubisco is under-saturated in C₃ plants, and the oxygenase activity gives rise directly to the competing process of photorespiration. Photorespiratory rates in C₃ plants increase steeply with increasing temperature and give rise to a distinct temperature optimum for net photosynthesis, above which plant yields decline steeply. Increased carbon loss via photorespiration at higher temperatures is attributable mainly to the declining specificity of Rubisco for CO_2 relative to O_2 ($S_{c/o}$). In fact, it has been proposed that the very slow turnover of Rubisco ($k_{cat} \approx 3$ s^{-1}) is a direct consequence of the enzyme's particular reaction mechanism, in which $S_{c/o}$ is maximized by tight binding of the transition-state intermediate [7]. Land plants also depend on the enzyme rubisco activase which removes tightly binding inhibitors at the active site of Rubisco and thus prevents the loss of its catalytic activity. The cascade of side-reactions performed by Rubisco is yet to be fully understood although recent achievements in mathematical modelling of Rubisco reactions offer the theoretical background for predicting 'side-effects' by simulating the overall kinetic behaviour [9]. Another corollary of low k_{cat} and of the large size of the holoenzyme (560 kDa) is that Rubisco comprises up to 50% of soluble protein in photosynthetic tissues and is probably the most abundant enzyme on Earth [10].

In terrestrial plants with C₄ photosynthesis or crassulacean acid metabolism (CAM), and in many aquatic organisms, photorespiration is partially or completely suppressed by the operation of an auxiliary CO_2-concentrating mechanism. C₄ plants initially fix atmospheric carbon in the mesophyll cells using phospho*enol*pyr-

uvate carboxylase, an enzyme with a high effective affinity for CO_2 (HCO_3^- being the true substrate of the enzyme). Further four-carbon compounds (malate or aspartate) produced by this fixation are transported to the specialized bundle-sheath cells, where CO_2 is released and fixed by Rubisco. Rubisco from C₄ plants, which experiences ~10-fold higher CO_2 concentrations in bundle-sheath cells than does the enzyme in C₃ plants [11], has a lower affinity for CO_2 but a higher k_{cat} (≈ 4 s^{-1}). Having less specific but faster Rubisco and no photorespiration losses, C₄ plants require 60 to 75% less Rubisco to match the photosynthetic capacity of C₃ plants [12,13]. In fact, many C₄ plants such as maize, sugarcane and sorghum are among the most productive of all species cultivated agriculturally. Although C₄ plants appeared relatively recently in evolutionary terms and constitute only 3% of terrestrial plant species, they are already among the most successful and abundant groups in warm climates and are responsible for about 20% of terrestrial gross primary productivity [14,15].

C₄ photosynthesis evolved independently in at least 62 recognizable lineages of angiosperms and represents one of the most striking examples of a convergent biochemical adaptation in plants [16]. However, since its discovery, most attention has been devoted to the more numerous and agriculturally important C₄ monocots in the Poaceae, while C₄ eudicots have been studied less intensively. The family Amaranthaceae *sensu lato* (i.e. including Chenopodiaceae) [17,18] contains about 180 genera and 2500 species, of which approximately 750 are C₄ species [16], making it by far the largest C₄ family among eudicots and the third-largest among angiosperms (after Poaceae and Cyperaceae). C₄ photosynthesis evolved at least 15 times within Amaranthaceae [16] making this family a good model to study coevolution of C₄ photosynthesis and Rubisco. Notably, the Amaranthaceae exceed the Poaceae and Cyperaceae in the diversity of photosynthetic organ anatomy [19], and is the only angiosperm family containing terrestrial C₄ plants that lack Kranz anatomy, with three species having a single-cell rather than the more usual dual-cell C₄ system [20,21]. The predominantly tropical Amaranthaceae *sensu stricto* and primarily temperate and subtropical Chenopodiaceae have long been treated as two closely related families (see review in [19]) until the formal proposal that Chenopodiaceae should be included within the expanded Amaranthaceae based on a lack of separation between the two families in sequence data [17]. Amaranthaceae *sensu lato* (henceforth referred to as Amaranthaceae) constitutes the most diverse lineage of the Caryophyllales. Both C₃ and C₄ species from this family are adapted to a range of conditions from temperate meadows to the tropics, hot deserts and salt marshes. However, it has been shown that the abundance of C₄ Amaranthaceae is correlated with precipitation but not temperature, in contrast to the abundance of C₄ Poaceae and Cyperaceae, which is correlated with temperature but not precipitation [22].

Despite C₄ Amaranthaceae showing different suites of anatomical and biochemical adaptations as well as ecological preferences compared to C₄ Poaceae and Cyperaceae, like C₄ monocots they possess faster but less CO_2-specific Rubiscos than their C₃ relatives [3,5,23]. Thus, Rubisco of C₄ eudicots and monocots represents a notable example of convergent evolution of enzyme properties in phylogenetically distant groups. However, it is not known whether this functional convergence in Rubisco kinetics evolved via similar or different structural changes in protein [24]. Molecular adaptation can be inferred from comparison of the rates of non-synonymous (changing amino-acid protein sequence, d_N) and synonymous (resulting in no change at the protein level, d_S) mutations along a phylogenetic tree using maximum likelihood

and Bayesian frameworks [25]. Recently, such methodology has been applied to the chloroplast gene *rbcL*, which encodes the large subunit of Rubisco that forms the enzyme's active site, and showed that positive Darwinian selection is acting within most lineages of plants [6]. Only a small fraction of Rubisco residues appear to be under positive selection, while most residues have been under purifying selection [6]. Some of these residues have been shown to be under positive selection within C₄ lineages of Poaceae and Cyperaceae [26] and in the small Asteraceae genus, *Flaveria* [27], which contains both C₃ and C₄ species. However, no specific analysis has yet been made of Rubisco sequence evolution in a large group of C₄ eudicots. In this study, we investigate positive selection on the *rbcL* gene of plants from the Amaranthaceae family and, in particular, focus on coevolution of Rubisco and C₄ photosynthesis asking whether positive selection on the *rbcL* gene occured on branches leading to C₄ clades and/or within C₄ clades. Finally, we address the following question: which amino-acid replacements were associated with transitions from C₃ to C₄ photosynthesis in Amaranthaceae, and are these replacements unique to this lineage or shared with C₄ monocots and/or *Flaveria*?

Materials and Methods

Phylogenetic analysis

We obtained all Amaranthaceae *rbcL* nucleotide sequences available in GenBank and aligned them. Sequences shorter than 1341 base pairs and sequences with missing data were excluded. The resulting trimmed alignment consisted of 179 *rbcL* sequences of 1341 base pairs long which represented 94% of the *rbcL* coding region and corresponded to positions 64 to 1404 of the *rbcL* sequence of *Spinacia oleracea* (GenBank AJ400848). The analysed dataset consisted of 95 C₃ and 84 C₄ species (Table S1). Most of the included sequences came from four studies [19,28,29,30] and evenly represented all main lineages within the family (Fig. 1). Phylogeny was reconstructed using a maximum-likelihood inference (ML) conducted with RAxML version 7.2.6 [31] using the raxmlGUI interface [32]. We conducted five independent runs from different starting points to assess convergence within two likelihood units of the best tree, which was consistently selected. The parameters of partition were allowed to vary independently under the GTRGAMMA model of evolution as implemented in RAxML. ML nodal support was calculated by analysing 1000 bootstrap replicates. The best-scoring ML tree was used for tests of positive selection (see below).

Tests for positive selection

Positive, neutral, or purifying selection at the molecular level can be inferred by comparing rates of non-synonymous (d_N) and synonymous (d_S) mutations along a phylogenetic tree [33]. Under neutrality, the two rates are expected to be equal ($d_N/d_S = 1$), while purifying (negative) or adaptive (positive) selection is expected to deflate ($d_N/d_S < 1$) or inflate ($d_N/d_S > 1$) this ratio, respectively. One can use likelihood ratio tests to detect positive selection that affects only a subset of codons in a protein-coding gene, with positive selection indicated by accelerated nonsynonymous substitutions. Models assuming positive selection along all phylogeny or prespecified branches only (e.g. C₄ lineages in our case) can be employed within Phylogenetic Analysis by Maximum Likelihood (PAML) framework [33].

We used the *codeml* program in the PAML v.4.4 package [33] to estimate d_N/d_S ratio in the model M0, that allows for a single d_N/d_S value across the whole phylogenetic tree obtained previously (see *Phylogenetic analyses* section). Further, *codeml* was used to perform likelihood ratio tests (LRTs) for positive selection among amino

0.01

Polycnemum perenne
Nitrophila occidentalis
Hemichroa diandra
Bosea yervamora
Charpentiera ovata
Charpentiera obovata
Deeringia amaranthoides
Hemitsaedia glauca
Celosia trigyna
Celosia argentea
Chamissoa altissima
Amaranthus greggii
Amaranthus tricolor
Amaranthus blitum
Amaranthus hypochondriacus
Ptilotus manglesi
Pupalia lappacea
Calicorema capitata
Pandiaka angustifolia
Sericostachys scandens
Achyranthes aspera
Nototrichium humile
Aerva javanica
Iresine palmeri
Gomphrena elegans
Pseudoplantago friesii
Hebanthe occidentalis
Blutaparon vermiculare
Guilleminea densa
Gomphrena serrata
Gomphrena haageana
Tidestromia lanuginosa
Alternanthera pungens
Alternanthera caracasana
Alternanthera repens
Oreobliton thesioides
Beta vulgaris
Beta nana
Hablitzia tamnoides
Aphanisma blitoides
Patellifolia patellaris
Teloxys aristata
Suckleya suckleyana
Cycloloma atriplicifolium
Chenopodium botrys
Chenopodium ambrosioides
Chenopodium cristatum
Dysphania glomulifera
Chenopodium bonus-henricus
Chenopodium foliosum
Monolepis nuttalliana
Spinacia oleracea
Axyris prostrata
Ceratocarpus arenarius
Krascheninnikovia ceratoides
Chenopodium coronopus
Micrognyoecium tibeticum
Einadia nutans
Rhagodia drummondii
Chenopodium desertorum
Chenopodium auricomum
Micromonolepis pusilla
Chenopodium frutescens
Chenopodium acuminatum
Chenopodium sanctaeclarae
Chenopodium album
Chenopodium murale
Manochlamys albicans
Archiatriplex nanpinensis
Halimione pedunculata
Halimione verrucifera
Atriplex aucheri
Atriplex australasica
Atriplex patula
Atriplex halimus
Cremnophyton lanfrancoi
Atriplex coriacea
Atriplex glauca
Atriplex centralasiatica
Atriplex spongiosa
Atriplex rosea
Atriplex lentiformis
Atriplex lampa
Atriplex undulata
Atriplex parryi
Atriplex powelli
Atriplex phyllostegia
Atriplex serenana
Acroglochin chenopodioides
Agriophyllum aquarcoum
Corispermum filifolium
Anthochlamys multinervis
Suaeda maritima
Suaeda crassifolia
Suaeda altissima
Suaeda physophora
Suaeda microphylla
Bienertia cycloptera
Allenrolfea occidentalis
Halostachys belangeriana
Halopeplis amplexicaulis
Kalidium cuspidatum
Kalidium caspicum
Kalidium foliatum
Arthrocnemum macrostachyum
Sarcocornia utahensis
Salicornia europaea
Tecticornia disarticulata
Sclerostegia moniliformis
Tecticornia australasica
Pachycornia triandra
Salicornia dolichostachya
Halaracia indica
Halocharis hispida
Salsola vermiculata
Salsola implicata
Salsola micranthera
Salsola orientalis
Salsola dshungarica
Petrosimonia sibirica
Petrosimonia nigdeensis
Petrosimonia glaucescens
Petrosimonia squarrosa
Salsola affinis
Climacoptera brachiata
Halimocnemis villosa
Halimocnemis karelinii
Climacoptera lanata
Salsola sukaczevii
Salsola ferganica
Salsola heptapotamica
Nanophyton erinaceum
Salsola genistoides
Salsola arbuscula
Salsola kali
Salsola praecox
Salsola pellucida
Salsola paulsenii
Salsola chinghaiensis
Salsola zaidamica
Salsola collina
Salsola komarovi
Salsola ruthenica
Kochia americana
Bassia diffusa
Bassia dasyphylla
Maireana brevifolia
Sclerolaena obliquicuspis
Roycea divaricata
Dissocarpus paradoxus
Bassia sedoides
Camphorosma monspeliaca
Kochia densiflora
Chenoleoides tomentosa
Bassia prostrata
Panderia pilosa
Sympegma regelii
Halothamnus bottae
Salsola lancifolia
Salsola arbusculiformis
Rhaphidophyton regelii
Ofaiston monandrum
Salsola rosacea
Noaea mucronata
Anabasis brevifolia
Anabasis truncata
Anabasis eriopoda
Anabasis aphylla
Anabasis salsa
Anabasis elatior
Salsola foliosa
Girgensohnia oppositiflora
Halogeton glomeratus
Haloxylon ammodendron
Haloxylon persicum
Iljinia regelii
Haloxylon tamariscifolium
Horaninovia ulicina
Halogeton arachnoideus

Figure 1. Maximum likelihood phylogram based on *rbcL* sequences of 179 Amaranthaceae species. Numbers above the branches are ML bootstrap support percentages. Filled orange circles of the first, second and third columns after species names indicate presence of C$_4$ photosynthesis, serine at the position 281 and isoleucine at the position 309, respectively. The figure was composed using iTOL program [62]. doi:10.1371/journal.pone.0052974.g001

acid sites. The tree length value obtained from the model M0 was compared with tree length values obtained from other models to control for consistency among models. We performed two LRTs to compare null models which assume the same selective pressure along all branches of a phylogeny and do not allow positive selection ($d_N/d_S >1$) with nested models which do allow it [33]. The first LRT, M1a-M2a, compares the M1a model (Nearly Neutral) which allows $0\leq d_N/d_S \leq 1$ with the M2a model (Selection model; same as the M1a model plus an extra class under positive selection with $d_N/d_S >1$). The second LRT, M8a-M8, compares the M8a model which assumes a discrete beta distribution for d_N/d_S, which is constrained between 0 and 1 including a class with $d_N/d_S = 1$ with the M8 model which allows the same distribution as M8a but an extra class under positive selection with $d_N/d_S >1$.

Finally, we performed two branch-site tests of positive selection along prespecified foreground branches [33,34,35]. The first was the A model for basal C$_4$ branches only where positive selection was allowed only on branches leading to C$_4$ clades. The second was the A model for all C$_4$ branches where positive selection was allowed on branches leading to C$_4$ clades and branches within C$_4$ clades. The A1-A LRT compares the null model A1 with the nested model A. Both the A1 and A models allow d_N/d_S ratios to vary among sites and among lineages. The A1 model allows $0< d_N/d_S <1$ and $d_N/d_S = 1$ for all branches, and also two additional classes of codons with fixed $d_N/d_S = 1$ along prespecified foreground branches while restricted as $0< d_N/d_S <1$ and $d_N/$

$d_S = 1$ on background branches. The alternative A model allows $0< d_N/d_S <1$ and $d_N/d_S = 1$ for all branches, and also two additional classes of codons under positive selection with $d_N/d_S >1$ along prespecified foreground branches while restricted as $0< d_N/d_S <1$ and $d_N/d_S = 1$ on background branches. C$_4$ lineages were marked as foreground branches.

For all LRTs, the first model is a simplified version of the second, with fewer parameters, and is thus expected to provide a poorer fit to the data (lower maximum likelihood). The M1a, M8a and A1 models are null models which do not allow codons with $d_N/d_S >1$, whereas the M2a, M8 and A models are alternative models which do allow codons with $d_N/d_S >1$. The significance of the LRTs was calculated assuming that twice the difference in the log of maximum likelihood between the two models was distributed as a chi-square distribution with the degrees of freedom (df) given by the difference in the numbers of parameters in the two nested models [34,36]. For the M1a-M2a comparison df = 2, and for M8a-M8, A1-A and M0 vs 2-rates model comparisons df = 1. Each LRT was run two times using different initial d_N/d_S values (0.1 and 0.4) to test for suboptimal local peaks. To identify amino acid sites potentially under positive selection, the parameter estimates from M2a, M8 and A models were used to calculate the posterior probabilities that an amino acid belongs to a class with $d_N/d_S >1$ using the Bayes Empirical Bayes (BEB) approaches implemented in PAML [37]. Independently from *codeml* we used the SLR program which implements "sitewise likelihood-ratio" (SLR) method for detecting non-neutral evolution, a statistical test

Table 1. Analysis of the Amaranthaceae *rbcL* genes for positively selected sites.

Model with positive selection [a]				Null model [a]			LRT [d]	
	log-likelihood	Parameters [b]	Positively selected sites [c]		log-likelihood	Parameters [b]	2*l*	*P*-value
Analysis for positively selected sites common for C$_3$ and C$_4$ clades								
M2a	−10711.44	$\kappa = 3.00$, $p_0 = 0.93$, $\omega_0 = 0.02$, $p_s = 0.01$, $\omega_s = 2.62$	*32, 145, 279, 439*	M1a	−10729.19	$\kappa = 2.94$, $p_0 = 0.93$, $\omega_0 = 0.02$	35.5	<0.00001
M8	−10705.58	$\kappa = 2.94$, $p_0 = 0.96$, $p = 0.15$, $q = 3.04$, $\omega_s = 1.56$	*32,* 43, *145,* 225, 262, *279, 439,* 443	M8a	−10717.70	$\kappa = 2.90$, $p_0 = 0.94$, $p = 0.20$, $q = 5.42$	24.2	<0.00001
SLR	NA	$\kappa = 2.75$, $\omega = 0.10$	*32, 145, 225, <u>279,</u> 439*	NA	NA	NA	NA	NA
Analysis for positively selected sites specific for branches leading to C$_4$ clades								
A	−10729.13	$\kappa = 2.94$, $p_0 = 0.93$, $\omega_0 = 0.02$, $p_s = 0.00$, $\omega_s = $ NA	*no*	A1	−10729.13	$\kappa = 2.94$, $p_0 = 0.93$, $\omega_0 = 0.02$	0.0	1.00000
Analysis for positively selected sites specific for C$_4$ clades								
A	−10723.60	$\kappa = 2.94$, $p_0 = 0.92$, $\omega_0 = 0.02$, $p_s = 0.01$, $\omega_s = 3.15$	*281, 309*	A1	−10726.15	$\kappa = 2.94$, $p_0 = 0.92$, $\omega_0 = 0.02$	5.1	0.02384

[a]M1a (nearly neutral), M2a (positive selection), M8a (beta & $\omega = 1$) and M8 (beta & ω) are PAML site models; A1 and A are PAML branch site models; SLR is "sitewise likelihood-ratio" method.
[b]κ is transition/transversion rate ratio; ω is d_N/d_S ratio; ω_s is d_N/d_S ratio in a class under putative positive selection; p_0 and p_s are proportion of codons with $\omega<1$ and $\omega>1$, respectively; p and q are parameters of beta distribution in the range (0, 1); for the SLR test, the parameter values given are those optimal under M0.
[c]The sites listed are those at which positive selection is detected with a cutoff (significance level or posterior probability, as appropriate to the method used) >95%; those >99% are in italics. For the SLR test, the italic underlined sites are those at which there is still evidence for positive selection after correcting for multiple comparisons.
[d]LRT is likelihood ratio test, 2*l* is twice the difference of model log-likelihoods.
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that can identify sites under positive selection even when the strength of selection is low [38]. The SLR test [38] consists of performing a likelihood-ratio test on a sitewise basis, testing the null model (neutrality, $d_N/d_S = 1$) against an alternative model ($d_N/d_S \neq 1$). SLR method is a test of whether a given site has undergone selection or not, and the test statistic summarizes the strength of the evidence for selection rather than the strength of the selection itself [38]. The same input files with sequence alignment and species phylogeny were used for both codeml and SLR.

Analysis of correlated evolution on phylogenies

Closely related taxa are not independent data points and they consequently violate the assumptions of conventional statistical methods [39]. Thus, we used analysis of correlated evolution on phylogenies to test the significance of correlation between pairs of discrete characters: (1) the presence/absence of C₄ photosynthesis and (2) the presence/absence of particular amino-acid at sites found to be under positive selection along C₄ branches in the A model of codeml. For this purpose, we used the phylogeny obtained using RAxML (see above) and performed Pagel's test of correlated (discrete) character evolution [40] implemented in the Mesquite package (version 2.72) [41]. Test was performed separately for each Rubisco residue under positive selection along C₄ branches and Bonferroni correction was performed for simultaneous statistical testing.

Structural analysis of Rubisco

We used the published Rubisco protein structure from spinach (Spinacia oleracea, Amaranthaceae) from data file 1RBO [42] obtained from the RCSB Protein Data Bank. Throughout the paper, the numbering of Rubisco large subunit residues is based on the spinach sequence. The locations and properties of individual amino acids in the Rubisco structure were analysed using DeepView – Swiss-PdbViewer v.3.7 [43] and by CUPSAT [44].

Results

Phylogenetic analysis

The ML phylogenetic tree (Fig. 1) for rbcL sequences from 179 Amaranthaceae species was largely congruent with previously obtained phylogenies and accepted taxonomic subdivisions of the family [19,28,29,30,45,46,47,48]; however no statistical tests for topological similarity between our tree and previously published trees were performed because of different sizes and species compositions of datasets. A minimum of 16 independent origins of C₄ photosynthesis were represented in the Amaranthaceae phylogeny if conservative approach for observed polytomies had been taken (Fig. 1), which is consistent with the estimate by Sage et al. [16]. The other assumption of this estimate was that no reversals from C₄ to C₃ were allowed. Predominance of C₄ gains over reversals to C₃ is supported by both empirical data and theoretical work [49].

Tests for positive selection

Likelihood ratio tests (LRTs) for variation in d_N/d_S ratios and for positive selection [33] were applied to the dataset of rbcL sequences from 179 C₃ and C₄ Amaranthaceae species. LRTs that were run using two different initial d_N/d_S values (0.1 and 0.4) to test for suboptimal local peaks produced identical results. LRTs for positive selection [33] showed that the models assuming positive selection (M2a and M8) fit the data better than the nested models without positive selection (M1a and M8a; p-value <0.00001;

Table 1). To test whether selection occurs specifically in C₄ clades we used two branch site models (aka model A [33,34]), one of which allowed positive selection only on branches leading to C₄ clades and the other also allowed positive selection within the C₄ clades. Each of these models was compared to an alternative model that allowed for no positive selection and only the latter of the two models demonstrated better fit to data than the model without positive selection (p-value <0.05; Table 1).

Sites under positive selection

Four sites were identified as evolving under positive selection with a posterior probability >0.95 by BEB [37] implemented in the M2a model (residues 32, 145, 279, 439), but eight sites when BEB was implemented in the M8 model (all the same that in M2a plus sites 43, 225, 262, 443). Independent SLR analysis showed five sites evolving under positive selection (32, 145, 225, 279, 439), but only for one of them (site 279) evidence for positive selection remained significant after correcting for multiple comparisons. Two sites (residues 281 and 309) were shown to be under positive selection within C₄ clades while under relaxed or purifying selection within C₃ clades with a posterior probability >0.99 by BEB in the A model for C₄ branches. Both sites had only two alternative amino acids in this dataset (Table 2). One of the two alternative amino acids was more frequent among C₄ species, while the other was more frequent among C₃ species (Table 2), but there were no fixed differences between C₄ and C₃ species. We refer to amino acids more frequently associated with C₄ taxa as the 'C₄' amino acids, but only for the sake of brevity, as they are not invariantly associated with C₄ photosynthesis. Pagel's test of correlated character evolution [40] on phylogeny showed significant positive associations (p-value <0.05) between the presence of C₄ photosynthesis and the presence of 'C₄' amino acids at sites 281 and 309, shown to be under positive selection along C₄ branches.

Discussion

Widespread positive selection on Rubisco

As the performance of Rubisco can directly affect plant growth and crop yields, substantial efforts have been made to study its structure and function, with the ultimate aim of trying to improve Rubisco performance [50]. The last few years have brought new approaches to improving our understanding of Rubisco evolution and its genetic mechanisms. The initial molecular-phylogenetic analysis of rbcL showed that positive selection is widespread among all main lineages of land plants, but is restricted to a relatively small number of Rubisco amino acid residues within functionally important sites [6]. Following studies showed that rbcL is under positive selection in particular taxonomic groups [26,27,51,52,53,54,55,56]. Coevolution of residues is common in Rubisco of land plants as well as positive selection and there is an overlap between coevolving and positively selected residues [57]. Hence, phylogeny-based genetic analyses suggest there has been a constant fine-tuning of Rubisco to optimize its performance in specific conditions, in agreement with empirical observations that Rubisco enzymes from different organisms show diversity of kinetics better related to species ecology than phylogeny [4].

All eight residues shown under selection in Amaranthaceae using SLR and PAML models M2 and M8 were already shown to be under Darwinian selection in other groups of plants [6]. Five of these residues (145, 225, 262, 279 and 439) were among twenty most commonly selected Rubisco large subunit residues [6]. Findings in Amaranthaceae are in agreement with the previously described uneven distribution of putative fine-tuning residues in Rubisco [6]. Residues 43, 145, 225, 262 and 279 had only two

Table 2. Characteristics of amino-acid replacements under positive selection in the C$_4$ lineages of Amaranthaceae.

AA No.[a]	AA changes 'C$_3$'→'C$_4$'			Type of changes [b]	ΔH[c]	ΔP[d]	ΔV[e]	SA[f] (%)	ΔG[g] (kJ/mol)	RFPS (%) [h]	% C$_3$/% C$_4$ species [i]	Location of residue	Structural motifs within 5 Å	Inter-actions [j]
281	A	→	S	HN → UP	−2.6	1.1	0.4	0.00	DS (−10.6)	2.7	2.1/34.5	Helix 4	Helices 4, 5	DD
309	M	→	I	HN → HN	2.6	−0.5	3.8	8.50	S (−1.3)	19.6	0.0/16.7	Strand F	Strand E; Helices F, 5	ID

[a]Amino acid (AA) numbering is based on the spinach sequence after [63].
[b]Side chain type changes. Types abbreviations: H – hydrophobic; N – nonpolar aliphatic; P – polar uncharged; U – hydrophilic (after [64]).
[c]Hydropathicity difference [65].
[d]Polarity difference [66].
[e]van der Waals volume difference [67].
[f]Solvent accessibility calculated using the spinach structure (pdb file 1RBO) by CUPSAT [44].
[g]Overall stability of the protein predicted using the spinach structure (pdb file 1RBO) by CUPSAT [44]. DS – destabilizing, S – stabilizing.
[h]RFPS – relative frequency of the particular residue to be under positive selection in C$_3$ plants. Data from 112 *rbcL* datasets with detected positive selection from [6].
[i]Percentage of C$_3$ and C$_4$ species that have 'C$_4$' amino acid among the 95 C$_3$ species and 84 C$_4$ species of Amaranthaceae analysed.
[j]Interactions in which the selected residues and/or residues within 5 Å of them are involved. ID – intradimer interactions; DD – dimer-dimer interactions (after [63]).
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alternative amino acids in the analyzed dataset, while residues 32 and 439 had three and residue 443 had four alternative amino acids. Residue 145 is involved in dimer-dimer interactions, residue 225 is involved in interactions with small subunit, while residue 262 is involved in both [8]. C$_4$ photosynthesis has increased the availability of CO$_2$ for Rubisco in numerous independently evolved lineages of C$_4$ plants, including Amaranthaceae, driving selection for less specific but faster enzymes which have both higher $K_M(CO_2)$ and k_{cat} values [3,5,23]. In the present study, we found that model A assuming positive selection on C$_4$ branches provided a significantly better fit to the analysed Amaranthaceae dataset than the null model without selection (Table 1). We found no positive selection on branches which lead to C$_4$ clades of Amaranthaceae, but we found positive selection specific for all C$_4$ branches including branches which lead to C$_4$ clades and branches within C$_4$ clades (Table 1). This may be an argument in support of the hypothesis that C$_3$ ancestors of C$_4$ species, C$_3$–C$_4$ intermediates and C$_4$ species at the dawn of their origin have Rubisco with C$_3$ kinetics, but once C$_4$ pump is fully functional it creates a strong selective pressure for acquiring Rubisco with C$_4$ kinetics which then evolves during the stage of optimisation of C$_4$ photosynthesis [58].

Parallel amino-acid replacements in Rubisco from phylogenetically distant lineages

Bayesian analyses of *rbcL* sequences in a phylogenetic framework allowed us to identify two residues under directional selection along C$_4$ branches within Amaranthaceae (Table 2). There are no common trends in physicochemical properties of 'C$_4$' amino acids with respect to properties such as residue hydrophobicity, solvent accessibility, or location within the tertiary structure of the enzyme (Table 2). Alanine at the position 281 was replaced by serine at least eleven times within the studied species with nine of replacements taking place within C$_4$ clades and two replacements in C$_3$ species *Chenopodium bonus-henricus* and *Spinacia oleracea* (Fig. 1). Methionine at the position 309 was replaced by isoleucine at least four times, all of which within C$_4$ clades (Fig. 1). Only three C$_4$ species, *Atriplex spongiosa*, *A. rosea* and *Horaninovia ulicina*, had both 'C$_4$' amino acids simultaniously. Seven C$_4$ clades of which one was monospecific had 'C$_4$' amino acids, while nine C$_4$ clades of which six consisted of only one species did not have 'C$_4$' amino acids (Fig. 1). More frequent occurrence of 'C$_4$' amino acids in clades

consisting of many species compared to monospecific clades corresponds to our findings of stronger positive selection within C$_4$ clades (Table 1).

Interestingly, both selected residues in C$_4$ Amaranthaceae are among the eight residues selected in C$_4$ Cyperaceae and Poaceae [26] and the 'C$_4$' amino acid 309I is also among selected in C$_4$ *Flaveria* [27]. None of the 'C$_4$' amino acids is fixed among C$_4$ species, but they are more frequent among C$_4$ lineages, ranging from 17 to 35% in C$_4$ Amaranthaceae, and from 14 to 87% in C$_4$ Cyperaceae and Poaceae (Table 2; percentage for C$_4$ Cyperaceae and Poaceae calculated using numbers from [26]). Although 'C$_4$' amino acids are not fixed among all C$_4$ species, there is a significant positive association between their presence and C$_4$ photosynthetic type in Amaranthaceae. Given the existence of C$_4$ species without 'C$_4$' amino acids , it is likely that other as yet unidentified amino acids replacements may be involved in Rubisco adaptation. The model of sequence evolution used to identify Rubisco residues under positive selection within C$_4$ lineages averages selective pressure among selected branches (C$_4$ branches in our case) and hence allows detection only of the most typical substitutions, potentially missing ones that are unique for a particular branch. Other possible explanations are variation in Rubisco kinetic properties not only between C$_3$ and C$_4$ groups of species but also within these groups [3,4,5,23] and putative differences in other proteins which form the Rubisco complex (small subunit, Rubisco activase). Although the large subunits contain active sites, changes in small subunits may make significant contribution to kinetic properties of plant and algal Rubiscos [59], including differences observed between C$_3$ and C$_4$ plants [60], and the *rbcS* genes encoding small subunits have been shown under positive selection in C$_4$ *Flaveria* [27].

Identical amino-acids in Rubisco of C$_4$ Amaranthaceae and C$_4$ Cyperaceae and Poaceae, representing eudicots and monocots with significantly different anatomy and ecological preferences [22], constitute a remarkable example of parallel molecular evolution in phylogenetically distant groups. This example becomes even more interesting if C$_3$ plants are considered as well. Various groups of C$_3$ plants such as some aquatic species and C$_3$ species from cold habitats have faster but less CO$_2$-specific Rubisco compared with their C$_3$ relatives from terrestrial and warm conditions, respectively [3,23]. Hence, some groups of C$_3$ plants can arrive at the same evolutionary solutions for Rubisco fine-tuning as C$_4$ plants. Indeed, 'C$_4$' amino acids shown for C$_4$

Amaranthaceae in the present study and for C$_4$ monocots and *Flaveria* previously [26,27], have been reported to be under positive selection in various groups of C$_3$ plants by Kapralov and Filatov [6]. Moreover, residue 309 is among the most frequently positively selected sites in land plants, and although residue 281 itself is not, its close neighbours, residues 279 and 282, are among the most often positively selected ones [6]. Thus, we can conclude that both 'C$_4$' amino acids, 281S and 309I, evolved in parallel in various phylogenetically distant lineages of C$_3$ and C$_4$ plants in which faster but less specific Rubisco was needed.

The residue 309 is located on the interface of large subunits within a large subunit dimer, while the residue 281 is involved into dimer-dimer interactions (Table 2). Methionine at position 309 is replaced by the smaller and more hydrophobic isoleucine, which has a stabilising and favourable effect on overall molecule stability according to CUPSAT calculations using spinach pdb-structure [44], while A281S replacement decreases hydrophobicy and may be destabilising (Table 2).

Effects of A281S replacement on kinetics of land plants Rubisco has not been studied, while recent study by Whitney et al. [61] using mutagenic approach showed that M309I replacement in *Flaveria* changed Rubisco kinetics from "C$_3$-like" to "C$_4$-like" making the enzyme faster but less CO$_2$-specific. Importance of M309I replacement for changes in kinetics of *Flaveria* Rubisco was predicted using *in silico* approach similar to one used in the present study [27] and confirmed *in planta* by the study of Whitney et al. [61] making it a good case in support of further application of phylogeny-based methods for detecting residues under positive selection in Rubisco and elsewhere.

Towards the periodic table of functional amino-acid replacements in Rubisco

Continuing population growth creating increasing demand for food, coupled with future climate change and its potentially dire consequences such as biome collapse and crop failure, both call for an improved understanding of mechanisms allowing plant species to adapt the photosynthetic process to a wide range of conditions. Hence, there is a necessity for more phylogeny-based studies of genes encoding Rubisco from various lineages of phototrophs established in different conditions to better understand Rubisco evolution at the molecular level. The integration of phylogenetic and biochemical research is required to study how Darwinian selection has created a range of enzymes with different kinetic and physical properties tailored to function in virtually all ecosystems on our planet. Knowledge of the role of specific residues in Rubisco adaptation to the particular conditions may provide clues for engineering better enzymes suited to contemporary agricultural needs as well as helping to understand what modifications in the enzyme may have been (and perhaps will be) driven by adaptation to different environmental conditions.

Supporting Information

Acknowledgments

Author Contributions

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