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Environmentally driven evolution of Rubisco and improved photosynthesis and growth within the C3 genus Limonium (Plumbaginaceae)

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Summary

- Carbon assimilation by most ecosystems requires ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Its kinetic parameters are likely to have evolved in parallel with intracellular CO2 availability, with the result that faster forms of Rubisco occur in species with CO2-concentrating mechanisms.
- The Rubisco catalytic properties were determined and evaluated in relation to growth and carbon assimilation capacity in Mediterranean Limonium species, inhabiting severe stress environments.
- Significant kinetic differences between closely related species depended on two amino acid substitutions at functionally important residues 309 and 328 within the Rubisco large subunit. The Rubisco of species facing the largest CO2 restrictions during drought had relatively high affinity for CO2 (low Michaelis–Menten constant for CO2 ($K_c$)) but low maximum rates of carboxylation ($V_{\text{max}}$), while the opposite was found for species that maintained higher CO2 concentrations under similar conditions. Rubisco kinetic characteristics were correlated with photosynthetic rate in both well-watered and drought-stressed plants. Moreover, the drought-mediated decrease in plant biomass accumulation was consistently lower in species with higher Rubisco carboxylase catalytic efficiency ($k_{\text{cat}}/K_c$).
- The present study is the first demonstration of Rubisco adaptation during species diversification within closely related C3 plants, revealing a direct relationship between Rubisco molecular evolution and the biomass accumulation of closely related species subjected to unfavourable conditions.

Introduction

The photosynthetic processes that are most vulnerable to high temperature and drought remain unclear and somewhat controversial (Salvucci & Crafts-Brandner, 2004; Schrader et al., 2004; Yamori et al., 2010). However, factors related to the performance of a key photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), undoubtedly contribute to the restricted yields associated with elevated temperature and water deficit (Sharkey, 2005; Sage & Kubien, 2007). Rubisco provides a ubiquitous gateway for the entry of inorganic carbon (as CO2) into plant metabolism, yet cannot fully discriminate between CO2 and O2 as it catalyses the chemical combination of either gaseous substrate with an enediolate form of the Calvin cycle intermediate ribulose-1,5-bisphosphate (RuBP). The oxygenation of RuBP gives rise to the energy-dissipating process of photorespiration, and increases steeply in extent with increasing temperature and water stress, reducing plant productivity.

Significant progress has been made in understanding the components that determine the fundamental properties of Rubisco at a molecular level (Spreitzer & Salvucci, 2002; Parry et al., 2013), such as the driving forces behind the evolutionary adaptation of Rubisco (Raven, 2000; Tcherkez et al., 2006), the trade-offs involved in determining the magnitude of its kinetic parameters (Savir et al., 2010; Tcherkez, 2013; Galmés et al., 2014), and the underlying structural dynamics (Bracher et al., 2011; van Lun et al., 2011, 2014). Strong evidence for widespread amino acid sequence coevolution and for the role of positive selection in the evolutionary fine-tuning of Rubisco from both C3 and C4 plants has been reported in recent phylogenetic studies (Kapralov & Filatov, 2007; Christin et al., 2008; Kapralov et al., 2011, 2012; Wang et al., 2011; Young et al., 2012). Genetic transformation of Chlamydomonas has helped to elucidate the roles of particular Rubisco domains (Meyer et al., 2012; Esquivel et al., 2013), while new molecular tools have facilitated the modification of Rubisco in planta, enabling the effect of specific amino acid
substitutions on Rubisco kinetics to be evaluated (Whitney et al., 2011). However, there are still insufficient data to establish the way in which the kinetic properties of Rubisco impact upon the capacity for CO₂ assimilation and the overall plant carbon balance under challenging environmental conditions.

Extreme environments are particularly useful in the study of adaptive evolution in plants. Intense selection pressure not only increases the frequency of adaptation but can also lead to the emergence of alternative survival strategies (Agrawal et al., 2009; Adler et al., 2014). Species of the genus Limonium Mill. (Plumbaginaceae, Caryophyllales) inhabit Mediterranean coastal habitats, which are characterized by low soil osmotic potential as a result of negative water balance and the continuous influx of salt from marine spray. Low atmospheric and soil water potentials require strict control of water loss through transpiration, by means of stomatal closure. Diminished stomatal conductance (gₛ) is often accompanied by decreased leaf mesophyll conductance to CO₂ (gₘ), which in turn limits the availability of CO₂ to Rubisco in the chloroplast stroma, favouring RuBP oxygenation (Flexas et al., 2013). The high ambient temperatures and elevated solar irradiance typical of these xeric environments further increase photorespiration, which may compromise plant carbon balance and, ultimately, survival.

Here we integrate data on Rubisco kinetics, leaf photosynthesis and plant growth under irrigated and severe drought conditions for a number of species of the genus Limonium. Limonium species from the Balearic Islands are an ideal model system in which to study the link between drought tolerance and Rubisco kinetics for several reasons. First, the genus Limonium, with up to 300 species, represents one of the largest radiations in the Mediterranean Basin (Erben, 1993). The Balearic Islands are home to 42 Limonium species, most of them being endemics with a narrow distribution (Erben, 1993; Rosselló & Sáez, 2001) that have evolved as a recent radiation (reviewed in Lledó et al., 2005). Secondly, drought-tolerant Limonium species have adapted to the hostile environments of coastal margins (Galmés et al., 2011a), where biotic competition is virtually absent as a result of extreme abiotic stress factors such as lack of fresh water, high temperature and excess solar radiation. Finally, there is significant diversity in Rubisco kinetics among Limonium, with Limonium gibraltarii (Sennen) Sennen having among the highest specificities for CO₂ reported in terrestrial plants (Galmés et al., 2005). Furthermore, biochemical modelling predicts that significant yield increases would ensue if L. gibraltarii Rubisco were to replace the native wheat counterpart (Parry et al., 2011).

In the present work, we sampled all Limonium species inhabiting the Balearic Islands and assessed the amino acid sequence polymorphisms in the Rubisco large subunit (L-subunit). Full kinetic characterization of Rubisco was performed for 14 of these species, representing all of the amino acid polymorphisms found. Further, photosynthetic performance and plant growth were assessed for these species under well-watered (WW) and severe water stress (WS) conditions. Our results demonstrate a correlation between drought tolerance, photosynthetic performance and Rubisco catalytic efficiency. We also identified two L-subunit amino acid substitutions that were probably responsible for changes in Rubisco kinetic properties, and assessed the ecological success of Limonium species with different L-subunits.

Materials and Methods

Species sampling
All cited and described Limonium species reliably recognized in the Balearic Islands (Erben, 1993; Rosselló & Sáez, 2001) were included in the survey, resulting in sampling plants from 47 populations which represented 42 Limonium species (Fig. 1). Several populations were sampled for species occurring in different islands, occurring in extremes of an island, or showing notorious differences in plant morphology. The locations of sampled populations are shown in Fig. 1. In addition to the 47 populations of Limonium, we also sampled Myriolimon ferulaceum (L.) Lledó, Erben & M.B. Crespo, the monotypic genus sister to Limonium, which served as an outgroup. Fresh leaf tissue and seeds from five plants per population were sampled in the field and dried in silica gel for further DNA sequencing and the drought experiment, respectively.

rbcL sequences and phylogenetic analyses
Total DNA was extracted from fresh leaves using the DNeasy Plant MiniKit (Qiagen). The rbcL gene coding for the Rubisco L-subunit was amplified and sequenced using the forward primer rbcL1F (5’-ATGTCACCAAAAACAGAAT-3’) and a newly designed reverse primer (5’-AGTATCCATTGCGGCGAAT-3’), following the protocol in Kapralov & Filatov (2006). All polymorphic sites were checked against the original sequence chromatograms and doubtful regions were re-sequenced; sequences were compared with homologues from GenBank, and open reading frame (ORF) integrity was confirmed. Novel sequences have been submitted to GenBank under accession numbers KJ608007–KJ608054. Sequences were aligned and phylogenetic reconstructions were performed using maximum likelihood (ML) and maximum parsimony (MP) algorithms, both with 1000 bootstrap resamplings, implemented in MEGA 5.1 (Tamura et al., 2011), and Bayesian analyses (Markov chain Monte Carlo method), with 10⁶ bootstrap generations, implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The trees were rooted using M. ferulaceum. All methods produced trees with identical topology but minor differences in bootstrap values.

Identification of Rubisco residues under positive selection
We analysed variation in the ratio of nonsynonymous to synonymous substitution rates (dₚ/dₛ) across rbcL codons and/or particular branches of phylogenies using models M8 and A from the codeml program of the PAML package v.4 (Yang, 1997, 2007). Posterior probabilities that a certain amino acid belongs to a class with dₛ/dₚ > 1 and hence is under positive selection were calculated using the Bayes Empirical Bayes approaches implemented in PAML (Yang et al., 2005). Because all Balearic Limonium rbcL sequences were clustered into three groups without
nonsynonymous variation within them, we only used one sequence per group plus the sequence of *M. ferulaceum*. One needs at least seven sequences or preferably more to overcome the problem of false negatives in *codeml* (Anisimova *et al.*, 2001). Therefore, in addition to four *rbcL* sequences representing Balearic Plumbaginaceae, we used seven *rbcL* sequences from GenBank which represented Plumbaginaceae species occurring in communities of drought-resistant plants in regions with a Mediterranean climate similar to that experienced by Balearic *Limonium* species (Kubitzki *et al.*, 1993): *Armeria bottendorfensis* A. Schulz (Z97640), *Armeria splendens* Boiss. (Y16908), *Limoniastrum monopetalum* Boiss. (Z97642), *Limonium sinuatum* (L.) Mill. (Y16900), *Limonium spectabile* (Svent.) G. Kunkel & Sunding (Z97646), *Plumbago auriculata* Lam. (EU002283) and *Plumbago capensis* Thunb. (Y16906). Using such a broad outgroup also allowed the establishment of ancestral states for polymorphic amino acid residues. *PAML* analyses were run on an unrooted phylogeny (Fig. 2a; spinach (*Spinacia oleracea* L.) was not included in the analyses) which was reconstructed using ML and only synonymous sites to avoid distortion caused by possible positive selection on *rbcL* (Kapralov & Filatov, 2007). Models *M8* and *A* assumed selection across all branches and selected foreground branches only, respectively. Branches leading to all Balearic *Limonium* haplotypes and to derived haplotypes II and III only were selected as foreground branches in two separate tests for positive selection allowing *dN/dS* ratios to vary both among sites and among lineages.

**Drought treatment and growth analysis**

Based on the three different Rubisco L-subunit haplotypes detected within *Limonium*, the following 14 species were selected for the drought experiment: *Limonium balearicum*, *Limonium barceloi*, *Limonium companyonis* and *Limonium echiloides* representing haplotype I; *Limonium ejulabilis*, *Limonium grossii*, *Limonium leonardi-lorensii*, *Limonium magallufianum* and *Limonium retusum* representing haplotype II; *Limonium antonii-lorensii*, *Limonium atruchium*, *Limonium biforum*, *Limonium giberti* and *Limonium gymnesicum* representing haplotype III. Populations used for each species and their
locations are indicated in Fig. 1 (including species authorities). In these populations, seeds were collected from several plants. Thereafter, 10 plants per species were grown from seeds outdoors in 3-l pots with water supplied at field capacity under typical Mediterranean summer conditions. Three months after germination, and to the end of the experiment, five plants per species were subjected to severe water stress by maintaining the pots at 10–30% of field capacity (WS), whereas the remaining plants were maintained at field capacity (WW). Pots were weighed every 2–3 d to enable replacement of lost water. Three months after drought treatment application, plants were harvested to obtain total plant biomass.

Leaf gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange and chlorophyll fluorescence were measured in all species and treatments included in the drought experiment except for *L. artruchium* and *L. gymnesicum*, because of their small leaves. Measurements were performed 2 months after the onset of the drought treatment, that is, in leaves completely developed under treatment. The net CO₂ assimilation rate (Aₜₚ) was measured at mid-morning in fully expanded leaves using a gas-exchange analyser equipped with a leaf chamber fluorometer (Li-6400-40; Li-Cor Inc., Lincoln, NE, USA). Leaf chamber conditions consisted

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**Fig. 2** *Limonium* Rubisco large subunit (L-subunit) sequence variation and structural location of polymorphic residues. (a) Maximum likelihood tree inferred from the *rbcL* gene coding for the L-subunit for Plumbaginaceae species used in the analyses of positive selection. *Spinacia oleracea* was added as an outgroup and for comparative purposes; it was not included in the positive selection analysis. Bootstrap values (from 1000 replicates) are indicated at the nodes. Amino acid polymorphism of six Rubisco L-subunit residues that varied in Balearic *Limonium* is shown on the right. Residue numbers are shown above the table. Residues identical to those of the first sequence are shown as dots. (b) Location of the six polymorphic Rubisco L-subunit residues shown on the structure of spinach Rubisco (1UPM). Two L-subunits forming a functional dimer are highlighted in green and cyan. Residues forming two active sites are shown in orange; six polymorphic residues are shown as green and cyan spheres for L-subunits B and L, respectively.
of a photosynthetic photon flux density of 1500 μmol m⁻² s⁻¹ (with 10% blue light to induce stomatal aperture) and an ambient CO₂ concentration of 400 μmol mol⁻¹. Leaf temperature during measurements was maintained at 25°C.

The quantum efficiency of the photosystem II (PSII)-driven electron transport was determined using the equation:

$$\Phi_{\text{PSII}} = \frac{F'_m - F_s}{F'_m}$$  \hspace{1cm} \text{Eqn 1}

($F_s$, the steady-state fluorescence in the light; $F'_m$, the maximum fluorescence obtained with a light-saturating pulse (10 000 μmol quanta m⁻² s⁻¹) (Genty et al., 1989)). As $\Phi_{\text{PSII}}$ represents the number of electrons transferred per photon absorbed by PSII, the rate of electron transport (ETR) can be calculated as:

$$\text{ETR} = \Phi_{\text{PSII}} \cdot \text{PPFD} \cdot \alpha \cdot \beta$$  \hspace{1cm} \text{Eqn 2}

($\alpha$, the leaf absorbance; $\beta$, the distribution of absorbed energy between the two photosystems). The product $\alpha \cdot \beta$ was determined from the relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{CO₂}}$, obtained by varying light intensity under nonphotorespiratory conditions in a nitrogen atmosphere containing <1% (v/v) O₂ (Valentini et al., 1995).

$\Phi_{\text{CO₂}}$ was calculated from gas-exchange measurements as $(A_N + R_l)/\text{PPFD}$, assuming that the rate of nonphotorespiratory CO₂ evolution in the light ($R_l$) was half of the dark mitochondrial respiration rate, $R_{\text{dark}}$ (Galmés et al., 2011b). $R_{\text{dark}}$ was measured at pre-dawn at an ambient CO₂ concentration ($C_a$) of 400 μmol mol⁻¹ and an air temperature of 25°C. The gross CO₂ assimilation rate ($A_g$) was obtained as $A_N + R_l$.

From combined gas-exchange and chlorophyll $a$ fluorescence measurements, mesophyll conductance for CO₂ ($g_m$) was estimated according to Harley et al. (1992) as:

$$g_m = A_N/(C_g - (\Gamma^* \times (\text{ETR} + 8 \times (A_N + R_l))))/(\text{ETR} - 4 \times (A_N + R_l)))$$  \hspace{1cm} \text{Eqn 3}

The chloroplast CO₂ compensation point ($\Gamma^*$) was calculated from the in vitro Rubisco specificity factor ($S_{\text{cha}}$) values for each species as:

$$\Gamma^* = 0.50/S_{\text{cha}}$$  \hspace{1cm} \text{Eqn 4}

($O$, the partial pressure of oxygen concentration in air (200 mbar)). Finally, from $g_m$ estimates, the substomatal CO₂ concentration ($C_i$) values were transformed into chloroplastic CO₂ concentration ($C_g$) as:

$$C_g = C_i - (A_N/g_m)$$  \hspace{1cm} \text{Eqn 5}

**Rubisco catalytic characterization**

Rubisco kinetic parameters were measured in the 14 species used in the growth experiment. Rates of Rubisco $^{14}$CO₂ fixation using fresh leaf protein extract were measured in 7-ml septum-capped scintillation vials, containing reaction buffer (yielding final concentrations of 100 mM Bicine-NaOH, pH 8.0, 20 mM MgCl₂, 0.4 mM RuBP and c. 100 W-A units of carbonic anhydrase) and one of nine different concentrations of CO₂ (0–80 μM, each with a specific radioactivity of 3.7 × 10⁻¹⁰ Bq mol⁻¹), each at two concentrations of O₂ (0 and 21% (v/v)), as described previously (Parry et al., 2007). Assays (1.0 ml total volume) were started by the addition of activated leaf extract, and the maximum velocity for carboxylase activity ($V_{\text{max}}$) together with the Michaelis–Menten constant ($K_{\text{m}}$) for CO₂ ($K_C$) determined from the fitted data. The $K_a$ for the oxygenase activity was calculated from the relationship $K_{a}(21\%O_2) = K_{a}(0\%O_2) \times (1 + [O_2]/K_a)$. The [O₂] was assumed to be 265 μM, but corrected for partial pressure by taking account of the atmospheric pressure and water saturated vapour pressure. Replicate measurements ($n = 3–5$) were made using protein preparations from two to 10 different leaves of different individuals. For each sample, the maximum rate of carboxylation ($k_{\text{cat}}$) was extrapolated from the corresponding $V_{\text{max}}$ value after allowance was made for the Rubisco active site concentration, as determined by [¹⁴C]CPBP binding (Yokota & Canvin, 1985). Rubisco CO₂/O₂ specificity ($S_{\text{cha}}$) was measured as described previously (Galmés et al., 2005) using enzyme purified by polyethylene glycol (PEG) precipitation and ion exchange chromatography, and the values given for each species were the mean of five to 10 repeated determinations. The maximum oxygenation rate ($k_{\text{cat}}/K_a$) was calculated using the equation $S_{\text{cha}} = (k_{\text{cat}}/K_a)/(k_{\text{cat}}/K_o)$. All kinetic measurements were performed at 25°C.

**Results**

The molecular evolution of Rubisco in Balearic Limonium species

The 42 Limonium species (47 populations; Fig. 1) from the Balearic Islands possessed three Rubisco L-subunit amino acid haplotypes (haplotypes I, II and III) occurring in similar island habitats. Haplotype III, found in 29 species, was by far the most frequent in geographical distribution, while haplotypes I and II were found only in five and eight species, respectively (Fig. 1 and Supporting Information Fig. S1). The differences among haplotypes corresponded to polymorphisms at six L-subunit amino acid residues (Fig. 2a). Haplotypes I and II differed in four positions: F10Y, I309M, S328A and D340E; while haplotypes I and III differed in five positions: N95S, G245A, I309M, S328A and D340E (Fig. 2a). Phylogenetically, haplotype I appeared more basal than haplotypes II and III, when eight Plumbaginaceae species were included as the outgroup (Figs 2a, S1).

The implementing of Bayes Empirical Bayes analysis (Yang et al., 2005) revealed that residues 309 and 328 were under positive selection along the branch leading to the Balearic Limonium haplotypes II and III, with posterior probabilities exceeding 0.95 (Fig. 2a). Amino acid substitutions I309M and S328A were unique to haplotypes II and III. No residues were shown to be under positive selection along the branch leading to all Balearic Limonium haplotypes (Fig. 2a). Site-specific tests revealed that residue 340 was under positive selection across all
investigated Plumbaginaceae, with a posterior probability of 0.93 (Fig. 2a).

Reconstruction of ancestral sequences for interior nodes was reliable; mean posterior probabilities for the entire protein exceeded 99% for all nodes. It confirmed that amino acid substitutions I309M and S328A occurred only once within analysed phylogeny (Fig. 2a) along the branch leading to the Balearic Limonium haplotypes II and III. Amino acid substitution G245A also occurred only once along the branch leading to the Balearic Limonium haplotype III. However, three other amino acid substitutions within the Balearic Limonium were not unique to this group. Parallel substitution F10Y occurred three times: along the branch leading to Armeria species, along the branch leading to L. spectabile and L. sinuatum, and also along the branch leading to the Balearic Limonium haplotype II. Parallel substitution N95S occurred twice along the branch leading to Plumbago species and the branch leading to the Balearic Limonium haplotype III. Parallel substitution E340D occurred four times: along the branch leading to the Balearic Limonium haplotype I, along the branch leading to L. sinuatum, along the branch leading to L. monopetalum and along the branch leading to the Plumbago species.

Species with different L-subunit haplotypes have different Rubisco kinetics

In order to investigate the impact of L-subunit substitutions on the catalytic properties of Rubisco, full kinetic characterization was undertaken for four to five species per haplotype (Table 1).

Significant differences were observed between species. The Michaelis–Menten constant (Kc) for CO2 (Kc) varied between 7.0 μM for L. magallufianum and 10.7 μM for L. echioides (Table 1). The species with the lowest Michaelis–Menten constant for O2 (Kc) was L. magallufianum (297 μM), while the highest values were measured in L. leonardi-llorensii (438 μM). The maximum rate of carboxylation (kcat) varied 2-fold, with the lowest values measured in L. eulobus (2.0 s⁻¹) and the highest in L. echioides (3.9 s⁻¹). Less variation was found in the carboxylation-catalytic efficiency (kcat/Kc), indicative of the inverse correlation between the rate of carboxylation and the affinity for CO2. The Rubisco specificity factor (Sc) ranged between 103.3 mol mol⁻¹ in L. balearicum and 121.2 mol mol⁻¹ in L. gymnemicum (Table 1).

Significant kinetic differences were also found between the three haplotypes (Table 1), suggesting that the observed amino acid substitutions had catalytic consequences. Haplotype I Rubisco was faster (higher kcat), but less specific for CO2 (higher Kc and lower Sc) compared with haplotypes II and III (Table 1). Based on its higher value for kcat/Kc, haplotype I represented superior carboxylation efficiency.

Highly significant relationships were found between the maximum rates of carboxylation (kcat) and oxygenation (ko) (Table 1; ñ² = 0.67; P < 0.001), as well as between the carboxylase (kcat/Kc) and the oxygenase (ko/Kc) catalytic efficiencies (ñ² = 0.98; P < 0.001). The greater kcat and Kc values of haplotype I than of haplotypes II and III were paralleled by greater catalytic efficiencies (kcat/Kc) for both the carboxylase and oxygenase reactions (Table 1). However, the relative change in catalytic efficiency for

### Table 1 Comparison of Rubisco kinetics at 25°C for 14 species of Limonium, representing the three haplotypes identified

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Species</th>
<th>Kc (μM)</th>
<th>Kc (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Kc (s⁻¹)</th>
<th>Sc (mol mol⁻¹)</th>
<th>kcat (s⁻¹)</th>
<th>ko/Kc (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>L. balearicum</td>
<td>277 ± 3</td>
<td>346 ± 19</td>
<td>3.6 ± 0.1</td>
<td>0.38 ± 0.04</td>
<td>103.3 ± 1.5</td>
<td>1.3</td>
<td>4.6</td>
</tr>
<tr>
<td>L. barceloi</td>
<td>93.9 ± 0.6</td>
<td>346 ± 17</td>
<td>3.4 ± 0.1</td>
<td>0.37 ± 0.03</td>
<td>106.3 ± 2.0</td>
<td>1.2</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>L. companynis</td>
<td>8.9 ± 0.3</td>
<td>429 ± 28</td>
<td>3.3 ± 0.2</td>
<td>0.37 ± 0.01</td>
<td>111.8 ± 2.1</td>
<td>1.4</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>L. echioides</td>
<td>10.7 ± 0.2</td>
<td>427 ± 38</td>
<td>3.9 ± 0.1</td>
<td>0.37 ± 0.01</td>
<td>106.4 ± 1.8</td>
<td>1.5</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>9.7 ± 0.2</td>
<td>390 ± 18</td>
<td>3.6 ± 0.1</td>
<td>0.37 ± 0.01</td>
<td>106.7 ± 1.1</td>
<td>1.4 ± 0.1</td>
<td>4.4 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Rubisco kinetic parameters describe the Michaelis–Menten constants for CO2 (Kc) and O2 (ko), maximum rate of carboxylation (kcat) and oxygenation (ko), carboxylation catalytic efficiency (kcat/Kc), specificity factor (Sc), maximum rate of oxygenation (ko) and oxygenation catalytic efficiency (ko/Kc). Data are means ± SE of 3–10 replicates per species (n = 13–37 replicates per haplotype). Different lowercase and uppercase letters denote statistically significant differences by Duncan analysis (P < 0.05) among species and haplotypes, respectively. The maximum rate of oxygenation (ko) was calculated using the equation Sc = (kcat/Kc)/ko/Kc.
the oxygenase activity always exceeded that for the carboxylase activity, leading to a decline in $S_{\text{c/o}}$. Hence, $k_{\text{cat}}$ was negatively correlated with the affinity for CO$_2$ (i.e. $1/K_c$) and $S_{\text{c/o}}$ (Fig. 3). In other words, slower *Limonium* Rubiscos have increased specificity for CO$_2$. Overall, the kinetic data presented in this study are in agreement with the kinetic interrelationships described in the analysis of Savir *et al.* (2010).

The diversity in Rubisco kinetics was related to the availability of CO$_2$ and influenced the photosynthetic rate and biomass accumulation

The concentration of CO$_2$ in the chloroplast ($C_c$), estimated from *in vivo* measurements of leaf photosynthesis, was affected by water availability in most, but not all the species. Hence, the drought-induced decrease in $C_c$ between WW and WS plants ($C_{c\text{WW}} - C_{c\text{WS}}$) was bigger in species with haplotypes II and III, and smaller in species with haplotype I Rubiscos (Fig. 4). Note that $C_{c\text{WW}} - C_{c\text{WS}}$ correlated negatively with $k_{\text{cat}}$ ($r^2 = 0.67$; $P < 0.001$) and positively with $S_{\text{c/o}}$ ($r^2 = 0.72$; $P < 0.001$). Hence, species with haplotype I Rubisco, which showed the smallest change in $C_c$ between WW and WS plants, had higher $k_{\text{cat}}$ and lower $S_{\text{c/o}}$.

The application of severe drought stress affected both the photosynthetic capacity and biomass accumulation ($B$) in a species-specific manner (Fig. 5). Within each irrigation treatment, the variability in the light-saturated, gross CO$_2$ assimilation rate ($A_G$) among species correlated with differences in Rubisco kinetics. Hence, species with higher $A_G$ had Rubisco with higher $k_{\text{cat}}$ under both WW and WS (Fig. 5a). In agreement with the trade-off between $k_{\text{cat}}$ and $S_{\text{c/o}}$ (Fig. 3a), $A_G$ was inversely correlated to $S_{\text{c/o}}$ (Fig. 5b). Under WW conditions, the observed trend in the relationship between $B$ and the same Rubisco kinetic parameters was the inverse of that with $A_G$, showing a negative correlation with $k_{\text{cat}}$ and a positive correlation with $S_{\text{c/o}}$ (Fig. 5c,d, respectively). However, the correlation between $A_G$ and $B$ was not significant, either under control or under stress conditions. This apparent discrepancy serves to illustrate that isolated CO$_2$ exchange rates measured on individual, light-saturated leaves are not necessarily representative of the carbon flux at either whole-plant or canopy scales over the applicable growth period (120 d), particularly in plants with dense cushion-like architecture and considerable self-shading.
The Rubisco carboxylase catalytic efficiency ($k_{\text{cat}}^c/K_c$) has frequently been referred to as an indicator of enzyme performance. There was a positive correlation between $k_{\text{cat}}^c/K_c$ and the ratio of plant biomass under WS and WW conditions (Fig. 6), suggesting that the detrimental effects of drought on plant carbon gain were less in those species with higher $k_{\text{cat}}^c/K_c$.

**Discussion**

The present study shows that the stressful environments of the Mediterranean coast facilitated diversification of Rubisco within the genus *Limonium*, giving rise to three different Rubisco haplotypes coexisting in the Balearic Islands. Significantly different Rubisco kinetic properties among haplotypes were related to the differences in the concentration of CO2 within the chloroplast stroma, and correlated not only with the photosynthetic CO2 assimilation rate, but also with the response of plant growth to severe water stress. Rubisco L-subunit amino acid substitutions at positions 309 and 328 are likely to be responsible for the observed differences in kinetic properties, leading either to more CO2-specific (high $k_{\text{cat}}^c$) enzymes, or to less CO2-specific (high $K_c$) but faster (high $k_{\text{cat}}^c$) forms of Rubisco. Overall, this work represents the first integrative approach linking natural polymorphisms occurring in Rubisco, its kinetic parameters, *in vivo* leaf photosynthesis and whole-plant growth, under specific environmental conditions. Three of the observed amino acid substitutions were unique to the Balearic *Limonium* species, while the other three occurred as parallel substitutions along other branches too. The presence of the parallel amino acid substitutions is not surprising, given that all included Plumbaginaceae are drought-resistant species from Mediterranean climates with relatively similar genetic backgrounds. However, the existence of six polymorphic residues, three of which are unique to the western Mediterranean *Limonium* species, is surprisingly high.
given the conservative nature of Rubisco and the short period of divergence of Balearic Limonium species. Complete plastome replacements via interspecific introgression have been documented for numerous plant groups (reviewed in Avise, 2004), and strong positive selection acting on chloroplast genes may facilitate this process. Interspecific hybridization is common in the Balearic Limonium species (Erben, 1993) resulting in cytonuclear discordance (M. A. Conesa et al., unpublished data). Positive selection acting on the rbcL gene might have caused the three observed haplotypes to spread across all Balearic Limonium species. A similar situation was described for another island group, the Hawaiian Schiedea (Kapralov & Filatov, 2006), showing that evolution in small populations within confined geographical regions, such as islands, might be facilitated by the sharing of adaptive mutations by several closely related species.

Unlike Flaveria (Kubien et al., 2008), which contains C₃ and C₄ species, all Limonium species are strictly C₃, and therefore the presence of a carbon-concentrating mechanism cannot be the factor driving Rubisco diversification, although there could be significant variation in C₃ between C₃ plants (see below). Two amino acid substitutions at positions 309 and 328 were under positive selection along the branch leading to the Limonium species with derived haplotypes II and III, and gave rise to different Rubisco kinetics compared with the basal haplotype I. Phylogenetic analyses of the chloroplast rbcL gene encoding the Rubisco L-subunit showed that residues 309 and 328 were among the most frequently subjected to positive selection in both C₃ and C₄ plants (Kapralov & Filatov, 2007; Christin et al., 2008; Kapralov et al., 2011, 2012). Hybrid Rubisco assembling in tobacco (Nicotiana tabacum) plastids established that substitution M309Ile acted as the catalytic switch that caused the kinetic differences (similar to those reported here) between C₃ (309Met) and C₄ (309Ile) Flaveria species (Whitney et al., 2011). Residue 309 is at the C-terminal end of a short β-strand (βF) at the C-terminal side of the α/β-barrel between strand 5 and helix 5 of the barrel (Fig. 2b, Table S1). This location places residue 309 on the interface of L-subunits forming a catalytic L₂ dimer. In the Limonium species with haplotype I (higher k₉cat), residue 309 is Ile, but in haplotypes II and III (lower k₉cat), it is replaced by the larger and less hydrophobic 309Met (Table S1). Residue 328 is located within the loop-6 region of the α/β-barrel, which is close to the active site and has been shown to be important for catalysis and specificity (Chen & Spreitzer, 1989; Parry et al., 1992). In the Limonium species with haplotypes II and III, polar and uncharged 328Ser was replaced by the nonpolar and hydrophobic 328Ala (Table S1) – a change that in other species was accompanied by an increase in S₁₀ (Chen & Spreitzer, 1989; Parry et al., 1992).

The finding that haplotype I Rubiscos (309Ile and 328Ser) had higher k₉cat (Table 1), albeit a lower affinity for CO₂ (i.e. lower S₁₀ and higher Kᵣ) compared with the other haplotypes, is in agreement (1) with the observation of frequent positive selection at these sites (Kapralov & Filatov, 2007); and (2) with the fact that 309Ile and 328Ser are found in many (although not all) C₄ plants characterized by high k₉cat and low affinity for CO₂ (Christin et al., 2008; Kapralov et al., 2011, 2012). Thus, 309Ile and 328Ser are likely to be among the catalytic switches for faster and less specific Rubiscos in many groups of C₃ and C₄ plants. However, their effect on the kinetic properties may vary, depending on amino acid identity at other positions, as coevolution (the attainment of similar properties by means of alternative amino acid substitutions) has been shown to be a common phenomenon in Rubisco (Wang et al., 2011). Expression of recombinant Limonium Rubiscos in tobacco, incorporating directed mutations at these positions, may confirm their specific role in Rubisco catalysis, similar to the recent study involving Flaveria (Whitney et al., 2011).

Although kinetic differences among haplotypes are significant, there are cases when species belonging to different haplotypes have similar kinetics despite having different Rubisco L-subunit sequences, as well as species within the same haplotype (i.e. identical L-subunit sequences) having different kinetics. One of the possible explanations for this phenomenon is differences in small subunit (S-subunit) identity and the combinations of distinct S-subunits within the Rubisco holoenzyme. Although Rubisco active sites are located in the L-subunits, S-subunits can also affect catalysis (Spreitzer, 2003). Ishikawa et al. (2011) showed that significant changes in Rubisco kinetics occurred when the rice (Oryza sativa) S-subunit was replaced with the sorghum (Sorghum bicolor) S-subunit. More recently, Morita et al. (2014) showed that Rubisco catalytic properties were influenced by the differential expression of S-subunits in rice. Hexaploid wheat, Triticum aestivum, possesses at least 22 rbsS genes encoding different S-subunits (Galili et al., 1998). Many Balearic Limonium species are also recent polyploids (Erben, 1993), and may also possess large numbers of divergent rbsS genes.

The kinetic differences among haplotypes are explained on the basis of the inverse relationship between Rubisco velocity and CO₂ affinity (Fig. 3). This trade-off confirms that Rubisco evolution is flexible but essentially one-dimensional (Savit et al., 2010), that is, that Rubisco may specialize towards either increased k₉cat or increased S₁₀ because concomitant optimization of velocity and specificity is structurally impeded (Tcherkez, 2013). Among the potential forces driving the evolutionary adaptation of Rubisco, the availability of CO₂ for RuBP carboxylation has been crucial (Raven, 2000; Tcherkez et al., 2006). This trend was confirmed in Limonium and other Mediterranean species inhabiting extremely xeric environments, where the low soil and atmospheric water potentials require the operation of Rubisco at low C₃ for most of the growing season (Galmés et al., 2013). Under these highly selective conditions, Rubisco has evolved to increase its specificity for CO₂ (Galmés et al., 2005). The results in the present study clearly confirm the role exerted by C₃ in shaping Rubisco kinetics. Long-term growth of Limonium species under severe drought stress, similar to the environmental conditions of their natural habitat, caused a significant fall in C₃ in most species (Fig. 4). Under these stressful conditions, species showing the largest decrease in C₃ have a more CO₂-specific Rubisco, but a lower k₉cat, as a consequence of the trade-off between these properties.
On average, the fast and less CO₂-specific haplotype I Rubisco provided higher photosynthetic capacity, although this did not translate into higher whole-plant growth (Fig. 5). This apparent discrepancy can be explained in terms of the contrasting limitations governing the CO₂ assimilation rate (A) at the leaf and whole-plant levels. Leaf-level A, which was measured at saturating light intensities during discrete gas exchange measurements, was limited by Rubisco (Aₐ-limited photosynthesis) in all studied species (data not shown). According to the biochemical model of leaf photosynthesis (Farquhar et al., 1980), under Aₐ-limited photosynthesis kₑₛₐₙ – as part of Rubisco maximum carboxylation rate, Vₑ₉₉₉₉ – has a decisive role in determining A. By contrast, plant biomass is the integral of temporal and spatial CO₂ assimilation and respiration processes. Given the particular plant habit of Limonium plants, at any given moment of the day, a large proportion of the leaves in the canopy are likely to be exposed to subsaturating sunlight. Under these conditions, whole-plant photosynthesis is predominantly limited by RuBP regeneration (Aₐ-limited photosynthesis), and explains why plant biomass (B) is positively correlated to Sₑₛ₉₉₉₉ (Fig. 5d). B did not appear to be significantly correlated to either kₑₛ₉₉₉₉ or Sₑₛ₉₉₉₉ under WS (Cₑ decreased) but appeared to be so under WW conditions. If the ratio B₉₉₉₉₉₉/B₉₉₉₉₉₉ is indicative of whole-plant performance under drought, it is evident (Fig. 6) that Limonium Rubisco with higher carboxylase catalytic efficiency (kₑₛ₉₉₉₉/Kₑ) confers greater resilience to such severe stress conditions, as demonstrated by species from haplotype I (Fig. 6).

Our study is the first to use an interdisciplinary approach to investigate the relationship between Rubisco amino acid polymorphism and catalytic efficiency, plant photosynthetic performance, growth and drought tolerance in a group of closely related C₃ species. A wide-ranging assessment of the natural diversity of Rubisco-encoding genes, together with Rubisco kinetics and its consequences for plant photosynthesis and growth under contrasting environmental conditions, will be required if knowledge-based attempts to increase global crop yields by harnessing superior forms of Rubisco are to succeed (Parry et al., 2013).

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References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic tree inferred from the *rbcL* gene for all the *Limonium* species in the Balearic Islands.

Table S1 Changes in physical properties associated with amino acid substitutions in *Limonium rbcL*.

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