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Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves

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ABSTRACT

The specificity factor of Rubisco is a measure of the relative capacities of the enzyme to catalyse carboxylation and oxygenation of ribulose 1,5-bisphosphate and hence to control the relative rates of photosynthetic carbon assimilation and photorespiration. Specificity factors of purified Rubisco from 24 species of C_3 plants found in diverse habitats with a wide range of environmental growth limitations by both water availability and temperature in the Balearic Islands were measured at 25 °C. The results suggest that specificity factors are more dependent on environmental pressure than on phylogenetic factors. Irrespective of phylogenetic relationships, higher specificity factors were found in species characteristically growing in dryer environments and in species that are hemideciduous or evergreen. Effects of temperature on specificity factor of the purified enzyme from 14 species were consistent with the concept that higher specificity factors were associated with an increase in the activation energy for oxygenation compared to carboxylation of the 2,3-enediolate of RuBP to the respective transition state intermediates. The results are discussed in terms of selection pressures leading to the differences in specificity factors and the value of the observations for identifying useful genetic manipulation to change Rubisco polypeptide subunits.

Key-words: Balearic Islands; leaf habit; Mediterranean; Rubisco specificity factor; xericity.

INTRODUCTION

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) plays a central role in plant photosynthesis since it is involved in the uptake of CO_2 by photosynthetic organisms. Besides its carboxylase activity, Rubisco also acts as an oxygenase in a reaction involving competition between O_2 and CO_2 for reaction with RuBP (ribulose-1,5-bisphosphate). Thus, while photosynthesis is initiated by the carboxylase activity, the oxygenase activity catalyses the first reaction in the photorespiratory pathway (Ogren & Bowes 1971; Laing, Ogren & Hageman 1974), causing in C_3 plants the loss of up to 50% of carbon fixed by Rubisco and greatly decreasing the efficiency with which light energy is used (Zelitch 1973). The balance between the two competitive reactions is determined by the kinetic properties of Rubisco and the CO_2 and O_2 concentrations at the catalytic site of the enzyme (Laing *et al*. 1974):

$$v_c/v_o = (V_cK_o/V_oK_c)([CO_2]/[O_2])$$

where v_c and v_o are the velocities of carboxylation and oxygenation, respectively, V_c and V_o are the maximal velocities of the two reactions, and K_c and K_o the Michaelis constants for CO_2 and O_2. The substrate specificity factor, V_cK_o/V_oK_c, τ, determines the relative rates of the two reactions at any given CO_2 and O_2 concentrations. The specificity factor has increased during evolution to compensate for the gradual atmospheric shift from high CO_2 and low O_2 to low CO_2 and high O_2 (Jordan & Ogren 1981; Tortell 2000) and, as a result, higher plants and diatoms display in general much higher values of τ than cyanobacteria and algae.

Since excessive photorespiration appears to offer no essential function in the economy of the plant and involves considerable losses of carbon, the genetic modification of crop Rubiscos to increase the τ is an important strategy for increasing plant biomass (Larimer *et al*. 1987; Spreitzer 1993; Parry *et al*. 2003; Zhu, Portis & Long 2004). One way to achieve this may be to exploit any natural variation. Thus, the identification of organisms with high Rubisco τ is potentially an important step towards the improvement of the enzyme (Andrews & Lorimer 1987). Statistically meaningful variation in Rubisco τ among C_3 species has been confirmed (Gutteridge *et al*. 1986; Parry, Keys & Gutteridge 1989; Kent *et al*. 1992; Kane *et al*. 1994; Delgado *et al*. 1995; Kent & Tomany 1995). However, the exploration of natural variation in the kinetic properties of this enzyme among C_3 plants is not extensive and less than 100 species have been analysed (Jordan & Ogren 1981; 1983; Brooks & Farquhar 1985; Gutteridge *et al*. 1986; Keys 1986; Parry *et al*. 1989; Kent *et al*. 1992; Lee, Kostov & McFadden 1993; Kane *et al*. 1994; Delgado *et al*. 1995; Kent & Tomany 1995; Balaguer *et al*. 1996; Uemura *et al*. 1997; Bota *et al*.

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2002) from a plant population in excess of 300 000 species. This limited sampling of C_3 plants may be a significant limitation to crop improvement, since many major crops already have some of the highest specificity factors described up to now.

Improved knowledge of the natural variations of τ in higher plants is also necessary for modelling purposes and global predictions of productivity under present and future climates. Current models of leaf photosynthesis assume constant values among C_3 plants (Long & Bernacchi 2003) or derive them indirectly from gas exchange measurements (Bernacchi *et al*. 2001). Phylogenetic (Jordan & Ogren 1984) and environmental factors (Delgado *et al*. 1995; Kent & Tomany 1995) have been proposed to influence selection pressure for high Rubisco τ. For instance, differences in τ among distant phylogenetic groups (i.e. photosynthetic bacteria, cyanobacteria, higher plants, etc.) are much larger than between genera or species belonging to more closely related groups (Jordan & Ogren 1984). It has also been proposed that there is a strong correlation between plant physiological parameters and ecological plant traits (Reich, Walters & Ellsworth 1997; Wright *et al*. 2004). Since Rubisco τ decreases with increase of temperature, because the oxygenase catalytic efficiency (V_o/K_o) increases more with temperature than the carboxylase catalytic efficiency (V_c/K_c) (Jordan & Ogren 1984), Delgado *et al*. (1995) and Kent & Tomany (1995) hypothesized that hot environments associated with water stress, stomatal closure and low CO_2 concentrations at the site of Rubisco may impose increased selection pressure on Rubisco for improved specificity. Similarly, Austin (1999) reasoned that Rubisco with a higher specificity factor would have most advantage in a hot environment. Within the Balearic Islands there is a steep gradient of both precipitation (from 300 to 1500 mm) and temperature, which may impose different degrees of such environmental stresses. On the other hand, Gulías *et al*. (2003), showed that species endemic to the Balearic Islands have significantly lower photosynthetic capacities than more widespread species and proposed that one factor could be a lower τ in the endemic species to explain the observed differences. Geographical isolation could have favoured selection of distinctive properties for Rubisco.

Here we report a survey of τ measured on purified Rubiscos from 24 C_3 Mediterranean species having a variety of ecological, phylogenetic and morphological traits. The main objectives were: (1) to increase the number of taxa for which τ is known; (2) to elucidate the dependency of τ on ecological (life habit and habitat xericity), phylogenetic and evolutionary (endemicity) factors; and (3) to explore the temperature dependence of the catalytic activities of Rubiscos from different sources.

MATERIALS AND METHODS

Plant material

Twenty-four dicotyledonous species (Table 1) from different communities in the Balearic Islands were selected for study. The criteria used to select these species were based

Table 1. List of the species analysed, with their evolutionary history, life habit, xericity index and τ measured at 25 °C

Species	Evolutionary history	Life habit	Xericity index	τ at 25 °C
Diplotaxis ibicensis Pau	Endemic	Herb annual	1	95.6 ^{efg}
Urtica atrovirens ssp. *bianorii* (Knoche) Paira	Endemic	Herb annual	3	90.2 ^{abc}
Pimpinella bicknelli Briq.	Endemic	Herb annual	3	92.2 ^{abcde}
Paeonia cambessedesii Willk.	Endemic	Herb annual	3	94.1 ^{defg}
Beta maritima L. ssp. *marcosii* A. Juan & M. B. Crespo	Endemic	Herb evergreen	1	96.3 ^{fg}
Crepis triasii (Camb.) Nyman	Endemic	Herb evergreen	2	94.8 ^{defg}
Lysimachia minoricensis J. J. Rodr.	Endemic	Herb evergreen	3	93.8 ^{cdefg}
Digitalis minor L. *var. palauii* (G. Font) Hinz & Rosselló	Endemic	Herb evergreen	3	97.1 ^{g}
Digitalis minor L. *var. minor*	Endemic	Herb evergreen	3	91.0 ^{abcd}
Phlomis italica L.	Endemic	Woody hemi-deciduous	2	100.8 ^{hi}
Limonium magallufianum L. Llorens	Endemic	Woody evergreen	1	106.1 ^{j}
Rhamnus ludovici-salvatoris R. Chodat	Endemic	Woody evergreen	2	94.4 ^{defg}
Hypericum balearicum L.	Endemic	Woody evergreen	2	93.6 ^{cdefg}
Urtica membranacea Poiret	Non-endemic	Herb annual	2	102.4 ^{i}
Kundmannia sicula (L) D. C.	Non-endemic	Herb annual	2	89.2 ^{ab}
Helleborus foetidus L.	Non-endemic	Herb annual	3	88.7 ^{a}
Beta maritima L. ssp. maritima	Non-endemic	Herb evergreen	1	92.9 ^{bcdef}
Mentha aquatica L.	Non-endemic	Herb evergreen	3	97.2 ^{gh}
Lavatera maritima Gouan	Non-endemic	Woody hemi-deciduous	1	92.5 ^{abcdef}
Cistus albidus L.	Non-endemic	Woody hemi-deciduous	2	92.1 ^{abcde}
Limonium gibertii (Sennen) Sennen	Non-endemic	Woody evergreen	1	110.5 ^{k}
Limonium virgatum (Willd.) Fourr.	Non-endemic	Woody evergreen	1	100.7 ^{hi}
Rhamnus alaternus L.	Non-endemic	Woody evergreen	2	94.7 ^{defg}
Pistacia lentiscus L.	Non-endemic	Woody evergreen	2	97.2 ^{gh}

Different letters denote statistical differences at $P < 0.05$ by Duncan analysis.

on their evolutionary history, ecological characters and phylogenetic relationships. For evolutionary history, species were classified into: endemic species, those only occurring in the Balearic Islands, and non-endemic species, species that are not restricted to the Balearic Islands. Two different criteria were used to classify the species with respect to their ecology. Firstly, species were classified depending on their life habit into: herbaceous annuals, herbaceous evergreens, woody hemi-deciduous and woody evergreen species. Herbaceous annual species comprised all non-woody species that complete their life cycle in one year. Herbaceous evergreen species comprised all non-woody species that maintain functional leaves during the whole year. Woody hemi-deciduous species comprised all woody species that lose a certain amount of their leaves during the unfavourable season, depending on its length and severity. Woody evergreen species comprised all woody species that maintain their leaves during the whole year. The second ecological classification was made on the basis of habitat xericity. Group 1 comprised the species inhabiting the coastal, driest and hottest areas with annual precipitation typically below 400 L m^{-2}. Species typical of Mediterranean macchia with annual precipitation between 400 and 800 L m^{-2} were classified in group 2 together with some ruderal species. Group 3 comprised species inhabiting the wettest and coolest mountain areas with annual precipitation above 800 L m^{-2}, species growing only near open water sources, and species maintaining their leaves only during the wet season. Species were finally plotted in a phylogenetic tree from the specific categories of genera and families to the more general categories of orders and subclasses.

Most of the plant species were grown from seed. Specimens of *M. aquatica, D. minor* var. *minor*, *D. minor* var. *palauii* and *C. triasii* were collected in the field and propagated asexually. The resulting plants were grown in a glasshouse at Rothamsted Research, Harpenden, UK with supplementary lighting to give a photoperiod of 16 h. The minimum temperature was 25 °C in the photoperiod and 18 °C in the dark. Growth was in soil-based compost supplemented with slow-release fertilizer. Water was added sparingly by hand. For *L. virgatum, R. alaternus*, *R. ludovici-salvatoris*, *P. bicknelli*, *P. cambessedesii* and *H. foetidus*, young mature leaves were collected directly in the field and transported to England packed in dry ice.

Extraction and purification of Rubisco

Young but mature leaves (30–50 g) from up to 10 or more individuals from each species were immediately frozen in liquid nitrogen. For each species, the leaf material was pooled and ground to a powder in a mortar, buffer was added and grinding continued from time to time as the mixture thawed. After extensive preliminary tests, the most appropriate protein extraction media for Rubisco were found to be: (A) 0.1 M Bicine, 50 mM β-mercaptoethanol, 11 mM sodium diethyldithiocarbamate (Na-DIECA), 6% (w/v) polyethyleneglycol (PEG) 4000, 1 mM benzamidine, 1 mM ε-amino-*n*-caproic acid and 1 mM phenylmethylsulphonylfluoride (PMSF), at pH 8, and (B) containing 0.1 M HEPES, 3% (w/v) polyvinylpyrrolidone (PVP) 25, 6% (w/v) PEG 4000, 50 mM β-Mercaptoethanol, 2 mM dithiothreitol (DTT), 10% glycerol, 5 mM $MgCl_2$, 5 mM ethyleneglycol-bis(β-aminoetylether)-N,N,N′N′-tetraacetic acid (EGTA) and 2 mM PMSF, at pH 8.0. Buffer A was used to extract Rubisco from most of the species. Buffer B was used with *M. aquatica*, *P. lentiscus* and *C. albidus*. For *H. balearicum*, buffer A was used with the following modifications: the concentration of Bicine was increased to 0.2 M and the volume used was doubled. For *P. lentiscus*, the following modifications were made to buffer B: the concentration of HEPES was increased to 0.25 M and the volume used was doubled.

All the purification steps were carried out at 0–4 °C. Fully thawed but still cold homogenates were filtered through butter muslin and then centrifuged at 22 000 × *g* for 20 min. The supernatant liquid was decanted through 50 µm mesh nylon and PEG 4000 was added as a 60% (w/v) aqueous solution to the supernatant liquid to produce a final concentration of 20% (w/v). Furthermore, 1 M $MgCl_2$ was added to a final concentration of 20 mM followed by gentle mixing. After standing for 10 min the mixture was centrifuged again at 22 000 × *g* for 20 min. The pellet was re-suspended in 40 mL of Column buffer (10 mM Tris pH 8.0 with 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA and 1 mM KH_2PO_4) containing 1 mM each of PMSF, benzamidine and ε-amino-*n*-caproic acid. The suspension was centrifuged to remove insoluble material. The supernatant liquid was applied to an 88 × 1.6 cm column of Q Sepharose Fast Flow anion exchanger (Amersham Biosciences, UK Limited) previously equilibrated with column buffer and operated at 1 mL min^{-1}. The effluent was monitored for absorbance at 280 nm. The proteins were eluted using a linear gradient from 0 to 0.75 M NaCl in column buffer in 16 h and fractions were collected at 10 min intervals. Those fractions with high Rubisco activity were combined and desalted using a Sephadex G25 (Pharmacia) column 44 × 5 cm operated at 200 mL h^{-1} with de-salt buffer (bicine 5 mM, pH 8) and fractions collected at 3 min intervals. Finally, fractions containing high amounts of protein were pooled together, the concentration of Rubisco was estimated as $A_{280} \times 0.61$ mg mL^{-1} (Paulsen & Lane 1966), and the solution dispensed into vials and freeze dried.

Rubisco activity measurements

Rubisco activity was measured at different stages of the purifications by adding 10 or 25 μL of solution containing protein to 0.2 mL of a solution containing 1 mL 0.1 M $NaH^{14}CO_3$, 0.5 μC_i μmol^{-1}, 5 mL 0.2 M bicine containing 40 mM $MgCl_2$ pH 8.2 and 4 mL H_2O. After 3 min 10 μL 20 mM RuBP was added and after a further 1 min the reaction was stopped by adding 0.1 mL of 10 M formic acid. To activate the slowly activating form of Rubisco present in solution after desalting, reaction mixtures, less the RuBP, were heated at 37 °C for 40 min and then cooled to room

temperature before adding the RuBP and completing the assay. The acidified reaction mixes were dried down in an oven placed in a fume hood. After cooling, 0.4 mL of H_2O and 3.5 mL of Ultima Gold scintillation cocktail (Packard, Canberra, Australia) were added. ^{14}C in PGA (D-3-phosphoglycerate) was measured using a scintillation spectrometer.

Specificity factor determinations

For Rubisco from each species, between 6 and 12 measurements (to optimize the method precision) of specificity factor were made using an assay involving the total consumption of RuBP. For measurements at 25 °C the freeze-dried Rubisco samples from the 24 species were dissolved and desalted by centrifugation through G25 Sephadex columns (Helmerhorst & Stokes 1980) previously equilibrated with CO_2-free 0.1 M bicine pH 8.2 containing 20 mM $MgCl_2$. The desalted solutions were made 10 mM to $NaH^{14}CO_3$ and 0.4 mM to orthophosphate. These mixtures were incubated at 37 °C for 40 min to activate the Rubisco. Reaction mixtures were prepared in an oxygen electrode (Model DW1; Hansatech, Kings Lynn., UK) by first adding 0.95 mL of a solution of 100 mM bicine pH 8.2, 10 mM $MgCl_2$ containing 1.5 mg (7000 W-A units) per 100 mL of carbonic anhydrase and equilibrated with CO_2-free air at 25 °C. After adding 0.02 mL of 0.1 M $NaH^{14}CO_3$ the plug was fitted to the oxygen electrode vessel. Enough activated Rubisco was then added in 20 μL for the reaction to be completed within 5 min. The reaction was started by the addition of 10 μL of 15 mM RuBP to give a total reaction volume 1 mL. RuBP oxygenation was calculated from the oxygen consumption and carboxylation from the amount of ^{14}C incorporated into PGA when all the RuBP had been consumed (Parry *et al*. 1989). A sequence of reaction mixtures containing pure wheat Rubisco were interspersed with those containing Rubisco from the test species and the results normalized to the average value obtained from wheat Rubisco, 100.0 at 25 °C.

In addition, 14 species were selected for measurement of τ at 15 and 35 °C. The procedure followed was the same as at 25 °C except that the buffer was prepared and equilibrated with CO_2-free air at 15 or 35 °C as appropriate and the volume of 0.1 M $NaH^{14}CO_3$ used was 0.015 and 0.03 mL, respectively. The total volume of reaction mixtures was again 1 cm^3. The results were normalized to the average values for wheat Rubisco, 139.6 at 15 °C and 77.1 at 35 °C. From the slopes of the regressions between ln τ and 1000/RT, the difference in the free energy of activation to the transition state intermediates for the oxygenase and the carboxylase reactions of Rubisco ($\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$) were calculated according to Uemura *et al*. (1997).

Statistical analysis

An analysis of variance (Manugistics 1998) using one-way ANOVAs was made for τ with the following fixed effects: species at 15, 25 and 35 °C, evolutionary history, life habit, xericity index and phylogeny (from genera to classes). Finally, an analysis of variance was made for $\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$ in each species. Duncan tests at the 95% confidence limit were used to separate the means for τ and $\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$ within each treatment.

RESULTS

Species variation in specificity factor at 25 °C

Amounts and specific activities of purified Rubisco from the different species of plant varied (data not shown), but the 40 g of fresh leaf material used always yielded sufficient Rubisco and activity for τ determination. Table 1 shows τ measured at 25 °C. The values ranged from 88.7 for *H. foetidus* to 110.5 for *L. gibertii*. The Rubiscos from the two *Limonium* species, *L. gibertii* and *L. magallufianum*, had significantly higher τ than that of wheat ($P < 0.05$). The Rubiscos from the other species were similar to or lower than that of wheat. Significant differences were also found in Rubisco τ for species within the same genus. Within the *Limonium* genus, τ differed significantly ($P < 0.05$) between the three species analysed at 25 °C, *L. gibertii* (110.5), *L. magallufianum* (106.1) and *L. virgatum* (100.7). Even larger differences were observed for Rubiscos from the *Urtica* genus, with τ of 102.4 for *U. membranecea* and 90.2 for *U. atrovirens* ssp. *bianorii*. Statistically significant differences in τ were even observed between Rubiscos from two varieties of a single species, *D. minor*, 91.0 for *D. minor* var. *minor* and 97.1 for *D. minor* var. *palauii* ($P < 0.05$). By contrast, no significant differences were observed between the two varieties of *Beta maritima* analysed.

Ecological, phylogenetic and evolutionary influences on specificity factor

Significant differences in τ, $P < 0.05$, were also found among species from different habitats (Table 2). For instance, τ averaged 98.7 for those species inhabiting the driest environments (group 1), 96.4 for those species from intermediate environments (group 2) and 92.6 for those

Table 2. τ variation among life habit and xericity groups

	τ at 25 °C
Life habit	
Herb annual	93.5 [a]
Herb evergreen	94.5 [ab]
Woody hemi-deciduous	96.6 [bc]
Woody evergreen	99.5 [c]
Xericity index	
1	98.7 [c]
2	96.4 [b]
3	92.6 [a]

Different letters denote statistical differences at P <0.05 within each criteria by Duncan analysis.

species that do not suffer hot and dry summer seasons (group 3).

Significant differences were also observed in τ for Rubiscos from plants of different life habit. In woody evergreen species τ averaged 99.5, which was significantly higher ($P < 0.05$) than τ from both herbaceous annual and herbaceous evergreen species (93.5 and 95.5, respectively, Table 2). Woody hemi-deciduous species presented intermediate values (96.6). Therefore, in general, woody species showed higher values than herbs. In contrast, no significant differences in τ were observed between endemic and non-endemic species of the Balearic Islands at any of the temperatures analysed (data not shown). Even if the values obtained by Delgado *et al*. (1995) for other Balearic species are included in the analysis, the differences still remain non-significant. Finally, it has been impossible to elucidate any association of τ with phylogeny. From the analysis of variance made within each phylogenetic category (from genera to classes) no clear pattern could be detected between different phylogenetic branches, in respect to τ (data not shown).

Temperature dependence of specificity factor among different species

Several authors (Jordan & Ogren 1984; Brooks & Farquhar 1985; Larimer *et al*. 1987; Uemura *et al*. 1997) have reported that τ decreases with increasing temperature. Such a decrease was observed in all the species analysed in the present study and has been used to estimate the difference in activation energy to the transition state intermediates of the oxygenase and carboxylase reactions (Uemura *et al*. 1997). Significant differences were found in $\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$ ($P < 0.05$) between species (Table 3). The values calculated ranged from 5.4 to 5.6 kcal mol^{-1} for *Limonium* species to 4.7–4.8 kcal mol^{-1} for *Urtica* species.

DISCUSSION

There are substantial differences in τ for Rubisco purified from species from widely separated phylogenetic groups (Jordan & Ogren 1981, 1983) but much less variability of Rubisco τ from higher plants (Delgado *et al*. 1995; Kent & Tomany 1995). However studies have focused on a small number of species of cultivated plants. The variation in τ for Rubisco from species of higher plants from natural flora remains largely unknown. This work increases the range of known Rubisco values for C_3 plants and shows that the magnitude of τ from the plants appears to be related to the environments in which the plants grow, and to their life habits.

The Rubisco τ is greatest for plants confined to hot, arid and saline areas of the Balearic Islands with a xericity index of 1 in Table 2. Under these conditions both the high temperature and the low internal CO_2 concentrations, enforced by the need to conserve water, appear to have imposed a selection pressure for high Rubisco τ, in agreement with the hypothesis of Delgado *et al*. (1995) and Kent & Tomany (1995). Carbon isotope composition along climatic gradients in Mediterranean plants tends to show that life-averaged internal CO_2 concentrations are indeed lower in species inhabiting drier areas (Valentini, Scarascia-Mugnozza & Ehleringer 1992; Alessio *et al*. 2004). Gas exchange analysis of the species included in the present study demonstrates that *Limonium* species, for instance, have a lower internal CO_2 concentration than most of the other species (data not shown). The beneficial effects of increasing τ are predicted to be greater at higher temperatures (Austin 1999).

There is also a trend for an increase in τ from annual herbs, through evergreen herbs to hemi-deciduous and to evergreen shrubs. A reasonable hypothesis would be that with longevity of leaves there is an associated increased thickness and as a consequence decreased conductance for

Table 3. τ at 15, 25 and 35 °C, and differences in the activation energies of the transition states between the oxygenase and the carboxylase reactions for Rubisco ($\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$)

Species	τ at 15 °C	τ at 25 °C	τ at 35 °C	$\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$
Diplotaxis ibicensis	137.2 ^{d}	95.6	73.3 ^{bc}	5.6 ^{d}
Urtica atrovirens ssp. *bianorii*	121.5 ^{ab}	90.2	71.4 ^{b}	4.7 ^{a}
Beta maritima ssp. *marcosii*	130.9 ^{cd}	96.3	73.4 ^{bc}	5.1 ^{abc}
Lysimachia minoricensis	116.8 ^{a}	93.8	65.3 ^{a}	5.2 ^{abc}
Limonium magallufianum	150.7 ^{e}	106.1	81.8 ^{e}	5.4 ^{cd}
Rhamnus ludovici-salvatoris	137.3 ^{d}	94.4	76.1 ^{cd}	5.2 ^{ab}
Urtica membranacea	130.5 ^{cd}	102.4	76.3 ^{cd}	4.8 ^{a}
Kundmannia sicula	126.7 ^{bc}	89.2	71.3 ^{b}	5.1 ^{abc}
Beta maritima ssp. *maritima*	131.1 ^{cd}	92.9	75.6 ^{cd}	4.9 ^{ab}
Mentha aquatica	124.1 ^{bc}	97.2	71.1 ^{b}	4.9 ^{abc}
Limonium gibertii	148.8 ^{e}	110.5	78.8 ^{d}	5.6 ^{d}
Rhamnus alaternus	131.6 ^{cd}	94.7	74.3 ^{bc}	5.0 ^{abc}
Hypericum balearicum	131.6 ^{cd}	93.6	72.1 ^{b}	5.3 ^{bc}
Pistacia lentiscus	129.5 ^{c}	97.2	72.9 ^{bc}	5.1 ^{abc}

$\Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}$ was calculated from $\ln \tau = \Delta G_O^{\ddagger} - \Delta G_C^{\ddagger}/RT$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}), and T is the absolute temperature of the reaction mixture. Different letters denote statistical differences at $P < 0.05$ within each column by Duncan analysis.

CO_2 to the chloroplast stroma. Again, a Rubisco with a higher τ could be regarded as conferring an advantage in supporting faster assimilation relative to photorespiration. On the other hand, the present results do not support the suggestion of Gulías *et al*. (2003) that low photosynthetic rates of species endemic to the Balearic Archipelago might be explained by lower values.

The range in magnitude of τ observed is sufficiently small (approximately 20%) that there is a need to consider whether the advantage in terms of increased carbon assimilation provides significant selection pressure. Current models of leaf photosynthesis (Farquhar, von Caemmerer & Berry 1980) allow for estimations of the effects of τ variation on photosynthesis rates. As an example, the calculations in Table 4 show the increases in carbon assimilation that would accrue if the Rubisco from *L. gibertii* replaced the native Rubisco of wheat and tobacco plants. The amounts of any increase depend on which of the four constants that make up τ is responsible for the difference between Rubisco from the recipient species and Rubisco from *L. gibertii*. The model predicts increases in tobacco net photosynthesis of 26, 30, 16 and 5% depending whether the difference is due to differences in K_c, V_c, K_o, and V_o, respectively. For wheat, photosynthesis may be increased by 11, 12, 6 and 2%, respectively, for changes due to the same parameters. Table 1 shows that there are also significant differences in τ between closely related species, for example, *L. gibertii* and *L. magallufianum* or *U. membranacea* and *U. bianori* ssp. *atrovirens*, which are less than the difference in τ between the species considered in Table 4, but we can conclude that selection pressures based on Rubisco τ are significant. Table 4 illustrates the importance of knowledge of the kinetic constants that make up τ, especially V_c, in predicting the effect of the properties of Rubisco on photosynthetic capacity. Determination of a true V_c (K_{cat}) for Rubiscos proved difficult for leaves of the species studied so we cannot comment on the possibility of a negative correlation (see Zhu *et al*. 2004) that may exist over the restricted range of specificity factors observed.

Table 4. Predicted increases in light saturated photosynthesis (%) for tobacco and wheat if their native Rubisco could be replaced with Rubisco from *Limonium gibertii*

	Difference in τ caused by change in:			
	K_c	V_c	K_o	V_o
	Increase in net photosynthesis (%)			
Tobacco	26	30	16	5
Wheat	11	12	6	2

The concentrations of CO_2 and O_2 at the chloroplast level were considered to be 7 and 265 μM, respectively. The calculation used equations 2.17 and 2.23 (von Caemmerer 2000), assuming Rubisco-limited photosynthesis and considering the number of Rubisco catalytic sites to be constant.

Since the catalytic properties of Rubisco are mainly determined by the structure of the large subunit polypeptide of the enzyme, and this is coded for in the many copies of the chloroplast genome, the selection of particular variants of the gene seem likely to be slow. Nevertheless, the Rubisco large subunit sequences of different *Limonium* species are not identical (Fig. 1). The conclusion must be that variations in the genes encoding rbcL Rubisco gene arise or exist and that the normal process of maternal inheritance (Birky 2001) allows selection of superior versions over a relatively short timescale. Alternatively the differences observed in τ maybe entirely the result of differences in sequence of the Rubisco small subunit polypeptide (Wang *et al*. 2001).

Even very modest changes may be extremely important, since the difference in the critical steps in the catalytic mechanism, the activation and stabilization of the transition state intermediates of the carboxylation and oxygenation reactions that determine the rate of oxygenation compared to carboxylation, have a free energy difference of less than the energy of one hydrogen bond (Spreitzer 1993; Spreitzer & Salvucci 2002). It may be assumed therefore that differences of even one amino acid residue could account for observed differences in τ. The identification of sequence differences in the genes for Rubisco from closely related species (Fig. 1), together with differences in the kinetic properties, including τ, needs to be extended so that at a cause and effect relationship might be established. Knowledge of gene sequences encoding Rubisco and Rubisco τ for C_3 plants may identify genetic modifications that will have great agronomic importance. This is important because introducing into higher terrestrial plants, genes for Rubisco from distantly related cyanobacterial and algal species have thus far failed to yield a fully functional enzyme, whereas the introduction of smaller changes have resulted in catalytically competent enzyme (Spreitzer & Salvucci 2002; Parry *et al*. 2003; Zhu *et al*. 2004). In conclusion, the present data demonstrate that significant variability can be found in τ among closely related C_3 species, and that this variability is related to environmental pressure factors. This finding opens the possibility of genetic engineering to improve τ for more efficient crops – particularly for arid and semiarid agriculture. If a larger τ is to be found among C_3 species, the present survey suggests that it should be found among evergreen woody species from arid environments.

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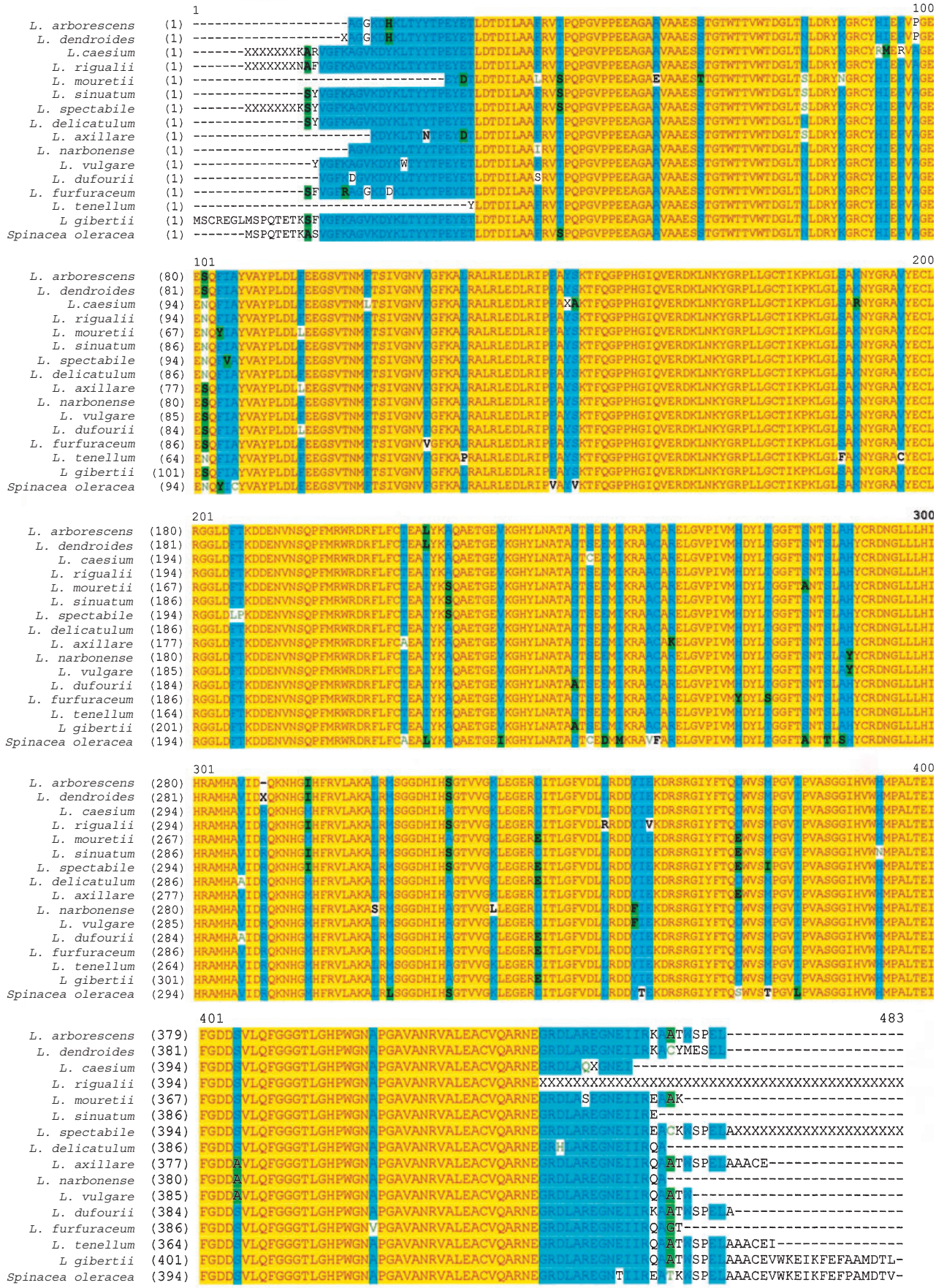
1 100
L. arborescens (1)
L. dendroides (1)
L.caesium (1)
L. rigualii (1)
L. mouretii (1)
L. sinuatum (1)
L. spectabile (1)
L. delicatulum (1)
L. axillare (1)
L. narbonense (1)
L. vulgare (1)
L. dufourii (1)
L. furfuraceum (1)
L. tenellum (1)
L gibertii (1)
Spinacea oleracea (1)
101 200
L. arborescens (80)
L. dendroides (81)
L.caesium (94)
L. rigualii (94)
L. mouretii (67)
L. sinuatum (86)
L. spectabile (94)
L. delicatulum (86)
L. axillare (77)
L. narbonense (80)
L. vulgare (85)
L. dufourii (84)
L. furfuraceum (86)
L. tenellum (64)
L gibertii (101)
Spinacea oleracea (94)
201 300
L. arborescens (180)
L. dendroides (181)
L. caesium (194)
L. rigualii (194)
L. mouretii (167)
L. sinuatum (186)
L. spectabile (194)
L. delicatulum (186)
L. axillare (177)
L. narbonense (180)
L. vulgare (185)
L. dufourii (184)
L. furfuraceum (186)
L. tenellum (164)
L gibertii (201)
Spinacea oleracea (194)
301 400
L. arborescens (280)
L. dendroides (281)
L. caesium (294)
L. rigualii (294)
L. mouretii (267)
L. sinuatum (286)
L. spectabile (294)
L. delicatulum (286)
L. axillare (277)
L. narbonense (280)
L. vulgare (285)
L. dufourii (284)
L. furfuraceum (286)
L. tenellum (264)
L gibertii (301)
Spinacea oleracea (294)
401 483
L. arborescens (379)
L. dendroides (381)
L. caesium (394)
L. rigualii (394)
L. mouretii (367)
L. sinuatum (386)
L. spectabile (394)
L. delicatulum (386)
L. axillare (377)
L. narbonense (380)
L. vulgare (385)
L. dufourii (384)
L. furfuraceum (386)
L. tenellum (364)
L gibertii (401)
Spinacea oleracea (394)

Figure 1. Alignment (Vector NTI, Invitrogen) of Rubisco large subunit amino acid sequences from *Limonium arborescens* (translation of AF206789), *L. dendroides* (CAB10739), *L. caesium* (CAB10738), *L. rigualii* (CAB10740), *L. mouretii* (CAA76531), *L. sinuatum* (CAA76531), *L. spectabile* (CAB10741), *L. delicatulum* (CAA76533), *L. axillare* (CAB86155), *L. narbonense* (CAB86158), *L. vulgare* (CAA76534), *L. dufourii* (CAB86156), *L. furfuraceum* (CAA76532), *L. tenellum* (CAB86159), *L. gibertii* (translated from AJ786659) and *Spinacia oleracea* (CAB88737) with accession numbers given in parenthesis. Yellow boxes indicate fully conserved residues among species. Blue boxes indicate partially conserved residues, while the exceptions are indicated in green (similar type of amino acids) or white (different type of amino acids).

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