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Short title: Variability of Rubisco kinetics in crops

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Article title: Rubisco catalytic properties and temperature response in crops

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One sentence summary:

Variability in Rubisco kinetic parameters and their temperature dependency determine differences in the photosynthetic efficiency in the most important crops worldwide.

List of author contributions: J.G. conceived the project; C.H-C. and J.G. designed the experiment; C.H-C. performed the experiments; C.H-C., M.V.K. and JG analyzed the data and wrote the article.

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ABSTRACT

Rubisco catalytic traits and their thermal dependence are two major factors limiting the CO_2 assimilation potential of plants. In this study, we present the profile of Rubisco kinetics for twenty crop species at three different temperatures. The results largely confirmed the existence of significant variation in the Rubisco kinetics among species. Although some of the species tended to present Rubisco with higher thermal sensitivity (e.g., *Oryza sativa*) than others (e.g., *Lactuca sativa*), interspecific differences depended on the kinetic parameter. Comparing the temperature response of the different kinetic parameters, the Rubisco Michaelis-Menten constants for CO_2 (K_c and K_c^{air}) presented higher energy of activation (ΔH_a) than the maximum carboxylation rate (k_{cat}^c) and the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*). The analysis of the Rubisco large subunit sequence revealed the existence of some sites under adaptive evolution in branches with

specific kinetic traits. Because Rubisco kinetics and their temperature dependency were species-specific, they largely affected the assimilation potential of Rubisco from the different crops, especially under those conditions (i.e., low CO_2 availability at the site of carboxylation and high temperature) inducing Rubisco-limited photosynthesis. As an example, at 25 ºC, Rubisco from *Hordeum vulgare* and *Glycine max* presented, respectively, the highest and lowest potential for CO_2 assimilation at both high and low chloroplastic CO_2 concentrations. In our opinion, this information is relevant to improve photosynthesis models and should be considered in future attempts to design more efficient Rubiscos.

INTRODUCTION

The reported stagnation in the annual gains of cereal yields in the last decade clearly indicates that the expected demand for increased yield - at least 50% by 2050 (FAO forecasts) - will not be met by conventional breeding (Zhu et al., 2010). Future improvements will come from novel bioengineering approaches specifically focussed on processes limiting crop productivity that have not been addressed so far (Parry et al., 2012; Ort et al., 2015). A number of specific modifications to the primary processes of photosynthesis that could increase canopy carbon assimilation and production through step changes include the modification of the catalytic properties of Rubisco (Murchie et al., 2009; Whitney et al., 2011; Parry et al., 2013; Ort et al., 2015). First, biochemical models indicate that CO_2 fixation rates are limited by Rubisco activity under physiologically relevant conditions (Farquhar et al., 1980; von Caemmerer, 2000; Rogers, 2014). Second, Rubisco's catalytic mechanism exhibits important inefficiencies which compromise photosynthetic productivity: it is a slow catalyst – forcing plants to accumulate large amounts of the protein – and unable to distinguish between CO_2 and O_2 – starting a wasteful side reaction with oxygen that leads to the release of previously fixed CO_2, NH_2 and energy (Roy and Andrews, 2000). These inefficiencies not only limit the rate of CO_2 fixation, but also the capacity of crops for an optimal use of resources, principally water and nitrogen (Flexas et al., 2010; Parry et al., 2012).

Rubisco kinetic parameters has been described *in vitro* at 25 °C for about 250 species of higher plants, of which only c.a. 8% are crop species (e.g., Yeoh et al., 1981; Sage, 2002; Bird et al., 1982; Ishikawa et al., 2009; Prins et al., 2016). This amount of data revealed the existence of significant variability in the main Rubisco kinetic parameters both among C_3 (Yeoh et al., 1980, 1981; Bird et al., 1982; Jordan and Ogren, 1983; Parry et al., 1987; Castrillo, 1995; Delgado et al., 1995; Kent and Tomany, 1995; Balaguer et al., 1996; Bota et al., 2002; Galmés et al., 2005, 2014a, 2014c; Ghannoum et al., 2005; Ishikawa et al., 2009) and between C_3 and C_4 species (Kane et al., 1994; Sage, 2002; Kubien et al., 2008; Perdomo et al., 2014). The existence of Rubiscos with different catalytic performance implies that the success – in terms of photosynthetic improvement – of Rubisco engineering approaches in crops will depend on the specific performance of the native enzyme from each crop species. Nevertheless, our knowledge on the actual variability in Rubisco kinetics is still narrow, not only because of the limited number of species that have been examined so far, but mainly because complete Rubisco kinetic characterization (including the main parameters) has been performed in very few species.

Recent modelling confirmed that Rubisco is not perfectly optimized to deliver maximum rates of photosynthesis, and indicated that Rubisco optimization depends on the environmental conditions under which the enzyme operates (Galmés et al., 2014b). In particular, Rubisco catalytic parameters are highly sensitive to changes in temperature. For instance, the maximum carboxylase turnover rate (k_{cat}^{c}) increases exponentially with temperature (Sage, 2002; Galmés et al., 2015). However, at temperatures higher than the photosynthetic thermal optimum, the increases in k_{cat}^{c} are not translated into increased CO_2 assimilation because of the decreased affinity of Rubisco for CO_2, i.e., higher Michaelis-Menten constant for CO_2 (K_c) and lower specificity factor ($S_{c/o}$), and the decreased CO_2/O_2 concentration ratio in solution (Hall and Keys, 1983; Jordan and Ogren, 1984). These changes favour the RuBP oxygenation by Rubisco relative to carboxylation, increasing the flux through photorespiration and, ultimately, reducing the potential growth at high temperatures (Jordan and Ogren, 1984).

Beyond the discernment of the existing variability in Rubisco kinetics at a standard temperature, the knowledge on the temperature dependence of Rubisco kinetics, and the existence of variability in the thermal sensitivity among higher plants is of key importance for modelling purposes. The number and diversity of plant species for which Rubisco kinetic parameters have been tested *in vitro* at a range of physiologically relevant temperatures are still very scarce (e.g. Laing et al., 1974; Badger and Collatz, 1977; Badger, 1980; Monson et al., 1982; Hall and Keys, 1983; Jordan and Ogren, 1984; Lehnherr et al., 1985; Uemura et al., 1997; Zhu et al., 1998; Sage et al., 2002; Galmés et al., 2005; Haslam et al., 2005; Yamori et al., 2006; Perdomo et al., 2015; Prins et al., 2016), and mostly restricted to a few kinetic parameters – actually, there is no study examining the temperature dependencies of the main kinetic constants on the same species. The limited number of data reported so far suggests the existence of interspecific differences in the temperature dependence of some Rubisco kinetic parameters, like k_{cat}^{c} (Chabot et al., 1972; Weber et al., 1977; Sage, 2002) or $S_{c/o}$ (Zhu et al., 1998; Galmés et al., 2005). Actually, differences in the energy of activation of k_{cat}^{c} and $S_{c/o}$ seems to be ascribed to the thermal conditions typically encountered by the species in their native habitat (Galmés et al., 2005), as well as to the photosynthetic mechanism (Perdomo et al., 2015).

The variability in the response of Rubisco kinetics to changes in temperature, if confirmed, is of paramount importance. The mechanistic models of photosynthesis at leaf, canopy and ecosystem levels are based on the kinetic properties of Rubisco (Farquhar et al., 1980; von Caemmerer, 2000; Bernacchi et al., 2002) and the accuracy of these photosynthetic models depends on knowing the Rubisco kinetic parameters and their species-specific equations for the Rubisco-temperature dependencies (e.g. Niinemets et al., 2009; Yamori and von Caemmerer, 2009; Bermúdez et al., 2012; Díaz-Espejo, 2013; von Caemmerer, 2013; Walker et al., 2013). The need for estimations of the temperature dependencies of Rubisco kinetic parameters becomes timely as modellers try to predict the impact of increasing temperatures on global plant productivity (Sage et al., 2008; Gornall et al., 2010). Ideally, surveying variations in Rubisco kinetics and their temperature dependence should incorporate a correlative analysis with variations in the L- and/or S-subunit amino acid sequence. Such a complementary research would permit deciphering what residue substitutions determine the observed variability in Rubisco catalysis.

In the present study, we examined Rubisco catalytic properties and their temperature dependence in twenty crop species, thereby constituting the largest published data set of its kind. The aims of this work were: i) to compare the Rubisco kinetic parameters among the most economically important crops, ii) to search for differences in the temperature response of the main kinetic parameters among these species, iii) to test whether crop Rubiscos are optimally suited for the conditions encountered in plant chloroplasts, and iv) to unravel key amino acid replacements putatively responsible for differences in Rubisco kinetics in crops.

RESULTS

The variability in Rubisco kinetics at 25 ºC among the most relevant crop species

When considering exclusively the 18 C_3 crop species, at 25 ºC, the Rubisco Michaelis-Menten constant for CO_2 under non-oxygenic (K_c) and 21% O_2 (K_c^{air}) varied c.a. two-fold and three-fold, respectively, and the maximum rates of Rubisco carboxylation (k_{cat}^{c}) varied c.a. two-fold (Table 1). For K_c and K_c^{air}, *Manihot esculenta* presented the lowest values (K_c = 6.1 μM, and K_c^{air} = 10.8 μM) and *Spinacia oleracea* the highest (K_c = 14.1 μM, and K_c^{air} = 26.9 μM). Values for k_{cat}^{c} varied between 1.4 s^{-1} (*Manihot esculenta*) and 2.5 s^{-1} (*Ipomoea batatas*). The Rubisco CO_2/O_2 specificity ($S_{c/o}$) was the kinetic parameter with the lowest variation among the C_3 species (Fig. S1), and ranged between 92.4 mol mol^{-1} (*Solanum lycopersicum*) and 100.8 mol mol^{-1} (*Beta vulgaris* and *Manihot esculenta*) (Table 1). *Brassica oleracea* and *Glycine max* presented the lowest value for the Rubisco carboxylase catalytic efficiency, calculated as k_{cat}^{c}/K_c (0.17 s^{-1} μM^{-1}), and *Coffea arabica* presented the lowest value for the k_{cat}^{c}/K_c^{air} ratio (0.08 s^{-1} μM^{-1}). With regard to the oxygenase catalytic efficiency (calculated as k_{cat}^{o}/K_o), *Spinacia oleracea* displayed the lowest value (1.76 s^{-1} nM^{-1}). *Hordeum vulgare* presented the highest values for the Rubisco carboxylase and oxygenase catalytic efficiencies (k_{cat}^{c}/K_c = 0.28 s^{-1} μM^{-1}, k_{cat}^{c}/K_c^{air} = 0.17 s^{-1} μM^{-1} and k_{cat}^{o}/K_o = 3.01 s^{-1} nM^{-1}).

When data from the two C_4 species (*Saccharum* × *officinarum* and *Zea mays*) were included in the comparison at 25 ºC, the range of variability increased for all parameters (Table 1). Rubisco from the two C_4 species presented higher k_{cat}^{c} but lower affinity for CO_2 (i.e., higher K_c and K_c^{air}, and lower $S_{c/o}$) than Rubisco from C_3 crops. On average, k_{cat}^{c}/K_c and k_{cat}^{o}/K_o of C_4 Rubiscos were 62 % and 70 % of those of C_3 crop Rubiscos, respectively.

The temperature response of Rubisco kinetics in crops and trade-offs between catalytic traits

Both the range of variation and the species showing the extreme values of Rubisco kinetics at 15 ºC and 35 ºC were similar to those described at 25 ºC, with some exceptions. As at 25 ºC, among the C_3 crops, Rubisco from *Manihot esculenta* presented the lowest values for K_c and K_c^{air} at 15 ºC and 35 ºC, while the highest values were measured on Rubisco from *Spinacia oleracea* (Table S1). The lowest and highest values for k_{cat}^{c} at 15 ºC were those of Rubisco from *Cucurbita maxima* and *Hordeum vulgare*, respectively. The degree of dispersion of the data and the range of variation between the maximum and the minimum values for K_c, K_c^{air} and k_{cat}^{c} increased with the increment in the assay temperature (Table S1 and Fig. S1). Regarding $S_{c/o}$, values ranged between 116.1 mol mol^{-1} (*Brassica oleracea*) and 132.2 mol mol^{-1} (*Cucurbita maxima*) at 15 ºC, and between 74.2 mol mol^{-1} (*Oryza sativa*) and 85.0 mol mol^{-1} (*Manihot esculenta*) at 35 ºC (Table S1). As for $S_{c/o}$, the range of variation for k_{cat}^{c}/K_c and k_{cat}^{o}/K_o was also narrowed with the increase in the assay temperature (Table S1 and Fig. S1).

Integrating all data across three assay temperatures, k_{cat}^{c} correlated positively with K_c for both C_3 ($r^2 = 0.82$, $p < 0.001$) and C_4 species ($r^2 = 0.94$, $p < 0.001$), with

Rubisco from C_4 species showing higher K_c for a given k_{cat}^c than that from C_3 species (Fig. 1A). The low interspecific variability in $S_{c/o}$ within each assay temperature determined a non-linear relationship between k_{cat}^c and $S_{c/o}$ when considering data from all temperatures together (Fig. 1B). At each temperature individually, Pearson's correlations between k_{cat}^c and K_c and $S_{c/o}$ were highly significant (Table 2) when considering both C_3 and C_4 together. The results from the Phylogenetically Independent Contrasts (PICs) analyses were in general more conservative compared to Pearson's correlations (Table 2), and some significant correlations were lost with PICs (e.g., $S_{c/o}$ *vs*. K_c or K_c^{air} at 25 ºC). Notably, when excluding the two C_4 species, PCCs decreased in almost all correlations (Table 2). Hence, the PCC between k_{cat}^c and K_c was no longer significant at 15 ºC, and the PCC between k_{cat}^c and $S_{c/o}$ was significant only at 15 ºC. Furthermore, when considering only C_3 species, the unique significant PICs between k_{cat}^c and K_c and $S_{c/o}$ were those found between k_{cat}^c and $S_{c/o}$ at 15 ºC and 25 ºC.

The energy of activation (ΔH_a) for K_c varied between 38.2 kJ mol^{-1} (*Solanum tuberosum*) and 83.1 kJ mol^{-1} (*Oryza sativa*; Table 3). *Ipomoea batatas* (40.7 kJ mol^{-1}) and *Manihot esculenta* (75.4 kJ mol^{-1}) were the species showing the lowest and highest values for ΔH_a of K_c^{air}. As for k_{cat}^c, ΔH_a varied between 27.9 kJ mol^{-1} (*Hordeum vulgare*) and 60.5 kJ mol^{-1} (*Medicago sativa*). Although the range of variation across C_3 species was similar for the energies of activation of both K_c and k_{cat}^c (2.2-fold), non-significant correlation was observed between ΔH_a for K_c and ΔH_a for k_{cat}^c in both conventional and phylogenetically independent analyses (r^2 = 0.11 and 0.15, respectively; $P > 0.05$). The lowest and highest values for ΔH_a of the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*, calculated from $S_{c/o}$) were measured in *Beta vulgaris* (19.8 kJ mol^{-1}) and *Glycine max* (26.5 kJ mol^{-1}), respectively. On average, Rubisco from C_3 crops presented significantly higher ΔH_a for K_c (60.9 ± 1.5 kJ mol^{-1}) and k_{cat}^c (43.7 ± 1.5 kJ mol^{-1}) than Rubisco from C_4 species ($K_c = 52.4 \pm 5.0$ kJ mol^{-1}, $k_{cat}^c = 30.6 \pm 1.6$ kJ mol^{-1}). By contrast, non-significant differences were observed in the average ΔH_a for Γ^* between C_3 (22.9 ± 0.4 kJ mol^{-1}) and C_4 species (25.0 ± 0.7 kJ mol^{-1}).

The CO_2 assimilation potential of Rubisco kinetics in crops

The CO_2 assimilation potential of Rubisco ($A_{Rubisco}$) was modelled at varying temperature and CO_2 availability at the catalytic site (C_c) using the species-specific kinetic data measured at each temperature (from Tables 1 and S2). The simulated value of $C_c = 250$ μbar is representative of that encountered in the chloroplast stroma of C_3 species under well-watered conditions (e.g., Bermúdez et al., 2012; Scafaro et al., 2012; Galmés et al., 2013). Under mild to moderate water stress, when no metabolic impairment is present, the decrease in the stomatal and leaf mesophyll conductances to CO_2 provokes a decrease in the concentration of CO_2 in the chloroplast (Flexas et al., 2006). We selected a value of 150 μbar to simulate the chloroplastic CO_2 concentration in water stressed plants.

Differences in $A_{Rubisco}$ across species were largely dependent on the temperature and the availability of CO_2 for carboxylation (Fig. 2). This fact was due to the different prevalence of RuBP-saturated (A_c) and RuBP-limited (A_j) rates governing $A_{Rubisco}$ under the contrasting temperature and C_c, assuming an invariable concentration of active Rubisco sites of 25 μmol m^{-2} for all species. At 15 ºC, A_c limited $A_{Rubisco}$ at C_c of 150 μbar in nine species (indicated by asterisks in Fig. 2). At 15 ºC and C_c of 250 μbar, only six species were A_c limited (*Capsicum annuum*, *Cucurbita maxima*, *Medicago sativa*, *Oryza sativa*, *Solanum tuberosum* and *Spinacia oleracea*). At 25 and 35 ºC, $A_{Rubisco}$ was A_c-limited in all C_3 species irrespective of C_c.

At 25 ºC, the *best* Rubisco was that from *Hordeum vulgare* at both C_c, while Rubisco from *Glycine max* yielded the lowest $A_{Rubisco}$ (Fig. 2). Rubisco from *Beta vulgaris* presented the best performance at 35 ºC irrespective of the CO_2 availability, while *Capsicum annuum* and *Saccharum* × *officinarum* Rubisco gave the lowest $A_{Rubisco}$ at C_c of 250 and 150 μbar, respectively. At 15 ºC and C_c of 250 μbar, the highest potential for CO_2 assimilation was found in Rubisco from *Glycine max*, *Manihot esculenta* and *Triticum aestivum*, while Rubisco from *Manihot esculenta* gave the highest $A_{Rubisco}$ at 15 ºC and C_c of 150 μbar. Rubisco from *Cucurbita maxima* displayed the lowest $A_{Rubisco}$ at 15 ºC, regardless of the CO_2 availability. It is interesting to note that Rubisco from the two C_4 species, in particular from *Saccharum* × *officinarum*, performed better than the average C_3 Rubiscos when $A_{Rubisco}$ was simulated according to the photosynthesis model for C_3 leaves (Farquhar et al., 1980), at 15 ºC and 25 ºC under C_c of 250 μbar (Fig. 2A). At lower C_c (150 μbar), the C_4 Rubiscos yielded higher $A_{Rubisco}$ values than the average C_3 Rubiscos at 15 ºC, and lower values at 35 ºC, being similar at 25 ºC (Fig. 2B).

To test the performance of the different Rubiscos in the context of C_4 photosynthesis, A_c was also modelled assuming C_c of 5000 μbar and E of 15 μmol m^{-2}. Under these conditions, the advantage of C_4-type Rubisco kinetics of *Saccharum* × *officinarum* and *Zea mays* - characterised by higher k_{cat}^c and K_c^{air} - became evident as providing higher A_c values at the three temperatures (data not shown). On average, at saturating CO_2 and lower concentration of Rubisco catalytic sites, C_4 Rubiscos yielded A_c of 35, 49 and 60 μmol m^{-2} s^{-1} at 15, 25 and 35 ºC, respectively, compared to C_3-Rubiscos average (10, 23 and 42 μmol m^{-2} s^{-1}, respectively).

Positively selected L-subunit residues: relationship with Rubisco kinetics

The phylogeny obtained with *rbcL, matK* and *ndhF* genes matched currently accepted angiosperm classification (Fig. S2) (Bremer et al., 2009).

When considering all species together, 10 L-subunit residues were under positive selection: 94, 262, 281, 309, 439, 446, 449, 470, 477 and amino acid insert between residues 468 and 469. Moreover, positive selection was identified in specific L-subunit residues along branches leading to species with high and low K_c, high k_{cat}^c and low $S_{c/o}$ at 25 ºC and low ΔH_a for K_c (Table 4). The residues under positive selection were located at different positions within the Rubisco tertiary structure and included functionally diverse sites participating in L-subunit intradimer and dimer-dimer interactions, interactions with small subunits (S-subunit) and with Rubisco activase (Table 4). No residue under positive selection was associated with ΔH_a for K_c^{air}, ΔH_a for k_{cat}^c or ΔH_a for $S_{c/o}$.

DISCUSSION

Main crops possess Rubiscos with different performance at 25 ºC

The kinetic data reported in the present study are consistent with the range previously reported for higher plants at 25 ºC (e.g., Yeoh et al., 1980, 1981; Bird et al., 1982; Jordan and Ogren, 1983; Kent and Tomany, 1995; Galmés et al., 2005, 2014a, 2014c; Ishikawa et al., 2009; Prins et al., 2016) (Table 1), and showing the existence of significant variation among species in the carboxylase catalytic efficiency under non-oxygenic (k_{cat}^c/K_c) and atmospheric conditions (k_{cat}^c/K_c^{air}). Recent reports related k_{cat}^c/K_c variation with the growth capacity in a group of closely related species with similar ecology (Galmés et al., 2014a), suggesting that improving this ratio would be an effective way to engineer a better Rubisco. Nevertheless, such an improvement becomes constrained by the trade-offs between k_{cat}^{c}, K_c and $S_{c/o}$ (Tcherkez et al., 2006; Savir et al., 2010; Galmés et al., 2014a, 2014c). Here, we demonstrate that these trade-offs, in particular k_{cat}^{c} *vs*. K_c, are hold when considering C_3 and C_4 species together, even after accounting for the phylogenetic signal in the data, and that they generally strengthen at increasing assay temperatures (Table 2). However, most of these trade-offs were lost when considering exclusively the C_3 species (Table 2), indicative that the broad-scale patterns of covariation between the Rubisco kinetic parameters may not hold at smaller scales, as previously observed in other angiosperm species (Galmés et al., 2014c).

The maximum carboxylase turnover rate of Rubisco (k_{cat}^{c}) from *Zea mays* and *Saccharum* × *officinarum* was 2-fold higher than that of the C_3 species, albeit at the expenses of 3 times less affinity for CO_2 (Table 1). This finding agrees with previously described trends between C_3 and C_4 species (Kubien et al., 2008; Ghannoum et al., 2005; Ishikawa et al., 2009), and with the fact that C_4 species present lower k_{cat}^{c}/K_c (Kubien et al., 2008; Perdomo et al., 2015).

Unlike other reports (Sage 2002; Ishikawa et al., 2009), the observed variation in the kinetic parameters at 25 ºC among C_3 species was apparently not related to the thermal climate of their respective domestication regions (data not shown). It should be noted that the origin, and hence the climatic conditions, of the selected varieties could be different to the species centre of domestication, and that the different crop varieties may have accumulated adaptive changes to local conditions by means of artificial selection (Meyer et al., 2012). Intraspecific variability in Rubisco catalytic traits has been reported in *Triticum aestivum* (Galmés et al., 2014c) and *Hordeum* *vulgare* (Rinehart et al., 1983), but how this variability among genotypes is related to adaptation of Rubisco to local environments remains elusive.

The Rubisco kinetic parameters of the main crops present different thermal sensitivity

The observed temperature response of the Rubisco kinetics parameters confirms well-described trends consisting in increases in k_{cat}^{c} and K_c and a decrease in $S_{c/o}$ with increasing assay temperature (Table 1 and Table S1) (Jordan and Ogren, 1984; Brooks and Farquhar, 1985; Uemura et al., 1997; Galmés et al., 2005; Prins et al., 2016).

The temperature dependency of full Rubisco catalytic constants was first provided for *Nicotiana tabacum*, using *in vivo*-based leaf gas exchange analysis (Bernacchi et al., 2001). After this report, all studies dealing with the temperature response of photosynthesis assumed the temperature dependency parameters of tobacco Rubisco, irrespective of the modelled species, from annual herbs to trees, and from cold to warm adapted species (e.g., Pons et al., 2009; Keenan et al., 2010; Yamori et al., 2010; Galmés et al., 2011; Bermúdez et al., 2012; Scafaro et al., 2012). Importantly, the present dataset constitutes the most unequivocal confirmation that different temperature sensitivities of Rubisco kinetic parameters exist among different species, and that extrapolating the temperature response of a unique model species to other plants induces errors when modelling the temperature response of photosynthesis. In this sense, the *in vitro* results of the present study support *in vivo* data showing different temperature dependency of Rubisco catalytic constants in *Arabidopsis thaliana* and *Nicotiana tabacum* (Walker et al., 2013).

In general, the Rubisco constant affinities for CO_2 (K_c and K_c^{air}) were more sensitive to changes in assay temperature (i.e., presented higher energies of activation, ΔH_a) than k_{cat}^c and Γ^* (Table 3), in agreement with a recent study (Perdomo et al., 2015). This fact is explained by the increase in the oxygenase catalytic efficiency (k_{cat}^o/K_o) at increasing temperature. However, it should be remarked that k_{cat}^o/K_o ratio was calculated from the measured parameters K_c, k_{cat}^c and $S_{c/o}$, and that direct measurements of the oxygenase activity of Rubisco, e.g., by mass spectrometry (Cousins et al., 2010), should be undertaken to confirm this trend.

As at 25 ºC, the differences in the temperature dependencies of Rubisco kinetic parameters among C_3 species were not related to the thermal environment of the species' domestication regions (data not shown). This finding contrasts with previous evidences suggesting that the temperature sensitivity of Rubisco kinetic properties have evolved to improve the enzyme's performance according to the prevailing thermal environment to which species are adapted (Sage, 2002; Galmés et al., 2005, 2015).

Although only two C_4 species were included in the present study, they presented lower ΔH_a for K_c and for k_{cat}^c than most of the C_3 species, in close agreement with trends recently observed by Perdomo et al. (2015) in *Flaveria* species (Table 3). A larger number of C_4 species need to be surveyed to verify the existence of differences in the temperature dependence of Rubisco kinetics between C_3 and C_4 species.

How do the species-specific properties of Rubisco kinetics and their temperature sensitivity impact the potential capacity of Rubisco to assimilate CO_2?

Modelling the effect of the species-specific Rubisco kinetics and temperature dependencies of Rubisco kinetics resulted in significant differences in the Rubisco CO_2 assimilation potential ($A_{Rubisco}$) among the studied C_3 crops (Fig. 2). This modelling exercise highlighted which species would mostly benefit from the genetic replacement of their native version of Rubisco by other foreign versions with improved performance. Notably, the modelling results clearly indicate that the performance of specific Rubiscos cannot be evaluated without considering the environmental conditions during catalysis, specifically the temperature and the CO_2 availability at the site of carboxylation (C_c). This fact results from the different temperature dependence of Rubisco kinetics among crops, and from the different impact that Rubisco kinetics have on the RuBP-saturated (A_c) and RuBP-limited (A_j) rates governing $A_{Rubisco}$. Hence, at 15 ºC and C_c of 250 μbar, $A_{Rubisco}$ was limited by A_j in most of the C_3 species (twelve out of eighteen), while it was limited by A_c in all C_3 species at 25 and 35 ºC irrespective of the C_c value.

Detailed examination of modelled $A_{Rubisco}$ suggests that future efforts to enhance Rubisco efficiency should be directed on the following C_3 species displaying the poorest performance: *Cucurbita maxima* and *Medicago sativa* at 15 ºC and both C_c, *Glycine max*, *Capsicum annuum* and *Coffea arabica* at 25 ºC and 250 μbar; *Glycine max*, *Spinacia oleracea*, *Capsicum annuum* and *Coffea arabica* at 25 ºC and 150 μbar; *Capsicum annuum*, *Solanum lycopersicum* and *Lactuca sativa* at 35 ºC and 250 μbar; and *Capsicum annuum* and *Solanum lycopersicum* at 35 ºC and 150 μbar.

In order to focus on the Rubisco catalytic traits, the modelling assumed invariable values for the concentration of active Rubisco sites (E = 25 μmol m^{-2} s^{-1}) and specific values for the rate of photosynthetic electron transport (J) and C_c. However, species adapt and plants acclimate to the prevailing thermal environment through changes in the concentration and/or activation of Rubisco and the rate of photosynthetic electron transport (Yamasaki et al., 2002; Yamori et al., 2011). Similarly, stomatal (g_s) and leaf mesophyll (g_m) conductances to CO_2 also vary in response to temperature (von Caemmerer and Evans, 2015). Considering the growth temperature effects on these parameters would have altered the equilibrium between A_c and A_j, and indirectly, the consequences of different Rubisco kinetic traits on the CO_2 assimilation potential. In the next future, we aim to increase the accuracy of the present simulation by examining and including the species-specific values for g_s, g_m, *E* and *J* at varying environmental conditions.

The analysis of positive selection in branches leading to specific Rubisco traits may reveal lineage specific amino acid substitutions

We found ten Rubisco L-subunit residues under positive selection (94, 262, 281, 309, 439, 446, 449, 469, 470, and 477; Table 4). With the exceptions of residues 469 and 477, these residues have been reported previously in other groups of plants, implying a relatively limited number of residues responsible for the Rubisco ‘fine-tuning’ (Kapralov and Filatov, 2007; Christin et al., 2008; Iida et al., 2009; Kapralov et al., 2011; Kapralov et al., 2012; Galmés et al., 2014a, 2014c). However, despite widespread parallel evolution of amino acid replacements in the Rubisco sequence, solutions found in particular groups of plants may be quite different. For instance, there are only two common residues under positive selection out of ten between this study and methodologically similar work with different sampling design published earlier (Galmés et al., 2014c). This fact raises questions of epistatic interactions and residue co-evolution within Rubisco (Wang et al., 2011) as well as residue co-evolution and complementarity between Rubisco and its chaperones (Whitney et al., 2015), which both may prevent evolution of identical amino acid replacements because of different genetic backgrounds.

We have not examined the species differences in the sequence of the Rubisco small subunit (S-subunit). Some of the species included in the present survey, like *Triticum aestivum*, possess a large number of S-subunit genes (*rbc*S) encoding different S-subunits (Galili et al., 1998). Previous reports have showed that species with identical L-subunits might have different Rubisco kinetics (Rosnow et al., 2015) as well as directly demonstrated that differences in the S-subunits might affect Rubisco catalytic traits (Ishikawa et al., 2011; Morita et al., 2014). Therefore, we cannot discard that the observed differences in Rubisco kinetics, and their temperature dependence, among the studied crops are partially due to differences in the S-subunits.

Conclusions

The present study confirms the significant variation in carboxylation efficiency and parameters that contribute to it among plant species, and for the first time provides full Rubisco kinetic profiles for the twenty most important crop species. Our dataset could be used as an input for the next generation of species-specific models of leaf photosynthesis and its response to climate change, leading to more precise forecasts of changes in crop productivity and yield. These data could help to decide in which crops CO_2 assimilation potential and carboxylation efficiency of Rubisco might be improved via re-engineering of native enzymes or by replacement with foreign ones as there is no a one size fits all solution. The design of future attempts of Rubisco engineering in crops should be based on surveys of Rubisco catalytic and genetic diversity with a particular stress on the relatives of crops in question. Growing knowledge of the Rubisco catalytic spectrum combined with the existing engineering toolkits for Rubisco (Whitney and Sharwood 2008) and its chaperones (Whitney et al., 2015) give us a hope that Rubisco efficiency and hence photosynthetic capacity of crops could be improved in a near future.

MATERIALS AND METHODS

Species selection and growth conditions

The following twenty crop species were selected for study: *Avena sativa* L. cv. Forridena, *Beta vulgaris* L. cv. Detroit, *Brassica oleracea* L. var. *italica* cv. Calabres, *Capsicum annuum* L. cv. Picante, *Coffea arabica* L. cv 'Catuaı´ Vermelho IAC 44, *Cucurbita maxima* D. cv. Totanera, *Glycine max* (L.) Merr cv. Ransom, *Hordeum vulgare* L. subsp. *vulgare* cv. Morex, *Ipomoea batatas* (L.) Lam var. *Rosa de Málaga*, *Lactuca sativa* L. cv. Cogollo de Tudela, *Manihot esculenta* C., *Medicago sativa* L. cv. Aragón, *Oryza sativa* L. cv. Bomba, *Phaseolus vulgaris* L. cv. Contender, *Saccharum* × *officinarum* (hybrid between *Saccharum officinarum* and *S. spontaneum*), *Solanum lycopersicum* L. cv. Roma VF, *Solanum tuberosum* L. cv. Erlanger, *Spinacia oleracea* L. cv. Butterfly, *Triticum aestivum* L. cv. Cajeme, *Zea mays* L. cv. Carella. These species represent the most important crops in terms of worldwide production (FAOSTAT, 2010). *Coffea arabica* was selected as being the most important commodity in the international agricultural trade (DaMatta 2004). Plants were grown from seeds under natural photoperiod in a glasshouse at the University of the Balearic Islands (Spain) during 2011 and 2012. Plants were grown in soil-based compost supplemented with slow-release fertilizer and frequently watered to avoid water stress. The air temperature in the glasshouse during the growth period was maintained between 15ºC and 30ºC.

Determination of the Rubisco Michaelis-Menten constant for CO_2 (K_c) and the maximum carboxylase turnover rate (k_{cat}^c)

The Rubisco Michaelis-Menten constant for CO_2 under 0% O_2 (K_c) and 21% O_2 (K_c^{air}) were determined in crude extracts obtained as detailed in Galmés et al., (2014a). Rates of $^{14}CO_2$-fixation were measured at 15 ºC, 25 ºC and 35 ºC using activated protein extracts in 7 mL septum capped scintillation vials containing reaction buffer (100 mM Bicine-NaOH pH 8.0, 20 mM $MgCl_2$, 0.4 mM RuBP and about 100 W-A units of carbonic anhydrase) previously equilibrated either with nitrogen (N_2) or a mixture of O_2 and N_2 (21:79). Nine different concentrations of $H^{14}CO_3^-$ (0.1 to 9.4 mM, each with a specific radioactivity of 3.7×10^{10} Bq mol^{-1}) were prepared in the scintillation vials as described previously (Galmés et al., 2014a). Assays at 35 ºC using Rubisco from C_4 species required increasing $H^{14}CO_3^-$ up to 17.7 mM to reach saturating CO_2 concentration in the aqueous-phase. Assays were started by the addition of 10 μL of protein extract and stopped after 1 min by injection of 0.1 mL 10 M formic acid. Acid-stable ^{14}C was determined by liquid scintillation counting (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, USA) following removal of acid-labile ^{14}C by evaporation. The Michaelis-Menten constants for CO_2 under 0% O_2 (K_c) and 21% O_2 (K_c^{air}) were determined from the fitted data as described elsewhere (Bird et al., 1982). Replicate measurements (n = 3-6) were made using different biological replicates for each species.

To obtain k_{cat}^{c}, the maximum rate of carboxylation was extrapolated from the Michaelis-Menten fit and divided by the number of Rubisco active sites in solution, quantified by [^{14}C] CABP binding (Yokota and Canvin 1985).

Additional control assays undertaken as detailed in Galmés et al. (2014a) confirmed that the observed acid stable ^{14}C signal was uniquely the result of Rubisco catalytic activity.

Determination of the Rubisco specificity for CO_2/O_2 ($S_{c/o}$)

The Rubisco CO_2/O_2 specificity ($S_{c/o}$) was measured on purified extracts obtained as in Gago et al., (2013). On the day of $S_{c/o}$ measurement, highly concentrated Rubisco solutions were desalted by centrifugation through G25 Sephadex columns 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 with $NaH^{14}CO^3$ (1.85×10^{12} Bq mol^{-1}) and 4 mM NaH_2PO_4, to activate Rubisco by incubation at 37.5°C for 40 min. Reaction mixtures were prepared in oxygen electrodes (Oxygraph, Hansatech instruments Ltd., Norfolk, UK) by first adding 0.95 mL of CO_2-free assay buffer (100 mM Bicine pH 8.2, 20 mM $MgCl_2$, containing 0.015 mg of carbonic anhydrase). After the addition of 0.02 mL of 0.1 M $NaH^{14}CO_3$ (1.85×10^{12} Bq mol^{-1}), the plug was fitted to the oxygen electrode vessel and enough activated Rubisco (20 μL) was added. The reaction was started by the injection of 10 μL of 25 mM RuBP to be completed between 2 and 7 min depending on the assay temperature. 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 (Galmés et al., 2014a). Measurements were performed at 15 ºC, 25 ºC and 35 ºC, with 3-9 biological replicates per each species and assayed temperature.

For all Rubisco assays, pH of the assay buffers was accurately adjusted at each temperature of measurement. The concentration of CO_2 in solution in equilibrium with HCO_3^- was calculated assuming a pK_a for carbonic acid of 6.19, 6.11 and 6.06 at 15 ºC, 25 ºC and 35 ºC, respectively. The concentration of O_2 in solution was assumed to be 305.0, 253.4 and 219.4 (nmol mL^{-1}) at 15 ºC, 25 ºC and 35 ºC, respectively (Truesdale and Downing 1954).

Temperature dependence parameters of Rubisco kinetics

To determine the temperature response of the Rubisco kinetic parameters from each species, values for K_c, K_c^{air} and $S_{c/o}$ were first converted from concentrations to partial pressures. For this, solubilities for CO_2 were considered to be 0.0450, 0.0340 and 0.0262 mol L^{-1} bar^{-1} at 15 ºC, 25 ºC and 35 ºC, respectively. In turn, solubilities for O_2 of 0.0016, 0.0013 and 0.0011 mol L^{-1} bar^{-1} were used at 15 ºC, 25 ºC and 35 ºC, respectively. The CO_2 compensation point in the absence of mitochondrial respiration (Γ*) was obtained from $S_{c/o}$ as in von Caemmerer (2000) using the above solubilities for O_2. Thereafter, values of K_c, Γ* and k_{cat}^c at the three temperatures were fitted to an Arrhenius-type equation (Badger and Collatz 1977; Harley and Tenhunen 1991):

$$Parameter = \exp\left[c - \frac{\Delta H_a}{RT_k}\right]$$

where c is a scaling constant, ΔH_a is the energy of activation, R is the molar gas constant (8.314 J K^{-1} mol^{-1}) and T_k is the absolute assay temperature.

CO_2 assimilation potential of crop Rubiscos at varying temperatures and CO_2 availability

According to the biochemical model of C_3 photosynthesis (Farquhar et al., 1980), the Rubisco CO_2 assimilation potential ($A_{Rubisco}$) is defined as the minimum of the RuBP-saturated (A_c) and RuBP-limited (A_j) CO_2 assimilation rates:

(1) $A_{Rubisco} = \min(A_c, A_j)$,

(2) $A_c = \frac{k_{cat}^{c} \, . \, E \, . \, (C_C - \Gamma^*)}{C_C + K_c^{air}}$

(3) $A_j = \frac{(C_C - \Gamma^*) \; J}{4C_C + 8\Gamma^*}$

$A_{Rubisco}$ was obtained for each species at three different temperatures, 15 ºC, 25 ºC and 35 ºC, and two different concentrations of CO_2 in the chloroplast stroma (C_c), 150 and 250 μbar, simulating situations of moderate water-stress and well-watered conditions in C_3 plants, respectively (Flexas et al., 2006). The Rubisco catalytic traits k_{cat}^{c}, Γ^* and K_c^{air} were taken from the species- and temperature-specific data obtained in the present study. The concentration of active Rubisco sites (E) was assumed invariable at 25 μmol m^{-2}. Values of the CO_2-saturated photosynthetic electron transport rates (J) were assumed 60, 150 and 212 μmol m^{-2} s^{-1} at 15 ºC, 25 ºC and 35 ºC, respectively, for all species. At 25 ºC, J = 150 μmol m^{-2} s^{-1} matches very well with a $J/(k_{cat}^{c} \cdot E)$ ratio of 1.5 (Egea et al., 2011). Values for J at 15 ºC and 35 ºC were obtained from the J temperature response described for tobacco in Walker et al. (2013).

Analysis of Rubisco L-subunit sites under positive selection

Full length DNA sequences of the Rubisco large subunit (L-subunit) encoding gene, *rbcL* (Fig. S3), and two additional chloroplast genes (*matK* and *ndhF*) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for the twenty studied species. Accession numbers information is given in the Table S2.

DNA sequences were translated into protein sequences for alignment using MUSCLE (Edgar 2004). The software MODELTEST 3.7 (Posada and Crandall 1998, Posada and Buckley 2004) was used to check for the best model before running the phylogenetic analyses. The species phylogeny was reconstructed using concatenated alignment of all three chloroplast genes and maximum-likelihood inference conducted with RAxML version 7.2.6 (Stamatakis 2006).

Amino acid residues under positive selection were identified using codon-based substitution models in comparative analysis of protein-coding DNA sequences within the phylogenetic framework (Yang 1997). Given the conservative assumption of no selective pressure at synonymous sites, codon-based substitution models assume that codons with the ratio of nonsynonymous/synonymous substitution rate (d_N/d_S) less than one evolve under purifying selection to keep protein function and properties, while codons with $d_N/d_S > 1$ evolve under positive Darwinian selection to modify properties of the given protein (Yang 1997).

The codeml program in the PAML v4.7 package (Yang 2007) was used to perform branch-site tests of positive selection along pre-specified foreground branches (Yang et al., 2005, Yang 2007). The codeml A model allows $0 \le d_N/d_S \le 1$ and $d_N/d_S = 1$ for all branches. The $d_N/d_S > 1$ is permitted only along pre-specified foreground branches and $0 \le d_N/d_S \le 1$ and $d_N/d_S = 1$ on background branches. Branches leading to species with high or low K_c, k_{cat}^c, $S_{c/o}$ and ΔH_a were marked as foreground branches. For the purpose of these tests, high or low K_c, k_{cat}^c and $S_{c/o}$ ranges were taken only at 25 °C because of high correlation between values for these kinetic parameters obtained at three different temperatures. ΔH_a for these kinetic parameters were also considered. The A model was used to identify the amino acid sites under positive selection and to calculate the posterior probabilities of an amino acid belongs to a class with d_N/d_S >1 using the Bayes empirical Bayes (BEB) approach implemented in PAML (Yang et al., 2005).

The Rubisco L-subunit residues were numbered based on the spinach sequence. The location of sites under positive selection was done using Rubisco protein structure from spinach (*Spinacia oleracea* L.) obtained from the RCSB Protein Data Bank (http://www.rcsb.org; file 1RCX; Karkehabadi et al., 2003).

Statistical analysis

Statistical analysis consisted of one-way ANOVA and correlation for linear regressions. For all the parameters studied, a univariate model of fixed effects was assumed. The univariate general linear model for unbalanced data (Proc. GLM) was applied and significant differences among species and groups of species were revealed by Duncan tests using IBM SPSS Statistics for Macintosh, Version 21.0. (Armonk, NY: IBM Corp software package). The relationships among the kinetic parameters and the temperature dependence parameters were tested with the square of the correlation coefficient observed for linear regressions using the tool implemented in R 3.1.1 (R Development Core Team 2014, http://www.R-project.org). All statistical tests were considered significant at $p < 0.05$.

The Pearson correlation coefficient was calculated between pairwise combinations of the kinetic parameters K_c, K_c^{air}, k_{cat}^c and $S_{c/o}$ at the three temperatures of measurement. However, correlations arising within groups of related taxa might reflect phylogenetic signal rather than true cause-effect relationships, because closely related taxa are not necessarily independent data points and could violate the assumption of randomized sampling employed by conventional statistical methods (Felsenstein 1985). To overcome this issue, tests were performed for the presence of phylogenetic signal in the data and trait correlations were calculated with phylogenetically independent contrasts using the AOT module of PHYLOCOM (Webb et al., 2008) using the species phylogeny based on the three chloroplast genes (see below). All these tests were considered significant at $p < 0.05$.

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Supplemental Material

Table S1. The Rubisco kinetic parameters measured at 15 ºC and 35 ºC for the selected crop species.

Table S2. List of crop species and GenBank accession numbers for *rbcL*, *matK* and *ndhF*.

Figure S1. Box plots depiction of Rubisco kinetic parameters (K_c, K_c^{air}, k_{cat}^{c} and $S_{c/o}$) at 15 °C, 25 °C and 35 °C when considering the 18 C_3 species alone.

Figure S2. Maximum likelihood phylogeny created using *rbcL*, *matK* and *ndhF* for the selected crop species.

Figure S3. Rubisco L-subunit amino acid alignment for the 20 crops species used in this study.

Table 1. Kinetic parameters of crop Rubiscos measured at 25 °C: the Michaelis-Menten constants for CO_2 under non-oxygenic (K_c) and 21% O_2 (K_c^{air}), the maximum carboxylation rate (k_{cat}^c), the specificity factor ($S_{c/o}$), and the carboxylation (k_{cat}^c/K_c and k_{cat}^c/K_c^{air}), and the oxygenation catalytic efficiencies (k_{cat}^o/K_o). The k_{cat}^o/K_o ratio was calculated as [(k_{cat}^c/K_c)/$S_{c/o}$*1000]. For each species, data are mean ± standard error (n = 3-9). Group averages were obtained from individual measurements on each species. Different letters denote statistical differences ($p < 0.05$) by Duncan analysis between C_3 and C_4 groups.

Species	K_c (µM)	K_c^{air} (µM)	k_{cat}^c (s^{-1})	$S_{c/o}$ (mol mol^{-1})	k_{cat}^c/K_c (s^{-1} μM^{-1})	k_{cat}^c/K_c^{air} (s^{-1} μM^{-1})	k_{cat}^o/K_o (s^{-1} nM^{-1})
C_3 species							
Avena sativa	10.8 ± 0.9	18.1 ± 2.0	2.3 ± 0.3	99.9 ± 3.0	0.21 ± 0.01	0.13 ± 0.03	2.14 ± 0.06
Beta vulgaris	10.8 ± 1.2	18.6 ± 1.1	2.0 ± 0.3	100.8 ± 2.0	0.19 ± 0.02	0.10 ± 0.01	1.94 ± 0.31
Brassica oleracea	11.8 ± 0.1	19.2 ± 0.3	2.1 ± 0.3	96.2 ± 1.3	0.17 ± 0.03	0.11 ± 0.02	1.81 ± 0.28
Capsicum annuum	9.6 ± 0.3	19.8 ± 1.5	1.9 ± 0.1	96.0 ± 4.5	0.20 ± 0.01	0.10 ± 0.01	1.98 ± 0.15
Coffea Arabica	11.0 ± 0.4	22.9 ± 2.4	2.1 ± 0.2	98.7 ± 3.8	0.19 ± 0.02	0.08 ± 0.01	1.98 ± 0.18
Cucurbita maxima	9.0 ± 0.5	19.2 ± 1.0	2.2 ± 0.2	98.4 ± 0.4	0.25 ± 0.04	0.12 ± 0.01	2.45 ± 0.31
Glycine max	8.6 ± 0.2	16.2 ± 0.7	1.5 ± 0.1	97.0 ± 1.1	0.17 ± 0.02	0.09 ± 0.01	1.76 ± 0.21
Hordeum vulgare	9.0 ± 0.6	14.9 ± 1.6	2.4 ± 0.2	99.2 ± 3.8	0.28 ± 0.02	0.17 ± 0.03	3.01 ± 0.19
Ipomoea batatas	12.0 ± 0.7	21.1 ± 1.0	2.5 ± 0.1	98.5 ± 6.6	0.20 ± 0.00	0.12 ± 0.01	1.96 ± 0.08
Lactuca sativa	11.1 ± 0.3	18.2 ± 1.4	2.2 ± 0.1	94.0 ± 1.9	0.19 ± 0.00	0.12 ± 0.01	2.06 ± 0.07

Manihot esculenta	6.1 ± 0.2	10.8 ± 0.6	1.4 ± 0.1	100.8 ± 0.9	0.23 ± 0.02	0.13 ± 0.01	2.24 ± 0.17
Medicago sativa	9.7 ± 1.6	16.4 ± 1.9	1.7 ± 0.1	95.6 ± 2.2	0.20 ± 0.02	0.11 ± 0.01	2.23 ± 0.36
Oryza sativa	8.0 ± 0.4	17.3 ± 2.4	2.1 ± 0.3	93.1 ± 1.2	0.26 ± 0.04	0.14 ± 0.03	2.73 ± 0.43
Phaseolus vulgaris	7.8 ± 0.3	14.0 ± 1.0	1.7 ± 0.2	99.7 ± 2.7	0.22 ± 0.02	0.13 ± 0.02	2.11 ± 0.17
Solanum lycopersicum	9.7 ± 0.4	16.6 ± 1.4	2.3 ± 0.2	92.4 ± 2.3	0.24 ± 0.02	0.14 ± 0.01	2.48 ± 0.20
Solanum tuberosum	9.6 ± 0.2	18.0 ± 0.8	2.0 ± 0.3	95.4 ± 2.3	0.22 ± 0.05	0.12 ± 0.03	2.32 ± 0.46
Spinacia oleracea	14.1 ± 0.8	26.9 ± 0.8	2.4 ± 0.1	97.0 ± 1.2	0.18 ± 0.01	0.09 ± 0.01	1.76 ± 0.13
Triticum aestivum	11.3 ± 0.4	16.0 ± 0.6	2.2 ± 0.2	100.1 ± 1.8	0.20 ± 0.02	0.14 ± 0.01	2.08 ± 0.24
C_3 average	*10.0 ± 0.3*^{a}	*18.0 ± 0.5*^{a}	*2.1 ± 0.1*^{a}	*97.5 ± 0.6*^{a}	*0.21 ± 0.01*^{a}	*0.12 ± 0.01*^{a}	*2.17 ± 0.07*^{a}
C_4 species							
Saccharum × officinarum	26.3 ± 4.0	31.7 ± 2.1	3.9 ± 0.3	82.2 ± 1.8	0.15 ± 0.02	0.13 ± 0.01	1.82 ± 0.35
Zea mays	31.6 ± 1.8	42.0 ± 2.8	4.1 ± 0.6	87.3 ± 1.4	0.11 ± 0.02	0.07 ± 0.01	1.22 ± 0. 20
C_4 average	*27.6 ± 2.3*^{b}	*36.1 ± 2.6*^{b}	*4.0 ± 0.5*^{b}	*84.4 ± 1.5*^{b}	*0.13 ± 0.02*^{b}	*0.10 ± 0.01*^{a}	*1.52 ± 0.23*^{b}

Table 2. Phylogenetically independent contrasts (PICs, upper part of the diagonals) and Pearson's correlation coefficients (PCCs, lower part of the diagonals) between the Rubisco kinetic parameters (K_c, K_c^{air}, k_{cat}^c and $S_{c/o}$) at 15 °C, 25 °C and 35 °C when considering the 20 C_3 and C_4 species together and the 18 C_3 species alone. Significant correlations are marked: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Data from C_3 and C_4 species analysed together

15 °C					25 °C					35 °C				
	K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$		K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$		K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$
K_c		0.826***	0.502*	-0.314	K_c		0.913***	0.819***	-0.202	K_c		0.960***	0.710***	-0.775***
K_c^{air}	0.927***		0.036	-0.099	K_c^{air}	0.946***		0.683***	0.037	K_c^{air}	0.962***		0.707***	-0.660**
k_{cat}^c	0.810***	0.645**		-0.660**	k_{cat}^c	0.941***	0.890***		-0.450*	k_{cat}^c	0.894***	0.858***		-0.634**
$S_{c/o}$	-0.498*	-0.361	-0.673**		$S_{c/o}$	-0.772***	-0.699***	-0.749***		$S_{c/o}$	-0.806***	-0.737***	-0.736***	

Data from C_3 species alone

15 °C					25 °C					35 °C				
	K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$		K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$		K_c	K_c^{air}	k_{cat}^c	$S_{c/o}$
K_c		0.900***	0.194	0.120	K_c		0.646**	0.256	0.057	K_c		0.907***	0.363	-0.357
K_c^{air}	0.892***		-0.118	0.394	K_c^{air}	0.829***		0.173	-0.006	K_c^{air}	0.816***		0.401	-0.155
k_{cat}^c	0.268	0.137		-0.787**	k_{cat}^c	0.698***	0.587*		-0.470*	k_{cat}^c	0.613**	0.476*		-0.285
$S_{c/o}$	0.025	0.145	-0.496*		$S_{c/o}$	-0.049	-0.162	-0.083		$S_{c/o}$	-0.386	-0.157	-0.259	

Table 3. The energy of activation (ΔH_a, kJ mol^{-1}) and *c* (dimensionless) values of the Rubisco Michaelis-Menten constants for CO_2 under non-oxygenic (K_c, µmol mol^{-1}) and 21% O_2 (K_c^{air}, µmol mol^{-1}), the maximum carboxylation rate (k_{cat}^{c}, s^{-1}) and the CO_2 compensation point in the absence of mitochondrial respiration (Γ^*,µmol mol^{-1}) for the twenty crop species. For each species, data are mean ± standard error (n = 3-9). Group averages were calculated from individual measurements on each species. Different letters denote statistical differences ($p < 0.05$) by Duncan analysis between C_3 and C_4 groups. Parameter concentrations of K_c (µM) and K_c^{air} (µM) in liquid phase (Table 1 and S2) were converted to gaseous phase partial pressures [K_c and/or K_c^{air} (µmol mol^{-1}) = parameter (µM) × K_h × Air Volume (L) / RT]. K_h is the hydrolysis constant (15 °C = 22.2, 25 °C = 29.4, 35 °C = 38.2). For the Air Volume (L): 15 °C = 23.7, 25 °C = 24.5, 35 °C = 25.4. The term Γ^* (µmol mol $^{-1}$) is derived from $0.5O/ S_{c/o}$.

	K_c		K_c^{air}		k_{cat}^{c}		Γ^*	
Species	*c*	ΔH_a	*c*	ΔH_a	*c*	ΔH_a	*c*	ΔH_a
C_3 species								
Avena sativa	31.3 ± 0.7	63.4 ± 2.0	26.0 ± 0.4	48.9 ± 1.2	17.6 ± 2.2	41.5 ± 5.5	13.3 ± 0.5	23.6 ± 1.4
Beta vulgaris	28.7 ± 1.7	57.0 ± 4.4	27.2 ± 0.7	51.8 ± 1.8	21.5 ± 3.7	51.2 ± 9.6	11.7 ± 0.4	19.8 ± 1.0
Brassica oleracea	28.1 ± 1.1	55.3 ± 2.8	26.5 ± 0.9	50.1 ± 2.4	18.8 ± 2.6	45.7 ± 6.5	12.6 ± 0.2	21.8 ± 0.5
Capsicum annuum	26.6 ± 1.5	51.8 ± 3.7	27.0 ± 1.6	51.2 ± 3.7	16.3 ± 2.8	39.2 ± 6.9	13.4 ± 0.7	24.1 ± 1.8
Coffea arabica	34.7 ± 0.3	71.5 ± 0.9	27.6 ± 1.8	52.2 ± 4.3	16.5 ± 2.6	39.0 ± 6.1	13.1 ± 0.5	23.4 ± 1.1
Cucurbita maxima	28.6 ± 0.8	57.0 ± 1.8	29.2 ± 1.1	56.8 ± 2.8	20.2 ± 1.0	48.7 ± 2.7	12.2 ± 0.9	21.1 ± 2.2

Glycine max	34.2 ± 0.5	71.1 ± 1.4	28.4 ± 1.2	55.3 ± 2.9	22.7 ± 2.5	55.2 ± 5.8	14.4 ± 1.7	26.5 ± 4.1
Hordeum vulgare	31.1 ± 1.1	63.4 ± 3.0	30.7 ± 1.9	60.9 ± 5.0	12.2 ± 1.6	27.9 ± 4.0	12.3 ± 0.2	21.2 ± 0.6
Ipomoea batatas	23.0 ± 0.7	42.4 ± 1.6	22.7 ± 1.2	40.7 ± 3.1	14.3 ± 1.5	33.4 ± 3.8	13.0 ± 0.3	22.8 ± 0.8
Lactuca sativa	28.3 ± 1.3	55.8 ± 3.2	29.0 ± 2.1	56.5 ± 5.2	14.1 ± 0.7	33.3 ± 1.7	12.3 ± 0.3	21.2 ± 0.9
Manihot esculenta	33.7 ± 1.4	70.8 ± 3.4	36.1 ± 1.1	75.4 ± 2.8	19.8 ± 1.6	47.4 ± 4.1	12.2 ± 0.2	21.1 ± 0.5
Medicago sativa	29.2 ± 1.3	58.8 ± 3.6	26.1 ± 0.4	49.5 ± 1.0	24.8 ± 1.1	60.5 ± 2.8	11.8 ± 0.2	20.1 ± 0.4
Oryza sativa	38.9 ± 0.8	83.1 ± 1.8	30.5 ± 1.2	60.5 ± 3.1	19.2 ± 1.8	46.4 ± 4.7	13.7 ± 0.5	24.6 ± 1.3
Phaseolus vulgaris	31.5 ± 0.8	64.6 ± 2.0	30.9 ± 2.7	61.7 ± 6.8	19.8 ± 2.1	47.7 ± 5.3	13.4 ± 0.6	24.1 ± 1.5
Solanum lycopersicum	30.8 ± 2.5	62.1 ± 6.3	36.0 ± 2.5	73.8 ± 6.4	14.7 ± 1.4	34.6 ± 3.6	12.5 ± 0.2	21.8 ± 0.5
Solanum tuberosum	21.1 ± 0.2	38.2 ± 0.5	24.4 ± 0.8	44.9 ± 1.9	19.2 ± 0.5	46.2 ± 1.1	13.7 ± 0.9	24.7 ± 2.2
Spinacia oleracea	34.3 ± 0.8	69.9 ± 2.2	25.1 ± 0.5	45.6 ± 1.1	20.2 ± 0.7	48.0 ± 1.8	13.5 ± 0.3	25.2 ± 1.0
Triticum aestivum	30.1 ± 0.5	60.4 ± 2.2	34.4 ± 2.2	70.1 ± 5.4	17.4 ± 1.7	41.2 ± 4.3	13.5 ± 0.2	24.2 ± 0.4
C_3 average	*30.2 ± 0.6*[a]	*60.9 ± 1.5*[a]	*28.8 ± 0.6*[a]	*55.9 ± 1.5*[a]	*18.3 ± 0.6*[a]	*43.7 ± 1.5*[a]	*13.0 ± 0.2*[a]	*22.9 ± 0.4*[a]
C_4 species								
Saccharum × officinarum	30.2 ± 1.9	58.3 ± 5.0	32.0 ± 1.0	62.3 ± 2.7	13.6 ± 1.5	30.2 ± 3.5	14.3 ± 0.6	25.8 ± 1.4
Zea mays	24.7 ± 3.4	44.5 ± 8.5	24.7 ± 3.4	44.5 ± 8.5	14.0 ± 0.9	31.0 ± 1.9	13.6 ± 0.1	24.3 ± 0.2
C_4 average	*27.9 ± 2.0*[a]	*52.4 ± 5.0*[b]	*28.9 ± 0.8*[a]	*53.7 ± 1.9*[a]	*13.7 ± 0.7*[b]	*30.6 ± 1.6*[b]	*14.0 ± 0.3*[a]	*25. 0 ± 0.7*[a]

Table 4. Amino acid replacements in the Rubisco large subunit (L-subunit) identified under positive selection by the Bayes Empirical Bayes (BEB) analysis implemented in the PAML package (Yang et al., 2005; Yang 2007) along branches of the phylogenetic tree leading to species with particular Rubisco properties**.**

Residue[a]	Amino acid changes	Location of residue	Interaction[b]
Branches leading to species with $K_c \geq 26.0$ μM and $k_{cat}^c \geq 3.9$ s^{-1} at 25 ºC (C_4 species)			
94**	D, E, K → P		ID, RA
446**	R → K	C-terminus	
469**	Insert of G or T before resi 469	C-terminus	ID
Branches leading to species with $k_{cat}^c \geq 2.5$ $s^{-}1$ at 25 ºC			
281**	A → S	Helix 4	DD, SS
Branches leading to species with $K_c \geq 10.8$ μM at 25 ºC			
439***	A → T, V	Helix G	
469*	Insert of G or T before residue 469	C-terminus	ID
470*	A, E → K, P, Q	C-terminus	ID
477**	S → E, G, P, Q	C-terminus	
Branches leading to species with $S_{c/o} \leq 94.0$ mol mol^{-1} at 25 ºC			
309**	M → I	βF Strand	ID
Branches leading to species with ΔH_a for $K_c \leq 56.0$ kJ mol^{-1}			
262**	V → A, T	Loop 3	S-subunit
439*	R → T, V	Helix G	
449**	C, S, T → A	C-terminus	
477**	K → E, G, P, Q	C-terminus	

[a] Residue numbering is based on the spinach sequence. Values for Bayesian Posterior Probabilities are: * > 0.90, ** > 0.95, *** > 0.99.

[b] Interactions in which the selected residues and/or residues within 5 Å of them are involved. ID – intradimer interactions; DD – dimer-dimer interactions; RA – interface for interactions with Rubisco

activase; SS – interactions with small subunits; interactions based on literature survey only are given in italics; after (Spreitzer and Salvucci 2002; Ott et al., 2000; Du et al., 2003).

Figure legends

Figure 1. The relationship between the turnover rate for the Rubisco carboxylase reaction (k_{cat}^c) with (A) the Michaelis–Menten affinity constant for CO_2 (K_c) and (B) the CO_2/O_2 specificity factor ($S_{c/o}$). Filled symbols correspond to C_3 species at 15 ºC (▲), 25 ºC (●) and 35 ºC (▼); open symbols correspond to C_4 species at 15 ºC (△), 25 ºC (○) and 35 ºC (▽). Each symbol represents the average value of a single species per temperature interaction.

Figure 2. Simulated CO_2 assimilation potential of Rubisco ($A_{Rubisco}$) for the C_3 and C_4 species at 15 ºC, 25 ºC and 35 ºC and at values for the chloroplastic CO_2 concentration (C_c) of (A) 250 μbar and (B) 150 μbar. Equations used to calculate $A_{Rubisco}$ were those described in the biochemical model of C_3 photosynthesis (Farquhar et al., 1980), as explained in Materials and Methods. The bars represent the minimum value of A_c- and A_j-limited $A_{Rubisco}$. Asterisks (*) above the bars indicate A_c-limited $A_{Rubisco}$ (absence of * indicate A_j-limited $A_{Rubisco}$). The rate of electron transport was considered 60, 150 and 212 μmol m^{-2} s^{-1} at 15 ºC, 25 ºC and 35 ºC, respectively. The concentration of active Rubisco sites was assumed invariable at 25 μmol m^{-2} for all the species and environmental conditions. The values used for the Rubisco kinetic parameters (k_{cat}^c, Γ^* and K_c^{air}) are those shown in Tables 1 and S2.

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