

1 **Short title:** Variability of Rubisco kinetics in crops

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8 **Article title:** Rubisco catalytic properties and temperature response in crops

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21 **One sentence summary:**

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

24

25 **List of author contributions:** J.G. conceived the project; C.H-C. and J.G. designed
26 the experiment; C.H-C. performed the experiments; C.H-C., M.V.K. and JG analyzed
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28

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37 **ABSTRACT**

38 Rubisco catalytic traits and their thermal dependence are two major factors limiting
39 the CO₂ assimilation potential of plants. In this study, we present the profile of
40 Rubisco kinetics for twenty crop species at three different temperatures. The results
41 largely confirmed the existence of significant variation in the Rubisco kinetics among
42 species. Although some of the species tended to present Rubisco with higher thermal
43 sensitivity (e.g., *Oryza sativa*) than others (e.g., *Lactuca sativa*), interspecific
44 differences depended on the kinetic parameter. Comparing the temperature response
45 of the different kinetic parameters, the Rubisco Michaelis-Menten constants for CO₂
46 (K_c and K_c^{air}) presented higher energy of activation (ΔH_a) than the maximum
47 carboxylation rate (k_{cat}^c) and the CO₂ compensation point in the absence of
48 mitochondrial respiration (Γ^*). The analysis of the Rubisco large subunit sequence
49 revealed the existence of some sites under adaptive evolution in branches with

50 specific kinetic traits. Because Rubisco kinetics and their temperature dependency
51 were species-specific, they largely affected the assimilation potential of Rubisco from
52 the different crops, especially under those conditions (i.e., low CO₂ availability at the
53 site of carboxylation and high temperature) inducing Rubisco-limited photosynthesis.
54 As an example, at 25 °C, Rubisco from *Hordeum vulgare* and *Glycine max* presented,
55 respectively, the highest and lowest potential for CO₂ assimilation at both high and
56 low chloroplastic CO₂ concentrations. In our opinion, this information is relevant to
57 improve photosynthesis models and should be considered in future attempts to design
58 more efficient Rubiscos.

59

60

61 **INTRODUCTION**

62 The reported stagnation in the annual gains of cereal yields in the last decade clearly
63 indicates that the expected demand for increased yield - at least 50% by 2050 (FAO
64 forecasts) - will not be met by conventional breeding (Zhu et al., 2010). Future
65 improvements will come from novel bioengineering approaches specifically focussed
66 on processes limiting crop productivity that have not been addressed so far (Parry et
67 al., 2012; Ort et al., 2015). A number of specific modifications to the primary
68 processes of photosynthesis that could increase canopy carbon assimilation and
69 production through step changes include the modification of the catalytic properties of
70 Rubisco (Murchie et al., 2009; Whitney et al., 2011; Parry et al., 2013; Ort et al.,
71 2015). First, biochemical models indicate that CO₂ fixation rates are limited by
72 Rubisco activity under physiologically relevant conditions (Farquhar et al., 1980; von
73 Caemmerer, 2000; Rogers, 2014). Second, Rubisco's catalytic mechanism exhibits
74 important inefficiencies which compromise photosynthetic productivity: it is a slow

75 catalyst – forcing plants to accumulate large amounts of the protein – and unable to
76 distinguish between CO₂ and O₂ – starting a wasteful side reaction with oxygen that
77 leads to the release of previously fixed CO₂, NH₂ and energy (Roy and Andrews,
78 2000). These inefficiencies not only limit the rate of CO₂ fixation, but also the
79 capacity of crops for an optimal use of resources, principally water and nitrogen
80 (Flexas et al., 2010; Parry et al., 2012).

81 Rubisco kinetic parameters has been described *in vitro* at 25 °C for about 250
82 species of higher plants, of which only c.a. 8% are crop species (e.g., Yeoh et al.,
83 1981; Sage, 2002; Bird et al., 1982; Ishikawa et al., 2009; Prins et al., 2016). This
84 amount of data revealed the existence of significant variability in the main Rubisco
85 kinetic parameters both among C₃ (Yeoh et al., 1980, 1981; Bird et al., 1982; Jordan
86 and Ogren, 1983; Parry et al., 1987; Castrillo, 1995; Delgado et al., 1995; Kent and
87 Tomany, 1995; Balaguer et al., 1996; Bota et al., 2002; Galmés et al., 2005, 2014a,
88 2014c; Ghannoum et al., 2005; Ishikawa et al., 2009) and between C₃ and C₄ species
89 (Kane et al., 1994; Sage, 2002; Kubien et al., 2008; Perdomo et al., 2014). The
90 existence of Rubiscos with different catalytic performance implies that the success –
91 in terms of photosynthetic improvement – of Rubisco engineering approaches in crops
92 will depend on the specific performance of the native enzyme from each crop species.
93 Nevertheless, our knowledge on the actual variability in Rubisco kinetics is still
94 narrow, not only because of the limited number of species that have been examined so
95 far, but mainly because complete Rubisco kinetic characterization (including the main
96 parameters) has been performed in very few species.

97 Recent modelling confirmed that Rubisco is not perfectly optimized to deliver
98 maximum rates of photosynthesis, and indicated that Rubisco optimization depends
99 on the environmental conditions under which the enzyme operates (Galmés et al.,

100 2014b). In particular, Rubisco catalytic parameters are highly sensitive to changes in
101 temperature. For instance, the maximum carboxylase turnover rate ($k_{\text{cat}}^{\text{c}}$) increases
102 exponentially with temperature (Sage, 2002; Galmés et al., 2015). However, at
103 temperatures higher than the photosynthetic thermal optimum, the increases in $k_{\text{cat}}^{\text{c}}$
104 are not translated into increased CO₂ assimilation because of the decreased affinity of
105 Rubisco for CO₂, i.e., higher Michaelis-Menten constant for CO₂ (K_{c}) and lower
106 specificity factor ($S_{\text{c/o}}$), and the decreased CO₂/O₂ concentration ratio in solution (Hall
107 and Keys, 1983; Jordan and Ogren, 1984). These changes favour the RuBP
108 oxygenation by Rubisco relative to carboxylation, increasing the flux through
109 photorespiration and, ultimately, reducing the potential growth at high temperatures
110 (Jordan and Ogren, 1984).

111 Beyond the discernment of the existing variability in Rubisco kinetics at a
112 standard temperature, the knowledge on the temperature dependence of Rubisco
113 kinetics, and the existence of variability in the thermal sensitivity among higher plants
114 is of key importance for modelling purposes. The number and diversity of plant
115 species for which Rubisco kinetic parameters have been tested *in vitro* at a range of
116 physiologically relevant temperatures are still very scarce (e.g. Laing et al., 1974;
117 Badger and Collatz, 1977; Badger, 1980; Monson et al., 1982; Hall and Keys, 1983;
118 Jordan and Ogren, 1984; Lehnerr et al., 1985; Uemura et al., 1997; Zhu et al., 1998;
119 Sage et al., 2002; Galmés et al., 2005; Haslam et al., 2005; Yamori et al., 2006;
120 Perdomo et al., 2015; Prins et al., 2016), and mostly restricted to a few kinetic
121 parameters – actually, there is no study examining the temperature dependencies of
122 the main kinetic constants on the same species. The limited number of data reported
123 so far suggests the existence of interspecific differences in the temperature
124 dependence of some Rubisco kinetic parameters, like $k_{\text{cat}}^{\text{c}}$ (Chabot et al., 1972; Weber

125 et al., 1977; Sage, 2002) or $S_{c/o}$ (Zhu et al., 1998; Galmés et al., 2005). Actually,
126 differences in the energy of activation of k_{cat}^c and $S_{c/o}$ seems to be ascribed to the
127 thermal conditions typically encountered by the species in their native habitat
128 (Galmés et al., 2005), as well as to the photosynthetic mechanism (Perdomo et al.,
129 2015).

130 The variability in the response of Rubisco kinetics to changes in temperature,
131 if confirmed, is of paramount importance. The mechanistic models of photosynthesis
132 at leaf, canopy and ecosystem levels are based on the kinetic properties of Rubisco
133 (Farquhar et al., 1980; von Caemmerer, 2000; Bernacchi et al., 2002) and the
134 accuracy of these photosynthetic models depends on knowing the Rubisco kinetic
135 parameters and their species-specific equations for the Rubisco-temperature
136 dependencies (e.g. Niinemets et al., 2009; Yamori and von Caemmerer, 2009;
137 Bermúdez et al., 2012; Díaz-Espejo, 2013; von Caemmerer, 2013; Walker et al.,
138 2013). The need for estimations of the temperature dependencies of Rubisco kinetic
139 parameters becomes timely as modellers try to predict the impact of increasing
140 temperatures on global plant productivity (Sage et al., 2008; Gornall et al., 2010).
141 Ideally, surveying variations in Rubisco kinetics and their temperature dependence
142 should incorporate a correlative analysis with variations in the L- and/or S-subunit
143 amino acid sequence. Such a complementary research would permit deciphering what
144 residue substitutions determine the observed variability in Rubisco catalysis.

145 In the present study, we examined Rubisco catalytic properties and their
146 temperature dependence in twenty crop species, thereby constituting the largest
147 published data set of its kind. The aims of this work were: i) to compare the Rubisco
148 kinetic parameters among the most economically important crops, ii) to search for
149 differences in the temperature response of the main kinetic parameters among these

150 species, iii) to test whether crop Rubiscos are optimally suited for the conditions
151 encountered in plant chloroplasts, and iv) to unravel key amino acid replacements
152 putatively responsible for differences in Rubisco kinetics in crops.

153

154

155 **RESULTS**

156 **The variability in Rubisco kinetics at 25 °C among the most relevant crop species**

157 When considering exclusively the 18 C₃ crop species, at 25 °C, the Rubisco
158 Michaelis-Menten constant for CO₂ under non-oxygenic (K_c) and 21% O₂ (K_c^{air})
159 varied c.a. two-fold and three-fold, respectively, and the maximum rates of Rubisco
160 carboxylation (k_{cat}^c) varied c.a. two-fold (Table 1). For K_c and K_c^{air} , *Manihot esculenta*
161 presented the lowest values ($K_c = 6.1 \mu\text{M}$, and $K_c^{\text{air}} = 10.8 \mu\text{M}$) and *Spinacia oleracea*
162 the highest ($K_c = 14.1 \mu\text{M}$, and $K_c^{\text{air}} = 26.9 \mu\text{M}$). Values for k_{cat}^c varied between 1.4 s^{-1}
163 (*Manihot esculenta*) and 2.5 s^{-1} (*Ipomoea batatas*). The Rubisco CO₂/O₂ specificity
164 ($S_{c/o}$) was the kinetic parameter with the lowest variation among the C₃ species (Fig.
165 S1), and ranged between $92.4 \text{ mol mol}^{-1}$ (*Solanum lycopersicum*) and $100.8 \text{ mol mol}^{-1}$
166 (*Beta vulgaris* and *Manihot esculenta*) (Table 1). *Brassica oleracea* and *Glycine*
167 *max* presented the lowest value for the Rubisco carboxylase catalytic efficiency,
168 calculated as k_{cat}^c/K_c ($0.17 \text{ s}^{-1} \mu\text{M}^{-1}$), and *Coffea arabica* presented the lowest value
169 for the $k_{\text{cat}}^c/K_c^{\text{air}}$ ratio ($0.08 \text{ s}^{-1} \mu\text{M}^{-1}$). With regard to the oxygenase catalytic
170 efficiency (calculated as k_{cat}^o/K_o), *Spinacia oleracea* displayed the lowest value (1.76
171 $\text{ s}^{-1} \text{ nM}^{-1}$). *Hordeum vulgare* presented the highest values for the Rubisco carboxylase
172 and oxygenase catalytic efficiencies ($k_{\text{cat}}^c/K_c = 0.28 \text{ s}^{-1} \mu\text{M}^{-1}$, $k_{\text{cat}}^c/K_c^{\text{air}} = 0.17 \text{ s}^{-1} \mu\text{M}^{-1}$
173 and $k_{\text{cat}}^o/K_o = 3.01 \text{ s}^{-1} \text{ nM}^{-1}$).

174 When data from the two C₄ species (*Saccharum × officinarum* and *Zea mays*)
175 were included in the comparison at 25 °C, the range of variability increased for all
176 parameters (Table 1). Rubisco from the two C₄ species presented higher $k_{\text{cat}}^{\text{c}}$ but
177 lower affinity for CO₂ (i.e., higher K_{c} and $K_{\text{c}}^{\text{air}}$, and lower $S_{\text{c/o}}$) than Rubisco from C₃
178 crops. On average, $k_{\text{cat}}^{\text{c}}/K_{\text{c}}$ and $k_{\text{cat}}^{\text{o}}/K_{\text{o}}$ of C₄ Rubiscos were 62 % and 70 % of those
179 of C₃ crop Rubiscos, respectively.

180

181 **The temperature response of Rubisco kinetics in crops and trade-offs between** 182 **catalytic traits**

183 Both the range of variation and the species showing the extreme values of Rubisco
184 kinetics at 15 °C and 35 °C were similar to those described at 25 °C, with some
185 exceptions. As at 25 °C, among the C₃ crops, Rubisco from *Manihot esculenta*
186 presented the lowest values for K_{c} and $K_{\text{c}}^{\text{air}}$ at 15 °C and 35 °C, while the highest
187 values were measured on Rubisco from *Spinacia oleracea* (Table S1). The lowest and
188 highest values for $k_{\text{cat}}^{\text{c}}$ at 15 °C were those of Rubisco from *Cucurbita maxima* and
189 *Hordeum vulgare*, respectively. The degree of dispersion of the data and the range of
190 variation between the maximum and the minimum values for K_{c} , $K_{\text{c}}^{\text{air}}$ and $k_{\text{cat}}^{\text{c}}$
191 increased with the increment in the assay temperature (Table S1 and Fig. S1).
192 Regarding $S_{\text{c/o}}$, values ranged between 116.1 mol mol⁻¹ (*Brassica oleracea*) and 132.2
193 mol mol⁻¹ (*Cucurbita maxima*) at 15 °C, and between 74.2 mol mol⁻¹ (*Oryza sativa*)
194 and 85.0 mol mol⁻¹ (*Manihot esculenta*) at 35 °C (Table S1). As for $S_{\text{c/o}}$, the range of
195 variation for $k_{\text{cat}}^{\text{c}}/K_{\text{c}}$ and $k_{\text{cat}}^{\text{o}}/K_{\text{o}}$ was also narrowed with the increase in the assay
196 temperature (Table S1 and Fig. S1).

197 Integrating all data across three assay temperatures, $k_{\text{cat}}^{\text{c}}$ correlated positively
198 with K_{c} for both C₃ ($r^2 = 0.82$, $p < 0.001$) and C₄ species ($r^2 = 0.94$, $p < 0.001$), with

199 Rubisco from C₄ species showing higher K_c for a given k_{cat}^c than that from C₃ species
200 (Fig. 1A). The low interspecific variability in $S_{c/o}$ within each assay temperature
201 determined a non-linear relationship between k_{cat}^c and $S_{c/o}$ when considering data from
202 all temperatures together (Fig. 1B). At each temperature individually, Pearson's
203 correlations between k_{cat}^c and K_c and $S_{c/o}$ were highly significant (Table 2) when
204 considering both C₃ and C₄ together. The results from the Phylogenetically
205 Independent Contrasts (PICs) analyses were in general more conservative compared
206 to Pearson's correlations (Table 2), and some significant correlations were lost with
207 PICs (e.g., $S_{c/o}$ vs. K_c or K_c^{air} at 25 °C). Notably, when excluding the two C₄ species,
208 PCCs decreased in almost all correlations (Table 2). Hence, the PCC between k_{cat}^c and
209 K_c was no longer significant at 15 °C, and the PCC between k_{cat}^c and $S_{c/o}$ was
210 significant only at 15 °C. Furthermore, when considering only C₃ species, the unique
211 significant PICs between k_{cat}^c and K_c and $S_{c/o}$ were those found between k_{cat}^c and $S_{c/o}$
212 at 15 °C and 25 °C.

213 The energy of activation (ΔH_a) for K_c varied between 38.2 kJ mol⁻¹ (*Solanum*
214 *tuberosum*) and 83.1 kJ mol⁻¹ (*Oryza sativa*; Table 3). *Ipomoea batatas* (40.7 kJ mol⁻¹)
215 and *Manihot esculenta* (75.4 kJ mol⁻¹) were the species showing the lowest and
216 highest values for ΔH_a of K_c^{air} . As for k_{cat}^c , ΔH_a varied between 27.9 kJ mol⁻¹
217 (*Hordeum vulgare*) and 60.5 kJ mol⁻¹ (*Medicago sativa*). Although the range of
218 variation across C₃ species was similar for the energies of activation of both K_c and
219 k_{cat}^c (2.2-fold), non-significant correlation was observed between ΔH_a for K_c and ΔH_a
220 for k_{cat}^c in both conventional and phylogenetically independent analyses ($r^2 = 0.11$ and
221 0.15, respectively; $P > 0.05$). The lowest and highest values for ΔH_a of the CO₂
222 compensation point in the absence of mitochondrial respiration (I^* , calculated from
223 $S_{c/o}$) were measured in *Beta vulgaris* (19.8 kJ mol⁻¹) and *Glycine max* (26.5 kJ mol⁻¹),

224 respectively. On average, Rubisco from C₃ crops presented significantly higher ΔH_a
225 for K_c ($60.9 \pm 1.5 \text{ kJ mol}^{-1}$) and k_{cat}^c ($43.7 \pm 1.5 \text{ kJ mol}^{-1}$) than Rubisco from C₄
226 species ($K_c = 52.4 \pm 5.0 \text{ kJ mol}^{-1}$, $k_{\text{cat}}^c = 30.6 \pm 1.6 \text{ kJ mol}^{-1}$). By contrast, non-
227 significant differences were observed in the average ΔH_a for Γ^* between C₃ ($22.9 \pm$
228 0.4 kJ mol^{-1}) and C₄ species ($25.0 \pm 0.7 \text{ kJ mol}^{-1}$).

229

230 **The CO₂ assimilation potential of Rubisco kinetics in crops**

231 The CO₂ assimilation potential of Rubisco (A_{Rubisco}) was modelled at varying
232 temperature and CO₂ availability at the catalytic site (C_c) using the species-specific
233 kinetic data measured at each temperature (from Tables 1 and S2). The simulated
234 value of $C_c = 250 \text{ }\mu\text{bar}$ is representative of that encountered in the chloroplast stroma
235 of C₃ species under well-watered conditions (e.g., Bermúdez et al., 2012; Scafaro et
236 al., 2012; Galmés et al., 2013). Under mild to moderate water stress, when no
237 metabolic impairment is present, the decrease in the stomatal and leaf mesophyll
238 conductances to CO₂ provokes a decrease in the concentration of CO₂ in the
239 chloroplast (Flexas et al., 2006). We selected a value of $150 \text{ }\mu\text{bar}$ to simulate the
240 chloroplastic CO₂ concentration in water stressed plants.

241 Differences in A_{Rubisco} across species were largely dependent on the
242 temperature and the availability of CO₂ for carboxylation (Fig. 2). This fact was due
243 to the different prevalence of RuBP-saturated (A_c) and RuBP-limited (A_j) rates
244 governing A_{Rubisco} under the contrasting temperature and C_c , assuming an invariable
245 concentration of active Rubisco sites of $25 \text{ }\mu\text{mol m}^{-2}$ for all species. At $15 \text{ }^\circ\text{C}$, A_c
246 limited A_{Rubisco} at C_c of $150 \text{ }\mu\text{bar}$ in nine species (indicated by asterisks in Fig. 2). At
247 $15 \text{ }^\circ\text{C}$ and C_c of $250 \text{ }\mu\text{bar}$, only six species were A_c limited (*Capsicum annuum*,

248 *Cucurbita maxima*, *Medicago sativa*, *Oryza sativa*, *Solanum tuberosum* and *Spinacia*
249 *oleracea*). At 25 and 35 °C, A_{Rubisco} was A_c -limited in all C_3 species irrespective of C_c .

250 At 25 °C, the *best* Rubisco was that from *Hordeum vulgare* at both C_c , while
251 Rubisco from *Glycine max* yielded the lowest A_{Rubisco} (Fig. 2). Rubisco from *Beta*
252 *vulgaris* presented the best performance at 35 °C irrespective of the CO_2 availability,
253 while *Capsicum annuum* and *Saccharum* \times *officinarum* Rubisco gave the lowest
254 A_{Rubisco} at C_c of 250 and 150 μbar , respectively. At 15 °C and C_c of 250 μbar , the
255 highest potential for CO_2 assimilation was found in Rubisco from *Glycine max*,
256 *Manihot esculenta* and *Triticum aestivum*, while Rubisco from *Manihot esculenta*
257 gave the highest A_{Rubisco} at 15 °C and C_c of 150 μbar . Rubisco from *Cucurbita maxima*
258 displayed the lowest A_{Rubisco} at 15 °C, regardless of the CO_2 availability. It is
259 interesting to note that Rubisco from the two C_4 species, in particular from
260 *Saccharum* \times *officinarum*, performed better than the average C_3 Rubiscos when
261 A_{Rubisco} was simulated according to the photosynthesis model for C_3 leaves (Farquhar
262 et al., 1980), at 15 °C and 25 °C under C_c of 250 μbar (Fig. 2A). At lower C_c (150
263 μbar), the C_4 Rubiscos yielded higher A_{Rubisco} values than the average C_3 Rubiscos at
264 15 °C, and lower values at 35 °C, being similar at 25 °C (Fig. 2B).

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

273

274 **Positively selected L-subunit residues: relationship with Rubisco kinetics**

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

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

287

288

289 **DISCUSSION**

290 **Main crops possess Rubiscos with different performance at 25 °C**

291 The kinetic data reported in the present study are consistent with the range previously
292 reported for higher plants at 25 °C (e.g., Yeoh et al., 1980, 1981; Bird et al., 1982;
293 Jordan and Ogren, 1983; Kent and Tomany, 1995; Galmés et al., 2005, 2014a, 2014c;
294 Ishikawa et al., 2009; Prins et al., 2016) (Table 1), and showing the existence of
295 significant variation among species in the carboxylase catalytic efficiency under non-
296 oxygenic (k_{cat}^c/K_c) and atmospheric conditions (k_{cat}^c/K_c^{air}). Recent reports related
297 k_{cat}^c/K_c variation with the growth capacity in a group of closely related species with

298 similar ecology (Galmés et al., 2014a), suggesting that improving this ratio would be
299 an effective way to engineer a better Rubisco. Nevertheless, such an improvement
300 becomes constrained by the trade-offs between $k_{\text{cat}}^{\text{c}}$, K_{c} and $S_{\text{c/o}}$ (Tcherkez et al., 2006;
301 Savir et al., 2010; Galmés et al., 2014a, 2014c). Here, we demonstrate that these
302 trade-offs, in particular $k_{\text{cat}}^{\text{c}}$ vs. K_{c} , are hold when considering C₃ and C₄ species
303 together, even after accounting for the phylogenetic signal in the data, and that they
304 generally strengthen at increasing assay temperatures (Table 2). However, most of
305 these trade-offs were lost when considering exclusively the C₃ species (Table 2),
306 indicative that the broad-scale patterns of covariation between the Rubisco kinetic
307 parameters may not hold at smaller scales, as previously observed in other
308 angiosperm species (Galmés et al., 2014c).

309 The maximum carboxylase turnover rate of Rubisco ($k_{\text{cat}}^{\text{c}}$) from *Zea mays* and
310 *Saccharum × officinarum* was 2-fold higher than that of the C₃ species, albeit at the
311 expenses of 3 times less affinity for CO₂ (Table 1). This finding agrees with
312 previously described trends between C₃ and C₄ species (Kubien et al., 2008;
313 Ghannoum et al., 2005; Ishikawa et al., 2009), and with the fact that C₄ species
314 present lower $k_{\text{cat}}^{\text{c}}/K_{\text{c}}$ (Kubien et al., 2008; Perdomo et al., 2015).

315 Unlike other reports (Sage 2002; Ishikawa et al., 2009), the observed variation
316 in the kinetic parameters at 25 °C among C₃ species was apparently not related to the
317 thermal climate of their respective domestication regions (data not shown). It should
318 be noted that the origin, and hence the climatic conditions, of the selected varieties
319 could be different to the species centre of domestication, and that the different crop
320 varieties may have accumulated adaptive changes to local conditions by means of
321 artificial selection (Meyer et al., 2012). Intraspecific variability in Rubisco catalytic
322 traits has been reported in *Triticum aestivum* (Galmés et al., 2014c) and *Hordeum*

323 *vulgare* (Rinehart et al., 1983), but how this variability among genotypes is related to
324 adaptation of Rubisco to local environments remains elusive.

325

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

328 The observed temperature response of the Rubisco kinetics parameters confirms well-
329 described trends consisting in increases in k_{cat}^c and K_c and a decrease in $S_{c/o}$ with
330 increasing assay temperature (Table 1 and Table S1) (Jordan and Ogren, 1984;
331 Brooks and Farquhar, 1985; Uemura et al., 1997; Galmés et al., 2005; Prins et al.,
332 2016).

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

347 In general, the Rubisco constant affinities for CO₂ (K_c and K_c^{air}) were more
348 sensitive to changes in assay temperature (i.e., presented higher energies of activation,
349 ΔH_a) than k_{cat}^c and I^* (Table 3), in agreement with a recent study (Perdomo et al.,
350 2015). This fact is explained by the increase in the oxygenase catalytic efficiency
351 (k_{cat}^o/K_o) at increasing temperature. However, it should be remarked that k_{cat}^o/K_o ratio
352 was calculated from the measured parameters K_c , k_{cat}^c and $S_{c/o}$, and that direct
353 measurements of the oxygenase activity of Rubisco, e.g., by mass spectrometry
354 (Cousins et al., 2010), should be undertaken to confirm this trend.

355 As at 25 °C, the differences in the temperature dependencies of Rubisco
356 kinetic parameters among C₃ species were not related to the thermal environment of
357 the species' domestication regions (data not shown). This finding contrasts with
358 previous evidences suggesting that the temperature sensitivity of Rubisco kinetic
359 properties have evolved to improve the enzyme's performance according to the
360 prevailing thermal environment to which species are adapted (Sage, 2002; Galmés et
361 al., 2005, 2015).

362 Although only two C₄ species were included in the present study, they
363 presented lower ΔH_a for K_c and for k_{cat}^c than most of the C₃ species, in close
364 agreement with trends recently observed by Perdomo et al. (2015) in *Flaveria* species
365 (Table 3). A larger number of C₄ species need to be surveyed to verify the existence
366 of differences in the temperature dependence of Rubisco kinetics between C₃ and C₄
367 species.

368

369 **How do the species-specific properties of Rubisco kinetics and their temperature**
370 **sensitivity impact the potential capacity of Rubisco to assimilate CO₂?**

371 Modelling the effect of the species-specific Rubisco kinetics and temperature
372 dependencies of Rubisco kinetics resulted in significant differences in the Rubisco
373 CO₂ assimilation potential (A_{Rubisco}) among the studied C₃ crops (Fig. 2). This
374 modelling exercise highlighted which species would mostly benefit from the genetic
375 replacement of their native version of Rubisco by other foreign versions with
376 improved performance. Notably, the modelling results clearly indicate that the
377 performance of specific Rubiscos cannot be evaluated without considering the
378 environmental conditions during catalysis, specifically the temperature and the CO₂
379 availability at the site of carboxylation (C_c). This fact results from the different
380 temperature dependence of Rubisco kinetics among crops, and from the different
381 impact that Rubisco kinetics have on the RuBP-saturated (A_c) and RuBP-limited (A_j)
382 rates governing A_{Rubisco} . Hence, at 15 °C and C_c of 250 μbar, A_{Rubisco} was limited by A_j
383 in most of the C₃ species (twelve out of eighteen), while it was limited by A_c in all C₃
384 species at 25 and 35 °C irrespective of the C_c value.

385 Detailed examination of modelled A_{Rubisco} suggests that future efforts to
386 enhance Rubisco efficiency should be directed on the following C₃ species displaying
387 the poorest performance: *Cucurbita maxima* and *Medicago sativa* at 15 °C and both
388 C_c , *Glycine max*, *Capsicum annuum* and *Coffea arabica* at 25 °C and 250 μbar;
389 *Glycine max*, *Spinacia oleracea*, *Capsicum annuum* and *Coffea arabica* at 25 °C and
390 150 μbar; *Capsicum annuum*, *Solanum lycopersicum* and *Lactuca sativa* at 35 °C and
391 250 μbar; and *Capsicum annuum* and *Solanum lycopersicum* at 35 °C and 150 μbar.

392 In order to focus on the Rubisco catalytic traits, the modelling assumed
393 invariable values for the concentration of active Rubisco sites ($E = 25 \mu\text{mol m}^{-2} \text{s}^{-1}$)
394 and specific values for the rate of photosynthetic electron transport (J) and C_c .
395 However, species adapt and plants acclimate to the prevailing thermal environment

396 through changes in the concentration and/or activation of Rubisco and the rate of
397 photosynthetic electron transport (Yamasaki et al., 2002; Yamori et al., 2011).
398 Similarly, stomatal (g_s) and leaf mesophyll (g_m) conductances to CO₂ also vary in
399 response to temperature (von Caemmerer and Evans, 2015). Considering the growth
400 temperature effects on these parameters would have altered the equilibrium between
401 A_c and A_j , and indirectly, the consequences of different Rubisco kinetic traits on the
402 CO₂ assimilation potential. In the next future, we aim to increase the accuracy of the
403 present simulation by examining and including the species-specific values for g_s , g_m ,
404 E and J at varying environmental conditions.

405

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

408 We found ten Rubisco L-subunit residues under positive selection (94, 262, 281, 309,
409 439, 446, 449, 469, 470, and 477; Table 4). With the exceptions of residues 469 and
410 477, these residues have been reported previously in other groups of plants, implying
411 a relatively limited number of residues responsible for the Rubisco ‘fine-tuning’
412 (Kapralov and Filatov, 2007; Christin et al., 2008; Iida et al., 2009; Kapralov et al.,
413 2011; Kapralov et al., 2012; Galmés et al., 2014a, 2014c). However, despite
414 widespread parallel evolution of amino acid replacements in the Rubisco sequence,
415 solutions found in particular groups of plants may be quite different. For instance,
416 there are only two common residues under positive selection out of ten between this
417 study and methodologically similar work with different sampling design published
418 earlier (Galmés et al., 2014c). This fact raises questions of epistatic interactions and
419 residue co-evolution within Rubisco (Wang et al., 2011) as well as residue co-
420 evolution and complementarity between Rubisco and its chaperones (Whitney et al.,

421 2015), which both may prevent evolution of identical amino acid replacements
422 because of different genetic backgrounds.

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

433

434 **Conclusions**

435 The present study confirms the significant variation in carboxylation efficiency and
436 parameters that contribute to it among plant species, and for the first time provides
437 full Rubisco kinetic profiles for the twenty most important crop species. Our dataset
438 could be used as an input for the next generation of species-specific models of leaf
439 photosynthesis and its response to climate change, leading to more precise forecasts
440 of changes in crop productivity and yield. These data could help to decide in which
441 crops CO₂ assimilation potential and carboxylation efficiency of Rubisco might be
442 improved via re-engineering of native enzymes or by replacement with foreign ones
443 as there is no a one size fits all solution. The design of future attempts of Rubisco
444 engineering in crops should be based on surveys of Rubisco catalytic and genetic
445 diversity with a particular stress on the relatives of crops in question. Growing

446 knowledge of the Rubisco catalytic spectrum combined with the existing engineering
447 toolkits for Rubisco (Whitney and Sharwood 2008) and its chaperones (Whitney et
448 al., 2015) give us a hope that Rubisco efficiency and hence photosynthetic capacity of
449 crops could be improved in a near future.

450

451

452 **MATERIALS AND METHODS**

453 **Species selection and growth conditions**

454 The following twenty crop species were selected for study: *Avena sativa* L. cv.
455 Forridena, *Beta vulgaris* L. cv. Detroit, *Brassica oleracea* L. var. *italica* cv. Calabres,
456 *Capsicum annuum* L. cv. Picante, *Coffea arabica* L. cv ‘Catuai’ Vermelho IAC 44,
457 *Cucurbita maxima* D. cv. Totanera, *Glycine max* (L.) Merr cv. Ransom, *Hordeum*
458 *vulgare* L. subsp. *vulgare* cv. Morex, *Ipomoea batatas* (L.) Lam var. *Rosa de Málaga*,
459 *Lactuca sativa* L. cv. Cogollo de Tudela, *Manihot esculenta* C., *Medicago sativa* L.
460 cv. Aragón, *Oryza sativa* L. cv. Bomba, *Phaseolus vulgaris* L. cv. Contender,
461 *Saccharum* × *officinarum* (hybrid between *Saccharum officinarum* and *S.*
462 *spontaneum*), *Solanum lycopersicum* L. cv. Roma VF, *Solanum tuberosum* L. cv.
463 Erlanger, *Spinacia oleracea* L. cv. Butterfly, *Triticum aestivum* L. cv. Cajeme, *Zea*
464 *mays* L. cv. Carella. These species represent the most important crops in terms of
465 worldwide production (FAOSTAT, 2010). *Coffea arabica* was selected as being the
466 most important commodity in the international agricultural trade (DaMatta 2004).
467 Plants were grown from seeds under natural photoperiod in a glasshouse at the
468 University of the Balearic Islands (Spain) during 2011 and 2012. Plants were grown
469 in soil-based compost supplemented with slow-release fertilizer and frequently

470 watered to avoid water stress. The air temperature in the glasshouse during the growth
471 period was maintained between 15°C and 30°C.

472

473 **Determination of the Rubisco Michaelis-Menten constant for CO₂ (K_c) and the**
474 **maximum carboxylase turnover rate (k_{cat}^c)**

475 The Rubisco Michaelis-Menten constant for CO₂ under 0% O₂ (K_c) and 21% O₂
476 (K_c^{air}) were determined in crude extracts obtained as detailed in Galmés et al.,
477 (2014a). Rates of ¹⁴CO₂-fixation were measured at 15 °C, 25 °C and 35 °C using
478 activated protein extracts in 7 mL septum capped scintillation vials containing
479 reaction buffer (100 mM Bicine-NaOH pH 8.0, 20 mM MgCl₂, 0.4 mM RuBP and
480 about 100 W-A units of carbonic anhydrase) previously equilibrated either with
481 nitrogen (N₂) or a mixture of O₂ and N₂ (21:79). Nine different concentrations of
482 H¹⁴CO₃⁻ (0.1 to 9.4 mM, each with a specific radioactivity of 3.7 × 10¹⁰ Bq mol⁻¹)
483 were prepared in the scintillation vials as described previously (Galmés et al., 2014a).
484 Assays at 35 °C using Rubisco from C₄ species required increasing H¹⁴CO₃⁻ up to
485 17.7 mM to reach saturating CO₂ concentration in the aqueous-phase. Assays were
486 started by the addition of 10 µL of protein extract and stopped after 1 min by injection
487 of 0.1 mL 10 M formic acid. Acid-stable ¹⁴C was determined by liquid scintillation
488 counting (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, USA)
489 following removal of acid-labile ¹⁴C by evaporation. The Michaelis-Menten constants
490 for CO₂ under 0% O₂ (K_c) and 21% O₂ (K_c^{air}) were determined from the fitted data as
491 described elsewhere (Bird et al., 1982). Replicate measurements ($n = 3-6$) were made
492 using different biological replicates for each species.

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

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

499

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

501 The Rubisco CO_2/O_2 specificity ($S_{c/o}$) was measured on purified extracts obtained as
502 in Gago et al., (2013). On the day of $S_{c/o}$ measurement, highly concentrated Rubisco
503 solutions were desalted by centrifugation through G25 Sephadex columns previously
504 equilibrated with CO_2 -free 0.1 M Bicine (pH 8.2) containing 20 mM MgCl_2 . The
505 desalted solutions were made 10 mM with $\text{NaH}^{14}\text{CO}_3$ (1.85×10^{12} Bq mol^{-1}) and 4 mM
506 NaH_2PO_4 , to activate Rubisco by incubation at 37.5°C for 40 min. Reaction mixtures
507 were prepared in oxygen electrodes (Oxygraph, Hansatech instruments Ltd., Norfolk,
508 UK) by first adding 0.95 mL of CO_2 -free assay buffer (100 mM Bicine pH 8.2, 20
509 mM MgCl_2 , containing 0.015 mg of carbonic anhydrase). After the addition of 0.02
510 mL of 0.1 M $\text{NaH}^{14}\text{CO}_3$ (1.85×10^{12} Bq mol^{-1}), the plug was fitted to the oxygen
511 electrode vessel and enough activated Rubisco (20 μL) was added. The reaction was
512 started by the injection of 10 μL of 25 mM RuBP to be completed between 2 and 7
513 min depending on the assay temperature. RuBP oxygenation was calculated from the
514 oxygen consumption and carboxylation from the amount of ^{14}C incorporated into
515 PGA when all the RuBP had been consumed (Galmés et al., 2014a). Measurements
516 were performed at 15 $^\circ\text{C}$, 25 $^\circ\text{C}$ and 35 $^\circ\text{C}$, with 3-9 biological replicates per each
517 species and assayed temperature.

518 For all Rubisco assays, pH of the assay buffers was accurately adjusted at each
519 temperature of measurement. The concentration of CO₂ in solution in equilibrium
520 with HCO₃⁻ was calculated assuming a pK_a for carbonic acid of 6.19, 6.11 and 6.06 at
521 15 °C, 25 °C and 35 °C, respectively. The concentration of O₂ in solution was
522 assumed to be 305.0, 253.4 and 219.4 (nmol mL⁻¹) at 15 °C, 25 °C and 35 °C,
523 respectively (Truesdale and Downing 1954).

524

525 **Temperature dependence parameters of Rubisco kinetics**

526 To determine the temperature response of the Rubisco kinetic parameters from each
527 species, values for K_c , K_c^{air} and $S_{c/o}$ were first converted from concentrations to partial
528 pressures. For this, solubilities for CO₂ were considered to be 0.0450, 0.0340 and
529 0.0262 mol L⁻¹ bar⁻¹ at 15 °C, 25 °C and 35 °C, respectively. In turn, solubilities for O₂
530 of 0.0016, 0.0013 and 0.0011 mol L⁻¹ bar⁻¹ were used at 15 °C, 25 °C and 35 °C,
531 respectively. The CO₂ compensation point in the absence of mitochondrial respiration
532 (I^*) was obtained from $S_{c/o}$ as in von Caemmerer (2000) using the above solubilities
533 for O₂. Thereafter, values of K_c , I^* and k_{cat}^c at the three temperatures were fitted to an
534 Arrhenius-type equation (Badger and Collatz 1977; Harley and Tenhunen 1991):

$$535 \quad \text{Parameter} = \exp \left[c - \frac{\Delta H_a}{RT_k} \right]$$

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

538

539 **CO₂ assimilation potential of crop Rubiscos at varying temperatures and CO₂**
540 **availability**

541 According to the biochemical model of C₃ photosynthesis (Farquhar et al., 1980), the
542 Rubisco CO₂ assimilation potential (A_{Rubisco}) is defined as the minimum of the RuBP-
543 saturated (A_c) and RuBP-limited (A_j) CO₂ assimilation rates:

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

545 (2) $A_c = \frac{k_{\text{cat}}^c \cdot E \cdot (C_c - \Gamma^*)}{C_c + K_c^{\text{air}}}$

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

547 A_{Rubisco} was obtained for each species at three different temperatures, 15 °C, 25
548 °C and 35 °C, and two different concentrations of CO₂ in the chloroplast stroma (C_c),
549 150 and 250 μbar , simulating situations of moderate water-stress and well-watered
550 conditions in C₃ plants, respectively (Flexas et al., 2006). The Rubisco catalytic traits
551 k_{cat}^c , Γ^* and K_c^{air} were taken from the species- and temperature-specific data obtained
552 in the present study. The concentration of active Rubisco sites (E) was assumed
553 invariable at 25 $\mu\text{mol m}^{-2}$. Values of the CO₂-saturated photosynthetic electron
554 transport rates (J) were assumed 60, 150 and 212 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 15 °C, 25 °C and 35
555 °C, respectively, for all species. At 25 °C, $J = 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ matches very well with
556 a $J/(k_{\text{cat}}^c \cdot E)$ ratio of 1.5 (Egea et al., 2011). Values for J at 15 °C and 35 °C were
557 obtained from the J temperature response described for tobacco in Walker et al.
558 (2013).

559

560 **Analysis of Rubisco L-subunit sites under positive selection**

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

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

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

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

590 acid belongs to a class with $d_N/d_S > 1$ using the Bayes empirical Bayes (BEB)
591 approach implemented in PAML (Yang et al., 2005).

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

596

597 **Statistical analysis**

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

608 The Pearson correlation coefficient was calculated between pairwise
609 combinations of the kinetic parameters K_c , K_c^{air} , k_{cat}^c and $S_{c/o}$ at the three temperatures
610 of measurement. However, correlations arising within groups of related taxa might
611 reflect phylogenetic signal rather than true cause-effect relationships, because closely
612 related taxa are not necessarily independent data points and could violate the
613 assumption of randomized sampling employed by conventional statistical methods
614 (Felsenstein 1985). To overcome this issue, tests were performed for the presence of

615 phylogenetic signal in the data and trait correlations were calculated with
616 phylogenetically independent contrasts using the AOT module of PHYLOCOM
617 (Webb et al., 2008) using the species phylogeny based on the three chloroplast genes
618 (see below). All these tests were considered significant at $p < 0.05$.

619

620

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631

632 **Supplemental Material**

633 **Table S1.** The Rubisco kinetic parameters measured at 15 °C and 35 °C for the
634 selected crop species.

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

637 **Figure S1.** Box plots depiction of Rubisco kinetic parameters (K_c , K_c^{air} , k_{cat}^c and $S_{c/o}$)
638 at 15 °C, 25 °C and 35 °C when considering the 18 C₃ species alone.

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

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

643

644

645

646 **Table 1.** Kinetic parameters of crop Rubiscos measured at 25 °C: the Michaelis-Menten constants for CO₂ under non-oxygenic (K_c) and 21% O₂ (K_c^{air}), the
647 maximum carboxylation rate (k_{cat}^c), the specificity factor ($S_{c/o}$), and the carboxylation (k_{cat}^c/K_c and $k_{\text{cat}}^c/K_c^{\text{air}}$), and the oxygenation catalytic efficiencies
648 (k_{cat}^o/K_o). The k_{cat}^o/K_o ratio was calculated as $[(k_{\text{cat}}^c/K_c)/S_{c/o} * 1000]$. For each species, data are mean \pm standard error (n = 3-9). Group averages were obtained
649 from individual measurements on each species. Different letters denote statistical differences ($p < 0.05$) by Duncan analysis between C₃ and C₄ groups.
650

Species	K_c (μM)	K_c^{air} (μM)	k_{cat}^c (s^{-1})	$S_{c/o}$ (mol mol^{-1})	k_{cat}^c/K_c ($\text{s}^{-1} \mu\text{M}^{-1}$)	$k_{\text{cat}}^c/K_c^{\text{air}}$ ($\text{s}^{-1} \mu\text{M}^{-1}$)	k_{cat}^o/K_o ($\text{s}^{-1} \text{nM}^{-1}$)
C ₃ species							
<i>Avena sativa</i>	10.8 \pm 0.9	18.1 \pm 2.0	2.3 \pm 0.3	99.9 \pm 3.0	0.21 \pm 0.01	0.13 \pm 0.03	2.14 \pm 0.06
<i>Beta vulgaris</i>	10.8 \pm 1.2	18.6 \pm 1.1	2.0 \pm 0.3	100.8 \pm 2.0	0.19 \pm 0.02	0.10 \pm 0.01	1.94 \pm 0.31
<i>Brassica oleracea</i>	11.8 \pm 0.1	19.2 \pm 0.3	2.1 \pm 0.3	96.2 \pm 1.3	0.17 \pm 0.03	0.11 \pm 0.02	1.81 \pm 0.28
<i>Capsicum annuum</i>	9.6 \pm 0.3	19.8 \pm 1.5	1.9 \pm 0.1	96.0 \pm 4.5	0.20 \pm 0.01	0.10 \pm 0.01	1.98 \pm 0.15
<i>Coffea Arabica</i>	11.0 \pm 0.4	22.9 \pm 2.4	2.1 \pm 0.2	98.7 \pm 3.8	0.19 \pm 0.02	0.08 \pm 0.01	1.98 \pm 0.18
<i>Cucurbita maxima</i>	9.0 \pm 0.5	19.2 \pm 1.0	2.2 \pm 0.2	98.4 \pm 0.4	0.25 \pm 0.04	0.12 \pm 0.01	2.45 \pm 0.31
<i>Glycine max</i>	8.6 \pm 0.2	16.2 \pm 0.7	1.5 \pm 0.1	97.0 \pm 1.1	0.17 \pm 0.02	0.09 \pm 0.01	1.76 \pm 0.21
<i>Hordeum vulgare</i>	9.0 \pm 0.6	14.9 \pm 1.6	2.4 \pm 0.2	99.2 \pm 3.8	0.28 \pm 0.02	0.17 \pm 0.03	3.01 \pm 0.19
<i>Ipomoea batatas</i>	12.0 \pm 0.7	21.1 \pm 1.0	2.5 \pm 0.1	98.5 \pm 6.6	0.20 \pm 0.00	0.12 \pm 0.01	1.96 \pm 0.08
<i>Lactuca sativa</i>	11.1 \pm 0.3	18.2 \pm 1.4	2.2 \pm 0.1	94.0 \pm 1.9	0.19 \pm 0.00	0.12 \pm 0.01	2.06 \pm 0.07

<i>Manihot esculenta</i>	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
<i>Medicago sativa</i>	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
<i>Oryza sativa</i>	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
<i>Phaseolus vulgaris</i>	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
<i>Solanum lycopersicum</i>	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
<i>Solanum tuberosum</i>	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
<i>Spinacia oleracea</i>	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
<i>Triticum aestivum</i>	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
<i>C₃ average</i>	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							
<i>Saccharum × officinarum</i>	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
<i>Zea mays</i>	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
<i>C₄ average</i>	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

651

652

653 **Table 2.** Phylogenetically independent contrasts (PICs, upper part of the diagonals) and Pearson's correlation coefficients (PCCs, lower part of the diagonals)
654 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 C₃ and C₄ species together and the 18 C₃
655 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

656 **Table 3.** The energy of activation (ΔH_a , kJ mol⁻¹) and c (dimensionless) values of the Rubisco Michaelis-Menten constants for CO₂ under non-oxygenic (K_c ,
657 $\mu\text{mol mol}^{-1}$) and 21% O₂ (K_c^{air} , $\mu\text{mol mol}^{-1}$), the maximum carboxylation rate (k_{cat}^c , s⁻¹) and the CO₂ compensation point in the absence of mitochondrial
658 respiration (Γ^* , $\mu\text{mol mol}^{-1}$) for the twenty crop species. For each species, data are mean \pm standard error (n = 3-9). Group averages were calculated from
659 individual measurements on each species. Different letters denote statistical differences ($p < 0.05$) by Duncan analysis between C₃ and C₄ groups. Parameter
660 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} ($\mu\text{mol mol}^{-1}$) =
661 parameter (μM) $\times K_h \times \text{Air Volume (L)} / \text{RT}$]. 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,
662 25 °C = 24.5, 35 °C = 25.4. The term Γ^* ($\mu\text{mol mol}^{-1}$) is derived from $0.5O / S_{c/o}$.
663

Species	K_c		K_c^{air}		k_{cat}^c		Γ^*	
	c	ΔH_a	c	ΔH_a	c	ΔH_a	c	ΔH_a
C ₃ species								
<i>Avena sativa</i>	31.3 \pm 0.7	63.4 \pm 2.0	26.0 \pm 0.4	48.9 \pm 1.2	17.6 \pm 2.2	41.5 \pm 5.5	13.3 \pm 0.5	23.6 \pm 1.4
<i>Beta vulgaris</i>	28.7 \pm 1.7	57.0 \pm 4.4	27.2 \pm 0.7	51.8 \pm 1.8	21.5 \pm 3.7	51.2 \pm 9.6	11.7 \pm 0.4	19.8 \pm 1.0
<i>Brassica oleracea</i>	28.1 \pm 1.1	55.3 \pm 2.8	26.5 \pm 0.9	50.1 \pm 2.4	18.8 \pm 2.6	45.7 \pm 6.5	12.6 \pm 0.2	21.8 \pm 0.5
<i>Capsicum annum</i>	26.6 \pm 1.5	51.8 \pm 3.7	27.0 \pm 1.6	51.2 \pm 3.7	16.3 \pm 2.8	39.2 \pm 6.9	13.4 \pm 0.7	24.1 \pm 1.8
<i>Coffea arabica</i>	34.7 \pm 0.3	71.5 \pm 0.9	27.6 \pm 1.8	52.2 \pm 4.3	16.5 \pm 2.6	39.0 \pm 6.1	13.1 \pm 0.5	23.4 \pm 1.1
<i>Cucurbita maxima</i>	28.6 \pm 0.8	57.0 \pm 1.8	29.2 \pm 1.1	56.8 \pm 2.8	20.2 \pm 1.0	48.7 \pm 2.7	12.2 \pm 0.9	21.1 \pm 2.2

<i>Glycine max</i>	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
<i>Hordeum vulgare</i>	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
<i>Ipomoea batatas</i>	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
<i>Lactuca sativa</i>	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
<i>Manihot esculenta</i>	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
<i>Medicago sativa</i>	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
<i>Oryza sativa</i>	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
<i>Phaseolus vulgaris</i>	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
<i>Solanum lycopersicum</i>	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
<i>Solanum tuberosum</i>	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
<i>Spinacia oleracea</i>	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
<i>Triticum aestivum</i>	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
<i>C₃ average</i>	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								
<i>Saccharum × officinarum</i>	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
<i>Zea mays</i>	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
<i>C₄ average</i>	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

664

665

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

Residue ^a	Amino acid changes	Location of residue	Interaction ^b
Branches leading to species with $K_c \geq 26.0 \mu\text{M}$ and $k_{\text{cat}}^c \geq 3.9 \text{ s}^{-1}$ at 25 °C (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 $k_{\text{cat}}^c \geq 2.5 \text{ s}^{-1}$ at 25 °C			
281**	A → S	Helix 4	DD, SS
Branches leading to species with $K_c \geq 10.8 \mu\text{M}$ at 25 °C			
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 $S_{\text{CO}_2} \leq 94.0 \text{ mol mol}^{-1}$ at 25 °C			
309**	M → I	βF Strand	ID
Branches leading to species with ΔH_a for $K_c \leq 56.0 \text{ kJ mol}^{-1}$			
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	

670

671 ^a Residue numbering is based on the spinach sequence. Values for Bayesian Posterior Probabilities are:

672 * > 0.90, ** > 0.95, *** > 0.99.

673 ^b Interactions in which the selected residues and/or residues within 5 Å of them are involved. ID –

674 intradimer interactions; DD – dimer-dimer interactions; RA – interface for interactions with Rubisco

675 activase; SS – interactions with small subunits; interactions based on literature survey only are given in
676 italics; after (Spreitzer and Salvucci 2002; Ott et al., 2000; Du et al., 2003).
677

678 **Figure legends**

679 **Figure 1.** The relationship between the turnover rate for the Rubisco carboxylase
680 reaction (k_{cat}^c) with (A) the Michaelis–Menten affinity constant for CO₂ (K_c) and (B)
681 the CO₂/O₂ specificity factor ($S_{c/o}$). Filled symbols correspond to C₃ species at 15 °C
682 (▲), 25 °C (●) and 35 °C (▼); open symbols correspond to C₄ species at 15 °C (△),
683 25 °C (○) and 35 °C (▽). Each symbol represents the average value of a single
684 species per temperature interaction.

685

686 **Figure 2.** Simulated CO₂ assimilation potential of Rubisco (A_{Rubisco}) for the C₃ and C₄
687 species at 15 °C, 25 °C and 35 °C and at values for the chloroplastic CO₂
688 concentration (C_c) of (A) 250 μbar and (B) 150 μbar. Equations used to calculate
689 A_{Rubisco} were those described in the biochemical model of C₃ photosynthesis (Farquhar
690 et al., 1980), as explained in Materials and Methods. The bars represent the minimum
691 value of A_c - and A_j -limited A_{Rubisco} . Asterisks (*) above the bars indicate A_c -limited
692 A_{Rubisco} (absence of * indicate A_j -limited A_{Rubisco}). The rate of electron transport was
693 considered 60, 150 and 212 μmol m⁻² s⁻¹ at 15 °C, 25 °C and 35 °C, respectively. The
694 concentration of active Rubisco sites was assumed invariable at 25 μmol m⁻² for all
695 the species and environmental conditions. The values used for the Rubisco kinetic
696 parameters (k_{cat}^c , I^* and K_c^{air}) are those shown in Tables 1 and S2.

697

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