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3 **Corresponding authors:** Douglas Orr (d.j.orr@lancaster.ac.uk), Elizabete Carmo-Silva

4 (e.carmosilva@lancaster.ac.uk); +44 (0)1524 594369.

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6 **Surveying Rubisco diversity and temperature response to improve crop**
7 **photosynthetic efficiency¹**

8

9 **Douglas J. Orr***, **André Alcântara²**, **Maxim V. Kapralov**, **P. John Andralojc**, **Elizabete Carmo-Silva**,

10 **Martin A.J. Parry**

11 Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK (DJO, AA, ECS, MAJP);

12 Rothamsted Research, Plant Biology and Crop Science, Harpenden, AL5 2JQ, UK (DJO, AA, PJA, ECS,

13 MAJP); Plant Sciences Division, Research School of Biology, Australian National University, Canberra,

14 ACT 0200, Australia (MVK); and School of Natural Sciences and Psychology, Liverpool John Moores

15 University, Liverpool, L3 3AF, UK (MVK)

16

17 **One sentence summary:** Species diversity in Rubisco catalysis shows consistencies in temperature
18 response. Some of the Rubiscos from diverse species can improve crop photosynthetic efficiency.

19

20 **List of author contributions:** DJO, MVK, PJA, ECS, MAJP designed research; ECS, PJA supervised the
21 experiments; DJO, AA performed the experiments; DJO, AA, MVK, ECS analysed data; and DJO, AA,
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23

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26 ²Present address: Gregor Mendel Institute of Molecular Plant Biology GmbH, Vienna, 1030, Austria.

27 *Address correspondence to d.j.orr@lancaster.ac.uk.

28 The author responsible for distribution of materials integral to the findings presented in this article in
29 accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Douglas
30 Orr (d.j.orr@lancaster.ac.uk).

31 **ABSTRACT**

32 The threat to global food security of stagnating yields and population growth makes increasing crop
33 productivity a critical goal over the coming decades. One key target for improving crop productivity and
34 yields is increasing the efficiency of photosynthesis. Central to photosynthesis is ribulose-1,5-
35 biphosphate carboxylase/oxygenase, Rubisco, which is a critical but often rate-limiting component. Here
36 we present full Rubisco catalytic properties measured at three temperatures for 75 plants species
37 representing both crops and undomesticated plants from diverse climates. Some newly characterised
38 Rubiscos were naturally 'better' compared to crop enzymes and have the potential to improve crop
39 photosynthetic efficiency. The temperature response of the various catalytic parameters was largely
40 consistent across the diverse range of species, though absolute values showed significant variation in
41 Rubisco catalysis, even between closely related species. An analysis of residue differences amongst the
42 species characterised identified a number of candidate amino acid substitutions that will aid in advancing
43 engineering of improved Rubisco in crop systems. This study provides new insights on the range of
44 Rubisco catalysis and temperature response present in nature, and provides new information to include in
45 models from leaf to canopy and ecosystem scale.

46

47 **Keywords:** Rubisco, photosynthesis, enzyme catalysis, carbon assimilation, natural diversity

48

49 **INTRODUCTION**

50 In a changing climate and under pressure from a population set to hit nine billion by 2050, global food
51 security will require massive changes to the way food is produced, distributed, and consumed (Ort et al.,
52 2015). To match rising demand agricultural production must increase by 50-70% in the next 35 years, and
53 yet the gains in crop yields initiated by the green revolution are slowing, and in some cases, stagnating
54 (Long and Ort 2010, Ray et al., 2012). Amongst a number of areas being pursued to increase crop
55 productivity and food production, improving photosynthetic efficiency is a clear target, offering great
56 promise (Parry et al., 2007; von Caemmerer et al., 2012; Price et al., 2013; Ort et al., 2015). As the
57 gatekeeper of carbon entry into the biosphere and often acting as the rate-limiting step of photosynthesis,
58 Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), the most abundant enzyme on the planet
59 (Ellis, 1979), is an obvious and important target for improving crop photosynthetic efficiency.

60 Rubisco is considered to exhibit comparatively poor catalysis, in terms of catalytic rate,
61 specificity, and CO₂ affinity (Tcherkez et al., 2006; Andersson, 2008), leading to the suggestion that even
62 small increases in catalytic efficiency may result in substantial improvements to carbon assimilation
63 across a growing season (Zhu et al., 2004; Parry et al., 2013; Galmés et al., 2014a; Carmo-Silva et al.,
64 2015). If combined with complimentary changes such as optimising other components of the Calvin
65 Benson or photorespiratory cycles (e.g. Raines, 2011; Peterhansel et al., 2013; Simkin et al., 2015),
66 optimised canopy architecture (Drewry et al., 2014), or introducing elements of a carbon concentrating
67 mechanism (Furbank et al., 2009; Lin et al., 2014a; Hanson et al., 2016; Long et al., 2016), Rubisco
68 improvement presents an opportunity to dramatically increase the photosynthetic efficiency of crop plants
69 (McGrath and Long, 2014; Long et al., 2015; Betti et al., 2016). A combination of the available strategies
70 is essential for devising tailored solutions to meet the varied requirements of different crops and the
71 diverse conditions under which they are typically grown around the world.

72 Efforts to engineer an improved Rubisco have not yet produced a 'super Rubisco' (Parry et al.,
73 2007; Ort et al., 2015). However, advances in engineering precise changes in model systems continue to
74 provide important developments that are increasing our understanding of Rubisco catalysis (Spreitzer et
75 al., 2005; Whitney et al., 2011a, 2011b; Morita et al., 2014; Wilson et al., 2016), regulation (Andralojc et
76 al., 2012; Carmo-Silva and Salvucci, 2013; Bracher et al., 2015) and biogenesis (Saschenbrecker et al.,
77 2007; Sharwood and Whitney, 2008; Lin et al., 2014b; Hauser et al., 2015; Whitney et al., 2015).

78 A complementary approach is to understand and exploit Rubisco natural diversity. Previous
79 characterisation of Rubisco from a limited number of species has not only demonstrated significant
80 differences in the underlying catalytic parameters, but also suggests that further undiscovered diversity
81 exists in nature and that the properties of some of these enzymes could be beneficial if present in crop
82 plants (Carmo-Silva et al., 2015). Recent studies clearly illustrate the variation possible amongst even

83 closely related species (e.g. Galmés et al., 2005; Kubien et al., 2007; Galmés et al., 2014b, 2014c;
84 Andralojc et al., 2014; Prins et al., 2016).

85 Until recently there have been relatively few attempts to characterise the consistency, or lack
86 thereof, of temperature effects on *in vitro* Rubisco catalysis (Sharwood and Whitney 2014), and often
87 studies only consider a subset of Rubisco catalytic properties. This type of characterisation is particularly
88 important for future engineering efforts, enabling specific temperature effects to be factored into any
89 attempts to modify crops for a future climate. In addition, the ability to co-analyse catalytic properties and
90 DNA or amino acid sequence provides the opportunity to correlate sequence and biochemistry to inform
91 engineering studies (e.g. Christin et al., 2008; Kapralov et al., 2011; Rosnow et al., 2015). Whilst the
92 amount of gene sequence information available grows rapidly with improving technology, knowledge of
93 the corresponding biochemical variation resulting has yet to be determined (Cousins et al., 2010; Carmo-
94 Silva et al., 2015; Sharwood and Whitney, 2014; Nunes-Nesi et al., 2016).

95 This study aimed to characterise the catalytic properties of Rubisco from diverse species,
96 comprising a broad range of monocots and dicots from diverse environments. The temperature
97 dependence of Rubisco catalysis was evaluated to tailor Rubisco engineering for crop improvement in
98 specific environments. Catalytic diversity was analysed alongside the sequence of the Rubisco large
99 subunit gene, *rbcL*, to identify potential catalytic switches for improving photosynthesis and productivity.
100 *In vitro* results were compared to the average temperature of the warmest quarter in the regions where
101 each species grows to investigate the role of temperature in modulating Rubisco catalysis.

102

103

104 **RESULTS**

105 **Variability in Rubisco catalysis across plant species**

106 Diversity in Rubisco catalytic properties determined at 20, 25 and 30°C was measured across 75 species
107 belonging to 10 families, expanding the range of previously characterised Rubiscos (Fig. 1; full dataset
108 available in Table S1). This is the largest dataset of complete Rubisco catalytic properties produced to
109 date. Analysis of variance revealed significant differences in carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$;
110 Supplemental Fig. S1) and specificity ($S_{\text{C/O}}$; Supplemental Fig. S2).

111 Carboxylation rates ($k_{\text{cat}}^{\text{c}}$) at 25°C ranged from 1.9 s⁻¹ in *Euphorbia helioscopia* (Euphorbiaceae)
112 to 7.1 s⁻¹ in the C₄-photosynthesis type annual grass *Eragrostis tef* (Poaceae). Affinity for CO₂ was highest
113 in *Oryza sativa* ssp. Indica ($K_{\text{c}} = 7 \mu\text{M}$ at 25°C), and lowest in C₄ grasses included in this study ($K_{\text{c}} \sim 34$ -
114 37 μM , *E. tef* and *Panicum* spp.). Across the diverse group of species analysed the CO₂/O₂ specificity
115 ($S_{\text{C/O}}$) showed a large range of values, from a 25°C high of 111 in the grass *Poa palustris* (Poaceae) to a
116 low of 82 in the C₄ dicot *Chrysanthellum indicum* (Asteraceae). C₃ plants surveyed ranged in $S_{\text{C/O}}$ from

117 111 to 91. Catalytic values generally agreed with previously reported ranges (e.g. Ishikawa et al., 2011;
118 Galmés et al., 2014b; Occhialini et al., 2015).

119 Modelling of leaf photosynthesis shows that the direct replacement of native Rubisco in a crop,
120 such as soybean (*Glycine max*), with two high performing monocot Rubiscos would support significant
121 improvements of leaf-level photosynthetic rates at current atmospheric CO₂ levels and high irradiance
122 (Fig. 2). Photosynthesis improvement was particularly evident at low internal CO₂ concentrations when
123 leaf photosynthesis is typically limited by Rubisco activity.

124

125 **Linking *rbcL* sequence variation with Rubisco biochemical diversity**

126 Accompanying the biochemical analysis of a large range of species with an analysis of variation in the
127 highly conserved chloroplast *rbcL* gene, which encodes the catalytic subunit of Rubisco, provides the
128 opportunity to identify amino acid replacements potentially responsible for changes in Rubisco catalysis.
129 Positive selection analysis identified residue positions that were correlated with particular catalytic
130 properties, namely: high carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$), high $k_{\text{cat}}^{\text{c}}$, low $K_{\text{c}}^{\text{air}}$, and high $S_{\text{C/O}}$. Five
131 Rubisco large subunit residues were associated with changes in particular catalytic characteristics across
132 the 75 species dataset (Fig. 3), with at least one residue linked to each parameter. The full list of residue
133 positions under positive selection, their structural location and possible molecular interactions is provided
134 in Supplemental Table S2.

135 Importantly, in a large analysis of sequence diversity alongside catalytic properties,
136 phylogenetically distant species may have acquired similar changes in Rubisco catalysis via different
137 amino acid substitutions, which makes finding common catalytic switches difficult. Thus, a subsequent
138 separate analysis of the monocot and dicot species subsets ($n = 39$ and 36 , respectively) was conducted.
139 Different sets of residues associated with catalytic changes were highlighted for these two groups with
140 little overlap (Fig. 3A and 3B). Amongst the six residues found within the monocots, three positions were
141 linked to high carboxylation efficiency, one to high $S_{\text{C/O}}$ and two to low $K_{\text{c}}^{\text{air}}$. In the dicot subset analysis,
142 two residue positions were associated with high catalytic rates ($k_{\text{cat}}^{\text{c}}$), whilst a further residue position was
143 linked to high carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$).

144

145 **Correlations between catalytic parameters at a range of temperatures**

146 Using phylogenetically independent contrast (PIC) analyses, correlation coefficients between catalytic
147 parameters for each measurement temperature were calculated (Fig. 4). The classical trade-off between
148 increasing $k_{\text{cat}}^{\text{c}}$ and decreasing CO₂ affinity (increased K_{c} or $K_{\text{c}}^{\text{air}}$) was evident (Tcherkez et al., 2006).
149 However, the significance and strength of this correlation varied at the different measurement
150 temperatures examined. At 20 and 25°C the strength and significance was high ($P \leq 0.01$), while at 30°C

151 there was no significant correlation between increasing $k_{\text{cat}}^{\text{c}}$ and CO₂ affinity (K_{c} or $K_{\text{c}}^{\text{air}}$). $S_{\text{C/O}}$ correlated
152 positively with $k_{\text{cat}}^{\text{c}}$, K_{c} and $K_{\text{c}}^{\text{air}}$, most significantly at 20 and 25°C, and negatively with carboxylation
153 efficiency at 25°C. The relationship between $k_{\text{cat}}^{\text{c}}$ and carboxylation efficiency was notably inconsistent
154 across the three measurement temperatures.

155 To explore how climate may correlate with Rubisco catalysis in diverse species, the temperature
156 of the warmest quarter of the year (T_{WQ}) where each species grows served as a proxy for conditions
157 during the main part of the growing season. T_{WQ} was negatively correlated with $S_{\text{C/O}}$ measured at 20 and
158 30°C (at 25°C the correlation was not significant; Fig. 4), indicating that Rubisco from species growing in
159 higher temperature climates had lower $S_{\text{C/O}}$. Oxygenation parameters (K_{o} and V_{o}) consistently showed a
160 significant positive correlation with T_{WQ} . Carboxylation efficiency was negatively correlated with T_{WQ} at
161 20 and 25°C, but the correlation was not significant for measurements at 30°C.

162

163 **Temperature response of Rubisco catalysis**

164 To examine the consistency of catalytic changes in response to temperature, the 75 species examined were
165 divided into five natural groups based on their phylogenetic relationships (indicated in Fig 3). A summary
166 of the catalytic properties for each group at each temperature is shown in Table I, and non-linear
167 regression analysis was used to assess the groups and species variation in temperature response
168 (Supplemental Fig. S3). There was variation in the temperature response of Rubisco catalysis for the
169 diverse species and groups analysed, but the trend of the response was consistent. The response of each
170 catalytic property to temperature in soybean (*Glycine max*) is provided as a representative example (Fig.
171 5). Group 3 consisted of a range of dicots, including *N. tabacum* and *Artemisia* spp., and could be fitted
172 with a single model that explained temperature response of $k_{\text{cat}}^{\text{c}}$ for the whole group (i.e. there was no
173 significant difference in temperature response of $k_{\text{cat}}^{\text{c}}$ between the species within group 3). For the other
174 groups and individual species, the temperature response of $k_{\text{cat}}^{\text{c}}$ was similarly explained by a linear model
175 and, while individual species displayed a consistent slope for the model generated, significant variation in
176 the intercept prevented the generation of a single model to explain the entire group. These results show
177 that the relative increase in $k_{\text{cat}}^{\text{c}}$ with temperature was consistent, despite the significant variation in
178 absolute values within groups.

179 A group level model for $K_{\text{c}}^{\text{air}}$ could be fitted to groups 2 and 3, but not groups 1, 4 and 5. Each of
180 the 75 species was modelled with a similar quadratic function; however, only groups 2 and 3 could have
181 all its members statistically explained by a single model. $K_{\text{c}}^{\text{air}}$ increased with temperature and the rate of
182 increase was lower above 25 °C, reflected in the representative function shown in Fig. 5A. As mentioned
183 above, $S_{\text{C/O}}$ decreased with temperature. Consistent with previous data, this decrease was non-linear and
184 for each species/group was best described by a quadratic function. The decrease in $S_{\text{C/O}}$ was generally

185 greater between 20-25°C than 25-30°C (Fig. 5B). In group 3, this response was reversed (greater decrease
186 between 25-30°C). Carboxylation efficiency ($k_{\text{cat}}^c/K_c^{\text{air}}$) was also described by a quadratic model with
187 efficiency being highest at 20 and 30°C, and consistently lower at 25°C. Though the drop in efficiency
188 around 25°C varied between species and groups, the quadratic effect was consistent across the range of
189 species, with variation evident in both the slope and intercept of the functions generated (Supplemental
190 Fig. S4).

191

192

193 **DISCUSSION**

194 **Significant variation in Rubisco catalysis amongst diverse species**

195 The present study represents the largest single survey of Rubisco catalysis to date. A large number of
196 studies have previously described Rubisco catalysis (reviewed in Parry et al., 2007; Whitney et al., 2011b;
197 Parry et al., 2013; Carmo-Silva et al., 2015). However, this still represents a very small fraction of known
198 lands plants (approximately 0.2% based on current literature). Unfortunately, many studies have also only
199 partially characterised Rubisco catalysis, with specificity ($S_{C/O}$) in particular lacking from most available
200 datasets (Sharwood and Whitney, 2014). The present study dramatically expands upon our knowledge of
201 Rubisco catalytic variability through full characterisation of 75 plant species, and provides a large
202 comparative dataset to inform future engineering efforts. The results presented here reinforce that, despite
203 the relatively highly conserved nature of the Rubisco large subunit gene *rbcL* (Kapralov and Filatov,
204 2007; Wang et al., 2011), key catalytic parameters vary significantly across diverse plant taxa.

205 Carboxylation rates in particular varied by almost 3-fold at 25°C. Leaf scale modelling predicted that
206 direct replacement strategies using newly characterised Rubiscos could substantially improve maximum
207 photosynthetic capacity, though this will likely require further advances in our ability to test foreign
208 Rubicos in tobacco based systems (Whitney et al., 2011a). Nevertheless this demonstrates the potential
209 gains in photosynthetic capacity through Rubisco substitution. This dataset characterising a broad range
210 of species at multiple temperatures will also be of use in modelling of photosynthesis at different scales
211 (Smith and Dukes, 2013), and complement *in planta* studies seeking to adapt models of various scales for
212 the increased temperatures expected in many regions in the coming decades (e.g. Bagley et al., 2015).

213

214 **Targeting improvements through mutagenesis**

215 The large subunit of Rubisco, encoded by the chloroplast *rbcL* gene, contains the catalytic sites and is
216 believed to be primarily, though not solely, responsible for the catalytic profile of the holoenzyme
217 (Sharwood et al., 2008). A number of residues were identified that warrant mutagenic testing in model
218 systems, including a number of new candidates not previously highlighted. The residues identified

219 differed dependent on the set of species included in the analysis, demonstrating the need to consider the
220 phylogenetic background of a target Rubisco when determining the potential impact of point mutations.
221 Careful consideration must also be given to avoiding effects on holoenzyme assembly and compatibility
222 with ancillary proteins or assembly chaperones (Carmo-Silva et al., 2015; Whitney et al., 2015). This
223 presents a promising avenue for future work in model systems, testing these residues either singly or in
224 combination, with previous studies having shown strong potential for modifying Rubisco catalysis with
225 targeted amino acid substitutions (e.g. Whitney et al., 2011b).

226

227 **The effect of temperature on Rubisco catalysis**

228 Few studies have explored the effect of temperature on Rubisco catalysis beyond model species
229 (Sharwood and Whitney, 2014, Sharwood et al., 2016), and none at the scale of the present study. Recent
230 work has begun to make important inroads into this area (Perdomo et al., 2015, Prins et al., 2016).
231 Analysis of the correlations between parameters at the three measurement temperatures largely agreed
232 with previous observations regarding the trade-off between increasing carboxylation rate ($k_{\text{cat}}^{\text{c}}$) and
233 decreasing CO_2 affinity (increasing K_c^{air}). However, the tight linking of these parameters was not evident
234 at 30°C. This ‘uncoupling’ at higher temperatures suggests the possibility of finding superior Rubiscos for
235 operating at relatively high temperatures. This study found a negative correlation between warmer
236 climates and specificity ($S_{\text{C/O}}$). Galmés et al. (2005) found that in hot and dry conditions in the
237 Mediterranean this correlation was positive, with high Rubisco specificity found for plants from this
238 region. This suggests a more complex relationship between climate and Rubisco specificity that is not
239 solely based on temperature, but also needs consideration of additional climatic data such as precipitation.

240 Higher temperature environments (T_{WQ}) did not consistently correlate with carboxylation
241 parameters across assay temperatures, but did correlate with increasing K_o and V_o . The observed
242 correlations suggest that Rubiscos from warmer climates are less efficient at lower temperatures. Fitting
243 mathematical models to the response of key parameters to measurement temperature resulted primarily in
244 non-linear models, the exception being carboxylation rate ($k_{\text{cat}}^{\text{c}}$). The type of model that best explained
245 temperature response of each parameter was consistent across species, though variation in the absolute
246 values for each species largely prevented fitting a single model to the species groupings. In many cases,
247 species within a group had parallel responses. This provides important new insights on the response of
248 Rubisco catalysis to temperature, and its consistency across diverse species, whilst further highlighting
249 the diversity of catalysis. It is important to note that a number of plant groups such as trees and basal
250 angiosperms remain either underrepresented in biochemical datasets, or have only just begun to be
251 surveyed (Galmés et al., 2014b), and provide potential areas where additional valuable information can be
252 gleaned from characterisation. Data is also lacking for crop species, with few represented in the

253 literature, and often with incomplete characterisation. This is an important gap in our knowledge that will
254 be important when targeting improvements to key crops. This study focused on C₃ species, the potential
255 for C₄ Rubiscos to respond differently has received increased interest recently (e.g. Boyd et al., 2015;
256 Perdomo et al., 2015), however there remains a need to characterise more Rubiscos from C₄ species for
257 thermal response.

258

259 **Tailored solutions are required for optimising crop carbon assimilation**

260 The variation in catalysis found during this study provides important information for future efforts to
261 engineer improved Rubisco in crops via either replacement with a foreign Rubisco (Fig. 2) or point
262 mutations of the endogenous gene (Fig. 3). In C₃ plants, 20-35°C is considered the optimum temperature
263 range for photosynthesis (Blankenship, 2014), and thus the effects of temperature on Rubisco catalysis
264 should be considered so that an appropriate Rubisco suited to the growth environment can be engineered
265 (Galmés et al., 2014a, 2015; Sharwood and Whitney, 2014). The subcellular environment of the crop is
266 also an important factor; it has been suggested that diversity in Rubisco catalysis may have evolved, at
267 least partly, as a consequence of the variability found in the subcellular environment of different plant
268 leaves (Tcherkez et al., 2006; Galmés et al., 2014c). This remains an important area requiring
269 investigation through the use of model systems such as tobacco, and an important consideration for co-
270 engineering improved Rubisco catalysis alongside large anatomical changes, e.g. the conversion of C₃
271 crops to C₄ photosynthesis (Driever and Kromdijk, 2013). Direct replacement of Rubisco will also likely
272 necessitate co-engineering of ancillary proteins to achieve maximum results, as demonstrated recently
273 through work with the co-chaperone RAF1 (Whitney et al., 2015). The recent introduction of a faster
274 cyanobacterial Rubisco that could sustain higher photosynthetic rates – albeit at high CO₂ concentrations
275 (Lin et al., 2014b; Occhialini et al., 2015) – confirms the feasibility and potential of interspecies Rubisco
276 substitutions.

277 The interaction of large and small subunits, and the potential of the small subunit to influence
278 catalysis also warrant further investigation. For example, in a recent study of close relatives of wheat, the
279 observed variability in catalysis appears unlikely to be related to differences in *rbcL*, and may be the
280 result of differences in Rubisco small subunit gene (*rbcS*) sequence (Prins et al., 2016). Wheat is known
281 to contain a large *rbcS* family (Spreitzer, 2003), however for many species the number and sequence
282 diversity of *rbcS* genes is unknown. The possible influence of environmental conditions on Rubisco small
283 subunit composition may also need to be considered (Cavanagh and Kubien, 2013). The introduction of
284 an *rbcS* gene from *Sorghum* into rice showed how the introduction of foreign small subunits can alter
285 catalysis (Ishikawa et al., 2011), and reinforces the need for more information on the variability of the
286 number, sequence and expression of *rbcS* gene-family members from wild species and crops of interest.

287

288 **CONCLUSION**

289 This study improves our understanding of the variability of Rubisco catalysis present in nature.
290 Interrogation of this large dataset provides new insights as to the consistency of the response of catalysis
291 to temperature across a broad range of species. Analysis of detailed biochemical characterisation
292 alongside sequence information suggests that targeted mutation of key residues and/or replacement of
293 crop Rubisco with superior existing enzymes will aid in efforts to engineer improved carbon assimilation
294 in key crops. This work highlights the importance of characterising the biochemistry of Rubisco at a
295 range of key temperatures alongside sequence information to improve our understanding of the
296 relationship between structure and function of this critical enzyme.

297

298 **MATERIALS AND METHODS**

299 **Plant material**

300 Seeds and plant material were kindly provided by: Royal Botanic Gardens Millennium Seed Bank (UK);
301 United States Department of Agriculture, Germplasm Resources Information Network (USDA-GRIN);
302 International Rice Research Institute (IRRI); Mike Birkett, Yi Chen, Belinda Townsend (Rothamsted
303 Research, UK); Guoxiong Chen (CAAS, Lanzhou, China); Mel Oliver (USDA, Plant Genetics Research).
304 Plants were grown in a glasshouse with a 16/8h day/night cycle with temperatures of 26/19°C. During the
305 day supplemental lighting was used to maintain a minimum light level of 200 $\mu\text{mol m}^2 \text{s}^{-1}$. Plants were
306 kept well-watered. For all analyses, samples of leaf material were taken from young, healthy plants and
307 immediately snap frozen in liquid nitrogen, then stored at -80°C.

308

309 **Climatic data**

310 Georeferenced co-ordinates for all species were downloaded from the Global Biodiversity Information
311 Facility (GBIF.org; accessed June-July 2015), and climate data (BioClim, worldclim.org/bioclim;
312 Hijmans et al., 2005) obtained using DIVA-GIS (diva-gis.org; Hijmans et al., 2001). Due to the
313 incompleteness of publically available distribution databases (Maldonado et al., 2015), studies on climate
314 niche typically use species mean values instead of climatic limits. This study used mean values of the
315 average temperature across the warmest quarter for each species as a proxy for the main growing season,
316 when most of the photosynthetic (and hence Rubisco) activity occurs. This value is referred to as T_{WQ}
317 (temperature of the warmest quarter) throughout the text, and values for each species are listed in
318 Supplemental Table S1.

319

320 **Rubisco catalytic properties**

321 Rubisco was extracted and its catalytic properties determined essentially as previously described (Prins et
322 al., 2016), with the following alterations: reactions were carried out in 0% and 21% O₂ conditions only,
323 with two technical replicates of each of these concentrations; and protein extracts were activated and
324 assayed immediately after extraction and desalting.

325

326 **Rubisco specificity factor**

327 Rubisco from each genotype was purified essentially as described by Prins et al. (2016), with the
328 exception that the final Sephacryl S-200 filtration step was found to be unnecessary for most of the
329 genotypes in this study. Testing confirmed that excluding this step did not influence the assay results.
330 Rubisco specificity (S_{CO}) was determined using the oxygen electrode method as described by (Parry et
331 al., 1989). For each species, at least four replicate measurements were made at each temperature. Values
332 were normalised to a value for *T. aestivum* at each temperature, as described by Parry et al. (1989).

333

334 **Rubisco content**

335 An aliquot of the soluble protein extracted for measuring catalytic constants was used to determine total
336 Rubisco content by ¹⁴C-CABP binding via either the method of Parry et al. (1997) or Whitney et al.
337 (1999). Testing confirmed that using one or the other method did not influence the quantification results.

338

339 ***rbcL* sequencing**

340 Genomic DNA was extracted from leaf tissue using the Qiagen DNEasy Plant Kit (Qiagen, UK).
341 Amplification of partial *rbcL* fragments equivalent to codons 1-463 (*ca.* 98% of the coding region) was
342 carried out using Phusion HF polymerase (Invitrogen, USA). Forward primer: (5'-
343 TAATTCATGAGTTGTAGGGAGGG-3'); paired with cp063R (Dong et al., 2013, 5'-
344 TTTCCATACTTCACAAGCAGCAGCTAG-3'). PCR products were then sequenced using the following
345 primers (Eurofins Genomics EU, Germany): DRS19 (5'-
346 GKGYYCCTATTGTAATGCATGACTACTTAAC-3'), *rbcL_F1*
347 (ATGTCACCACAAACAGAACTAAA) and *rbcL_F3* (CCRCCBCAYGGNATYCARG). At least two
348 independent PCR reactions were performed and had product sequenced for each genotype. Sequences
349 were submitted to EMBL (See supporting Table S3 for accession numbers).

350

351 **Rubisco L-subunit sites under positive selection**

352 DNA sequences of *rbcL* were aligned using MUSCLE (Edgar, 2004). The software MODELTEST 3.7
353 (Posada and Crandall, 1998; Posada and Buckley, 2004) was used to check for the best model before
354 running the phylogenetic analyses using maximum-likelihood inference conducted with RAxML version

355 7.2.6 (Stamatakis, 2006). Rubisco amino acid residues under positive selection associated with particular
356 kinetic traits were identified using codon-based substitution models in comparative analysis of protein-
357 coding DNA sequences within the phylogenetic framework using branch-site tests of positive selection
358 along pre-specified foreground branches in the PAML v.4.7 package (Yang, 2007) as described in
359 (Kapralov et al., 2011, 2012; Galmés et al., 2014b). Branches leading to species with high or low K_c^{air} ,
360 k_{cat}^c , K_o , k_{cat}^o and $S_{C/O}$ at 25°C were marked as foreground branches. The Rubisco L-subunit residues are
361 numbered based on the spinach sequence. The location of sites under positive selection was done using
362 Rubisco protein structure from spinach (*Spinacia oleracea* L.) obtained from the RCSB Protein Data
363 Bank (<http://www.rcsb.org>; file 1RCX; Karkehabadi et al., 2003).

364

365 **Phylogenetically Independent Contrasts (PIC)**

366 The Pearson correlation coefficient was calculated between pairwise combinations of the kinetic
367 parameters K_c , K_c^{air} , k_{cat}^c , K_o , V_o and $S_{C/O}$ at the three temperatures of measurement. Correlations arising
368 within groups of related taxa might reflect phylogenetic signal rather than true cause-effect relationships,
369 because closely related taxa are not necessarily independent data points and could violate the assumption
370 of randomized sampling employed by conventional statistical methods (Felsenstein, 1985). To overcome
371 this issue, tests were performed for the presence of phylogenetic signal in the data, and trait correlations
372 were calculated with phylogenetically independent contrasts using the AOT module of PHYLOCOM
373 (Webb et al., 2008) for the species phylogeny described above. All these tests were considered significant
374 at $P < 0.05$.

375

376 **Statistical analyses**

377 The 75 species were divided into five groups based on phylogenetic relationships (Fig. 3). To establish the
378 significance of variation between these groups (and the species within the groups), the variation with
379 temperature for each group was assessed using non-linear regression analysis and the fitting of an
380 asymptotic exponential/simple exponential model. The resulting best models were plotted. Analysis was
381 carried out using GenStat (VSN International, UK). The five C_4 species in this study were not included
382 when analysing temperature response. With the exception of $S_{C/O}$, all data were transformed via log
383 function to conform to the assumptions of the analysis.

384

385 **Supplemental Material**

386 The following supplemental materials are available.

387 Supplemental Table S1. Rubisco catalytic properties for 75 species measured at 20, 25, and 30°C.

388 Supplemental Table S2. Rubisco large subunit amino acid positions under positive selection.

389 Supplemental Table S3. EMBL accession codes for *rbcL* sequences.
390 Supplemental Table S4. Model parameters used for plotting temperature responses in Figures 5 and S3.
391 Supplemental Figure S1. Rubisco carboxylation efficiency ($k_{\text{cat}}^c/K_c^{\text{air}}$) at 20, 25 and 30°C.
392 Supplemental Figure S2. Rubisco specificity ($S_{C/O}$) at 20, 25 and 30°C.
393 Supplemental Figure S3. Temperature response of Rubisco catalytic parameters for the five groups.

394

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397 Alfred Keys (Rothamsted Research) for useful discussions, and numerous colleagues and institutions who
398 provided plant material or seeds of the species studied (listed in materials and methods).

399

400 **Table I. Key Rubisco catalytic parameters for five phylogenetic groups.**

401 $k_{\text{cat}}^{\text{c}}$, maximum carboxylation rate; $K_{\text{c}}^{\text{air}}$, Michaelis-Menten constant for CO_2 at atmospheric levels of O_2
 402 (21%); $S_{\text{C/O}}$, specificity for CO_2 vs. O_2 . For details of the species within each group see Fig. 3. Values are
 403 means \pm standard errors of the mean (n as indicated).

404

Group	n	$k_{\text{cat}}^{\text{c}}$ (s^{-1})			$K_{\text{c}}^{\text{air}}$ (μM)			$S_{\text{C/O}}$		
		20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C
1	34	2.3 \pm 0.1	3.7 \pm 0.2	5.7 \pm 0.3	19.4 \pm 0.9	28.6 \pm 1.2	34.4 \pm 1.7	114.9 \pm 0.8	104.7 \pm 0.6	92.6 \pm 0.5
2	5	2.3 \pm 0.2	3.9 \pm 0.3	5.6 \pm 0.1	14.8 \pm 1.7	31.0 \pm 2.9	40.1 \pm 3.6	110.2 \pm 1.9	99.4 \pm 2.2	86.8 \pm 0.9
3	4	2.3 \pm 0.1	4.0 \pm 0.3	7.2 \pm 0.3	18.8 \pm 3.9	39.5 \pm 4.5	52.6 \pm 8.3	110.0 \pm 4.4	101.3 \pm 3.1	88.5 \pm 1.9
4	8	1.9 \pm 0.1	3.1 \pm 0.3	4.8 \pm 0.3	16.4 \pm 2.2	27.4 \pm 1.9	30.3 \pm 1.8	107.2 \pm 1.1	99.8 \pm 1.6	92.1 \pm 1.3
5	18	1.9 \pm 0.1	3.2 \pm 0.2	5.2 \pm 0.2	15.8 \pm 1.0	25.9 \pm 1.3	33.1 \pm 2.4	107.7 \pm 1.1	97.6 \pm 1.2	87.2 \pm 1.1

405

406 **FIGURE LEGENDS**

407

408 **Figure 1.** Range of Rubisco (A) carboxylation rate ($k_{\text{cat}}^{\text{c}}$), (B) Michaelis-Menten constant for CO₂ (K_{c}),
409 and (C) specificity factor ($S_{\text{C/O}}$) at 20, 25 and 30°C. The range of values previously reported for C₃ plants
410 in the literature at 25°C (Lit 25°C) is shown for reference. Literature data is from a survey of publications
411 available as of January 2016. Box plot lines represent the median value and the 10, 25, 75 and 90th
412 percentiles.

413

414 **Figure 2.** Potential photosynthetic improvement in soybean (*Glycine max*) that would result from
415 replacement of native Rubisco with Rubisco from *Poa palustris* (yellow) or *Puccinellia maritima* (brown)
416 at 25°C. Rates of net CO₂ assimilation (A) were derived from the model of Farquhar *et al.* (1980) as
417 detailed in von Caemmerer (2000), and using *in vitro* measurements of Rubisco catalysis. Modelling
418 assumed: Rubisco content = 30 $\mu\text{mol m}^{-2}$; $R_{\text{d}} = 0.015 \times V_{\text{c,max}}$; $J = 1.75 \times V_{\text{c,max}}$; and O₂ = 21%.

419

420 **Figure 3.** Tree diagram illustrating Rubisco large subunit amino acid positions under positive selection
421 linked to superior Rubisco properties in (A) monocot species, and (B) dicot species. Eff; carboxylation
422 efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$). Dashed green lines indicate species groupings for analysis of temperature response.
423 Group 1, monocots, Poaceae/Musaceae (n=39); Group 2, Amaranthaceae (n=5); Group 3,
424 Asteraceae/Solanaceae (n=5); Group 4, Euphorbiaceae/Curcubitaceae (n=8); Group 5, Fabaceae (n=18).

425

426 **Figure 4.** Correlation coefficients of phylogenetically independent contrasts (PICs) calculated for
427 Rubisco catalytic parameters of 75 species, using data from measurements at 20, 25, or 30°C. Significant
428 correlations are marked: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

429

430 **Figure 5.** Temperature response of (A) carboxylation rate ($k_{\text{cat}}^{\text{c}}$) and CO₂ affinity in air ($K_{\text{c}}^{\text{air}}$), (B)
431 specificity factor ($S_{\text{C/O}}$) and carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$) in soybean (*Glycine max*).

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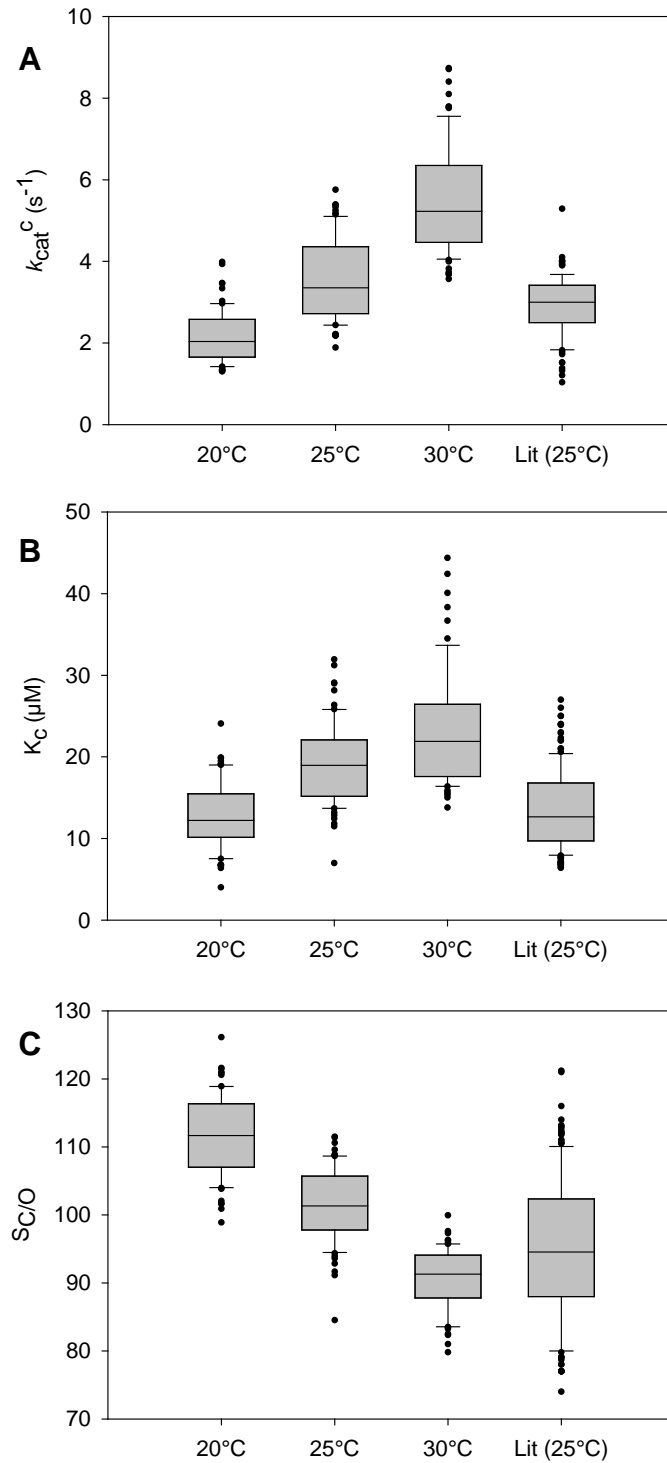


Figure 1. Range of Rubisco (A) carboxylation rate ($k_{\text{cat}}^{\text{C}}$), (B) Michaelis-Menten constant for CO_2 (K_{C}), and (C) specificity factor ($S_{\text{C/O}}$) at 20, 25 and 30°C. The range of values previously reported for C_3 plants in the literature at 25°C (Lit 25°C) is shown for reference. Literature data is from a survey of publications available as of January 2016. Box plot lines represent the median value and the 10, 25, 75 and 90th percentiles.

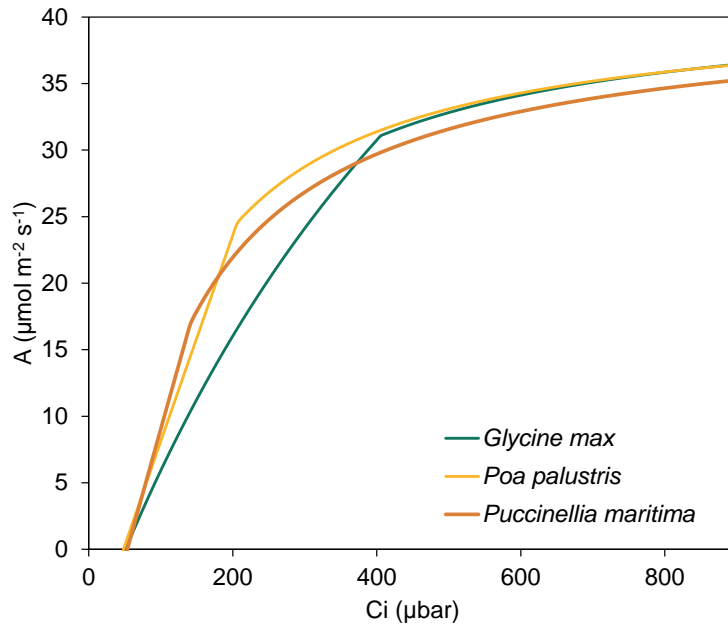


Figure 2. Potential photosynthetic improvement in soybean (*Glycine max*) that would result from replacement of native Rubisco with Rubisco from *Poa palustris* (yellow) or *Puccinellia maritima* (brown) at 25°C. Rates of net CO₂ assimilation (A) were derived from the model of Farquhar *et al.* (1980) as detailed in von Caemmerer (2000), and using *in vitro* measurements of Rubisco catalysis. Modelling assumed: Rubisco content = 30 μmol m⁻²; R_d = 0.015 × V_{c,max}; J = 1.75 × V_{c,max}; and O₂ = 21%.

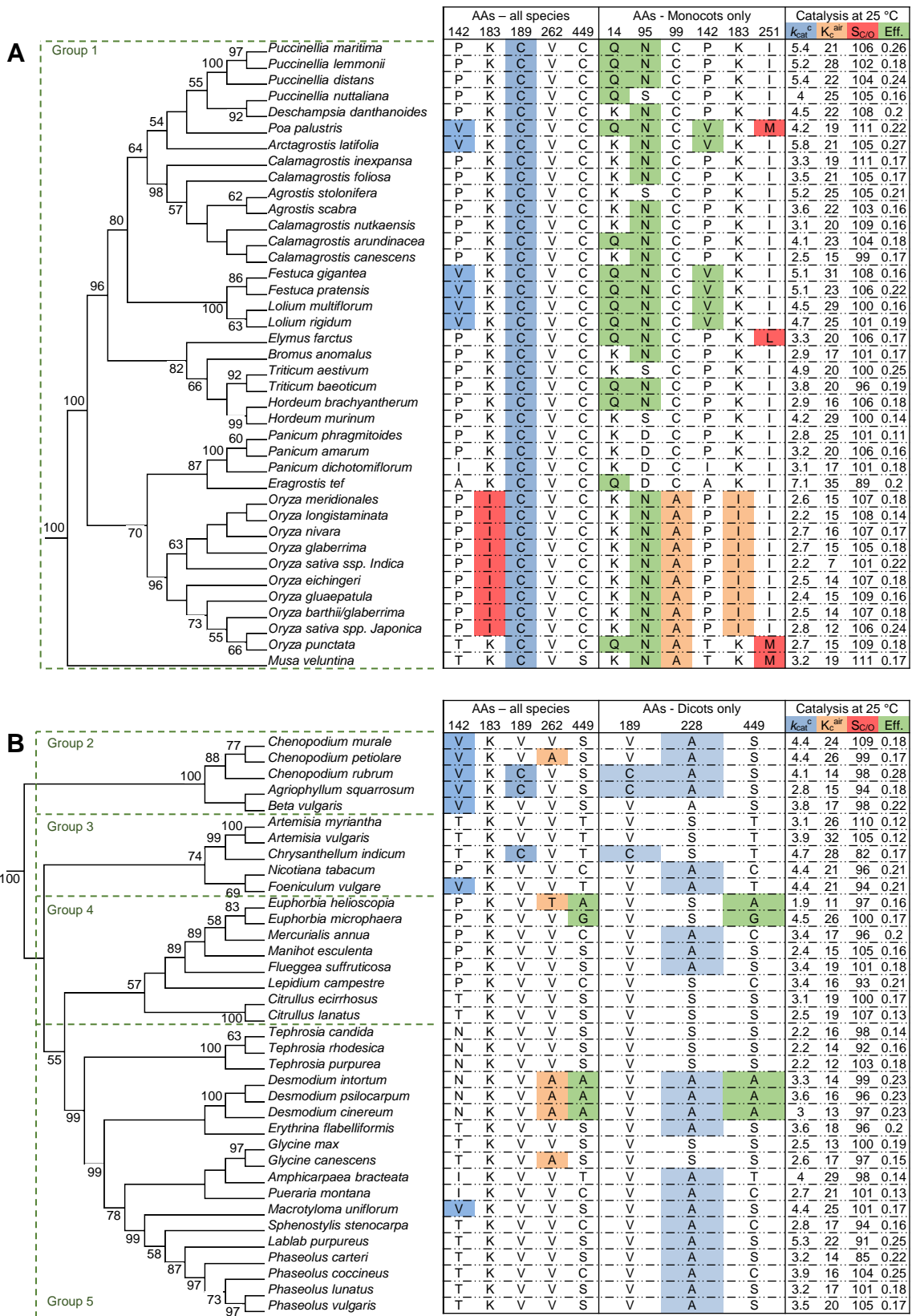


Figure 3. Tree diagram illustrating Rubisco large subunit amino acid positions under positive selection linked to superior Rubisco properties in (A) monocot species, and (B) dicot species. Eff; carboxylation efficiency (k_{cat}^c/K_c^{air}). Dashed green lines indicate species groupings for analysis of temperature response. Group 1, monocots, Poaceae/Musaceae (n=39); Group 2, Amaranthaceae (n=5); Group 3, Asteraceae/Solanaceae (n=5); Group 4, Euphorbiaceae/Cucurbitaceae (n=8); Group 5, Fabaceae (n=18).

A 20°C	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	$k_{\text{cat}}^c/K_C^{\text{air}}$	T_{WQ}
k_{cat}^c	0.730***	0.312**	-0.342**	-0.104	0.333**	0.652***	-0.775***
K_C		0.782***	0.529**	0.223*	0.209	-0.885***	0.538***
K_C^{air}			0.025	-0.265*	0.519***	-0.901***	-0.059
K_O				0.941***	-0.038	-0.132	0.742***
V_O					-0.130	0.194	0.626***
$S_{C/O}$						-0.171	-0.509***
$k_{\text{cat}}^c/K_C^{\text{air}}$							-0.307**

B 25°C	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	$k_{\text{cat}}^c/K_C^{\text{air}}$	T_{WQ}
k_{cat}^c	0.724***	0.673***	0.427***	-0.205	0.940***	-0.525***	-0.051
K_C		0.978***	0.302**	-0.639**	0.776***	-0.935***	0.208
K_C^{air}			0.110	-0.770**	0.765***	-0.927***	0.066
K_O				0.525***	0.202	-0.273*	0.716***
V_O					-0.445**	0.646***	0.284*
$S_{C/O}$						-0.567***	-0.100
$k_{\text{cat}}^c/K_C^{\text{air}}$							-0.338**

C 30°C	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	$k_{\text{cat}}^c/K_C^{\text{air}}$	T_{WQ}
k_{cat}^c	-0.028	0.034	-0.256**	0.210	0.106	0.206	-0.103
K_C		0.985***	0.244***	-0.731**	0.129	-0.977***	-0.096
K_C^{air}			0.099	-0.780**	0.071	-0.960***	-0.187
K_O				0.356*	0.061	-0.234**	0.826***
V_O					-0.231**	0.795***	0.637***
$S_{C/O}$						-0.173	-0.233**
$k_{\text{cat}}^c/K_C^{\text{air}}$							0.115

Figure 4. Correlation coefficients of phylogenetically independent contrasts (PICs) calculated for Rubisco catalytic parameters of 75 species, using data from measurements at 20, 25, or 30°C. Significant correlations are marked: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

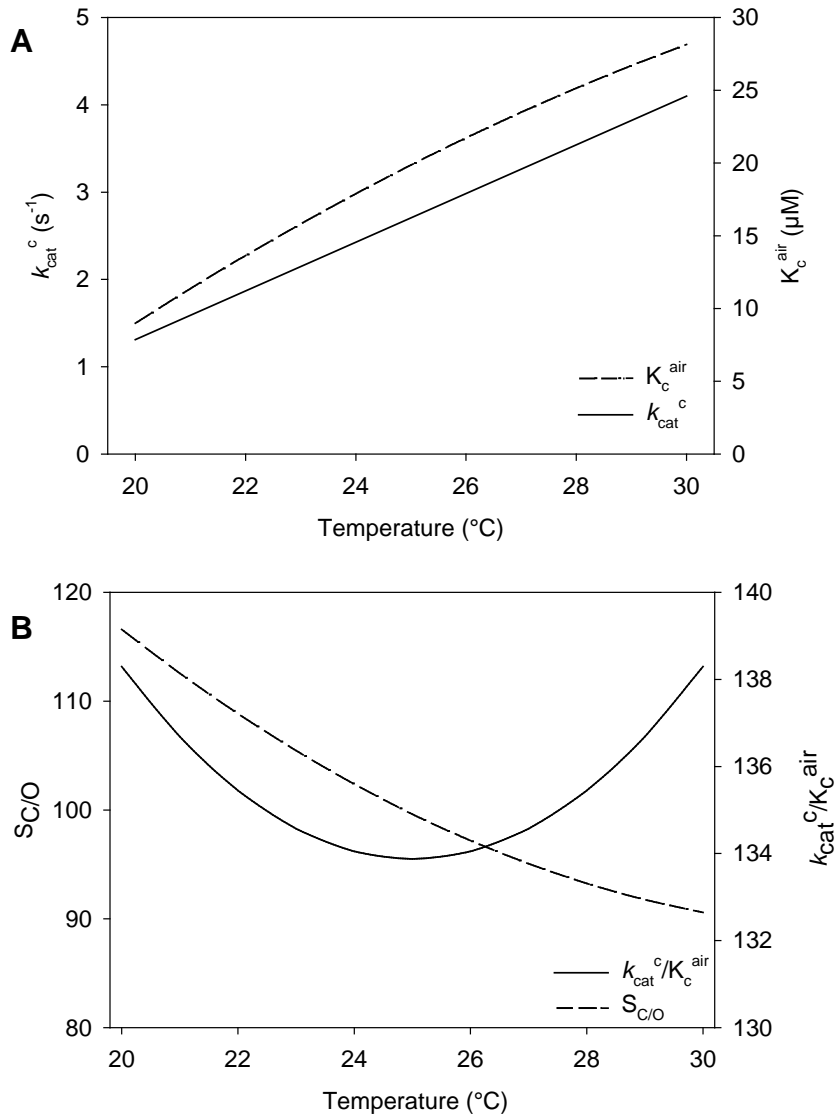


Figure 5. Temperature response of (A) carboxylation rate (k_{cat}^c) and CO₂ affinity in air (K_c^{air}), (B) specificity factor ($S_{C/O}$) and carboxylation efficiency (k_{cat}^c/K_c^{air}) in soybean (*Glycine max*).

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