

Orr et al. 2016. Rubisco catalytic diversity & temperature response

1 **Short title:** Rubisco catalytic diversity & temperature response

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

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17 **One sentence summary:** Species diversity in Rubisco catalysis shows consistencies in temperature
18 response, which can be used to improve crop photosynthetic efficiency.

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21 **List of author contributions:** DJO, MVK, PJA, ECS, MAJP designed research; ECS, PJA supervised the

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24

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32 **ABSTRACT**

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

47

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

49

50 **INTRODUCTION**

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

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

73 Efforts to engineer an improved Rubisco have not yet produced a 'super Rubisco' (Parry et al.,
74 2007; Ort et al., 2015). However, advances in engineering precise changes in model systems continue to
75 provide important developments that are increasing our understanding of Rubisco catalysis (Spreitzer et
76 al., 2005; Whitney et al., 2011a, 2011b; Morita et al., 2014; Wilson et al., 2016), regulation (Andralojc et
77 al., 2012; Carmo-Silva and Salvucci, 2013; Bracher et al., 2015) and biogenesis (Saschenbrecker et al.,
78 2007; Sharwood and Whitney, 2008; Lin et al., 2014b; Hauser et al., 2015; Whitney et al., 2015).

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

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

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

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

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106 **RESULTS**

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

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

113 Carboxylation rates ($k_{\text{cat}}^{\text{c}}$) at 25°C ranged from 1.9 s⁻¹ in *Euphorbia helioscopia* (Euphorbiaceae)
114 to 7.1 s⁻¹ in the C₄-photosynthesis type annual grass *Eragrostis tef* (Poaceae). Affinity for CO₂ was highest
115 in *Oryza sativa* ssp. Indica ($K_{\text{c}} = 7 \mu\text{M}$ at 25°C), and lowest in C₄ grasses included in this study ($K_{\text{c}} \sim 34$ -
116 37 μM , *E. tef* and *Panicum* spp.). Across the diverse group of species analysed the CO₂/O₂ specificity
117 ($S_{\text{C/O}}$) showed a large range of values, from a 25°C high of 111 in the grass *Poa palustris* (Poaceae) to a
118 low of 82 in the C₄ dicot *Chrysanthellum indicum* (Asteraceae). C₃ plants surveyed ranged in $S_{\text{C/O}}$ from
119 111 to 91. Catalytic values generally agreed with previously reported ranges (e.g. Ishikawa et al., 2011;
120 Galmés et al., 2014b; Occhialini et al., 2015).

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

126

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

128 Accompanying the biochemical analysis of a large range of species with an analysis of variation in the
129 highly conserved chloroplast *rbcL* gene, which encodes the catalytic subunit of Rubisco, provides the
130 opportunity to identify amino acid replacements potentially responsible for changes in Rubisco catalysis.
131 Positive selection analysis identified residue positions that were correlated with particular catalytic
132 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
133 Rubisco large subunit residues were associated with changes in particular catalytic characteristics across
134 the 75 species dataset (Fig. 3), with at least one residue linked to each parameter. The full list of residue
135 positions under positive selection, their structural location and possible molecular interactions is provided
136 in Supplemental Table S2.

137 Importantly, in a large analysis of sequence diversity alongside catalytic properties,
138 phylogenetically distant species may have acquired similar changes in Rubisco catalysis via different
139 amino acid substitutions, which makes finding common catalytic switches difficult. Thus, a subsequent

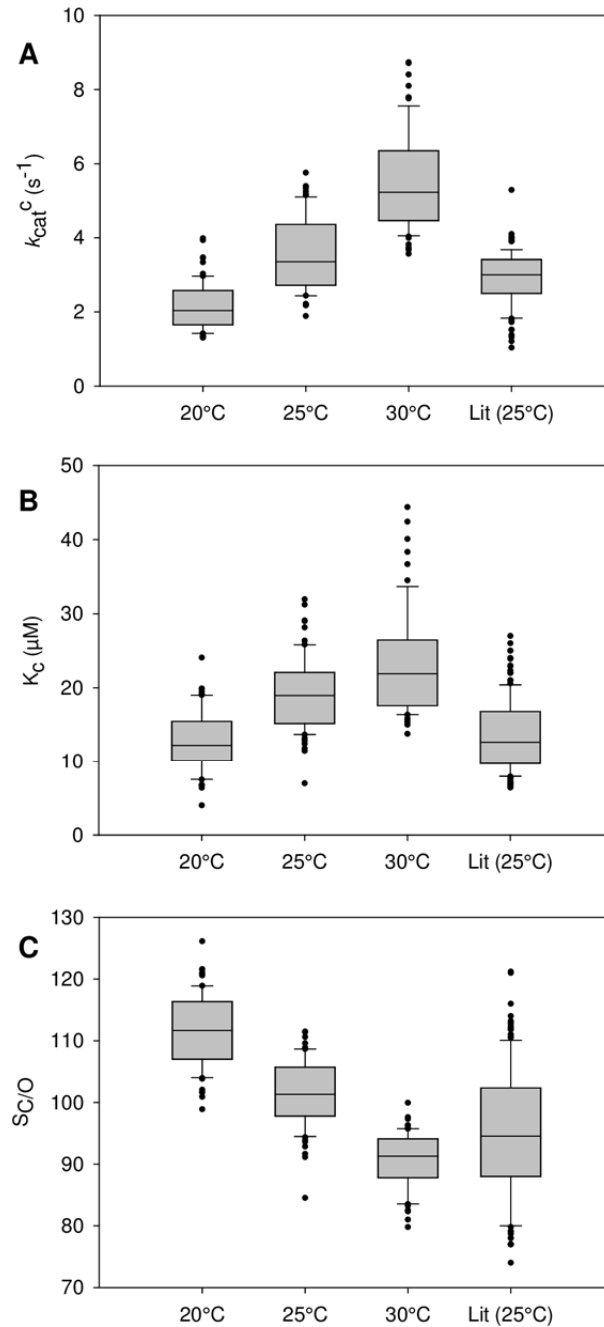


Figure 1. Range of Rubisco (A) carboxylation rate (k_{cat}^C), (B) Michaelis-Menten constant for CO_2 (K_c), and (C) specificity factor ($S_{C/O}$) at 20, 25 and 30°C. The range of values previously reported for C_3 plants in the literature at 25°C (Lit 25°C) is shown for reference. Literature data is 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.

140 separate analysis of the monocot and dicot species subsets ($n = 39$ and 36 , respectively) was conducted.
 141 Different sets of residues associated with catalytic changes were highlighted for these two groups with
 142 little overlap (Fig. 3A and 3B). Amongst the six residues found within the monocots, three positions were
 143 linked to high carboxylation efficiency, one to high $S_{C/O}$ and two to low K_c^{air} . In the dicot subset analysis,

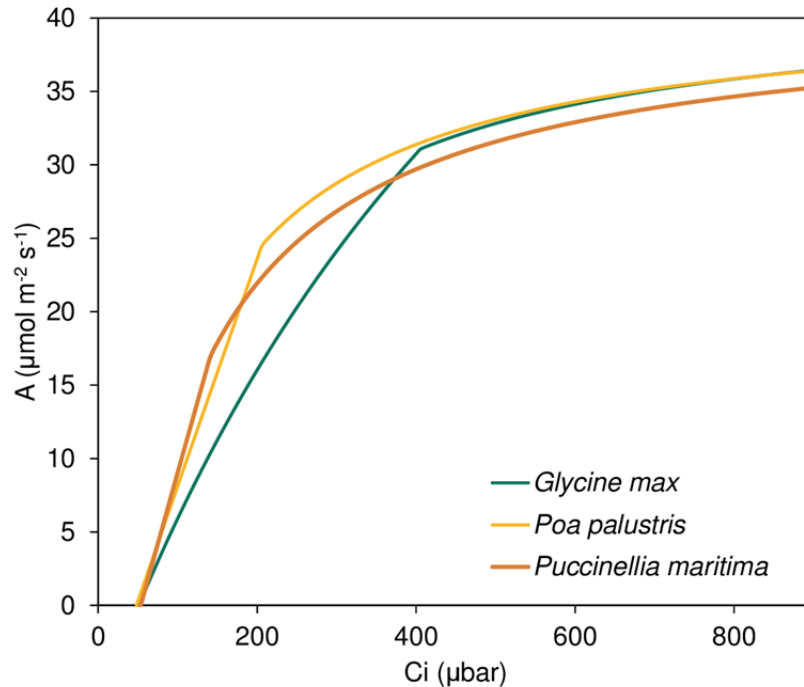


Figure 2. Potential photosynthetic improvement in soybean (*Glycine max*) that would result from replacement of native Rubisco with Rubisco from *Poa palustris* (yellow) or *Puccinellia maritima* (brown) at 25°C. Rates of net CO₂ assimilation (*A*) were derived from the model of Farquhar *et al.* (1980) 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%.

144 two residue positions were associated with high catalytic rates ($k_{\text{cat}}^{\text{c}}$), whilst a further residue position was
 145 linked to high carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_{\text{c}}^{\text{air}}$).

146

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

148 Using phylogenetically independent contrast (PIC) analyses, correlation coefficients between catalytic
 149 parameters for each measurement temperature were calculated (Fig. 4). The classical trade-off between
 150 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).

151 However, the significance and strength of this correlation varied at the different measurement
 152 temperatures examined. At 20 and 25°C the strength and significance was high ($P \leq 0.01$), while at 30°C
 153 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
 154 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
 155 efficiency at 25°C. The relationship between $k_{\text{cat}}^{\text{c}}$ and carboxylation efficiency was notably inconsistent
 156 across the three measurement temperatures.

157 To explore how climate may correlate with Rubisco catalysis in diverse species, the temperature
 158 of the warmest quarter of the year (T_{WQ}) where each species grows served as a proxy for conditions

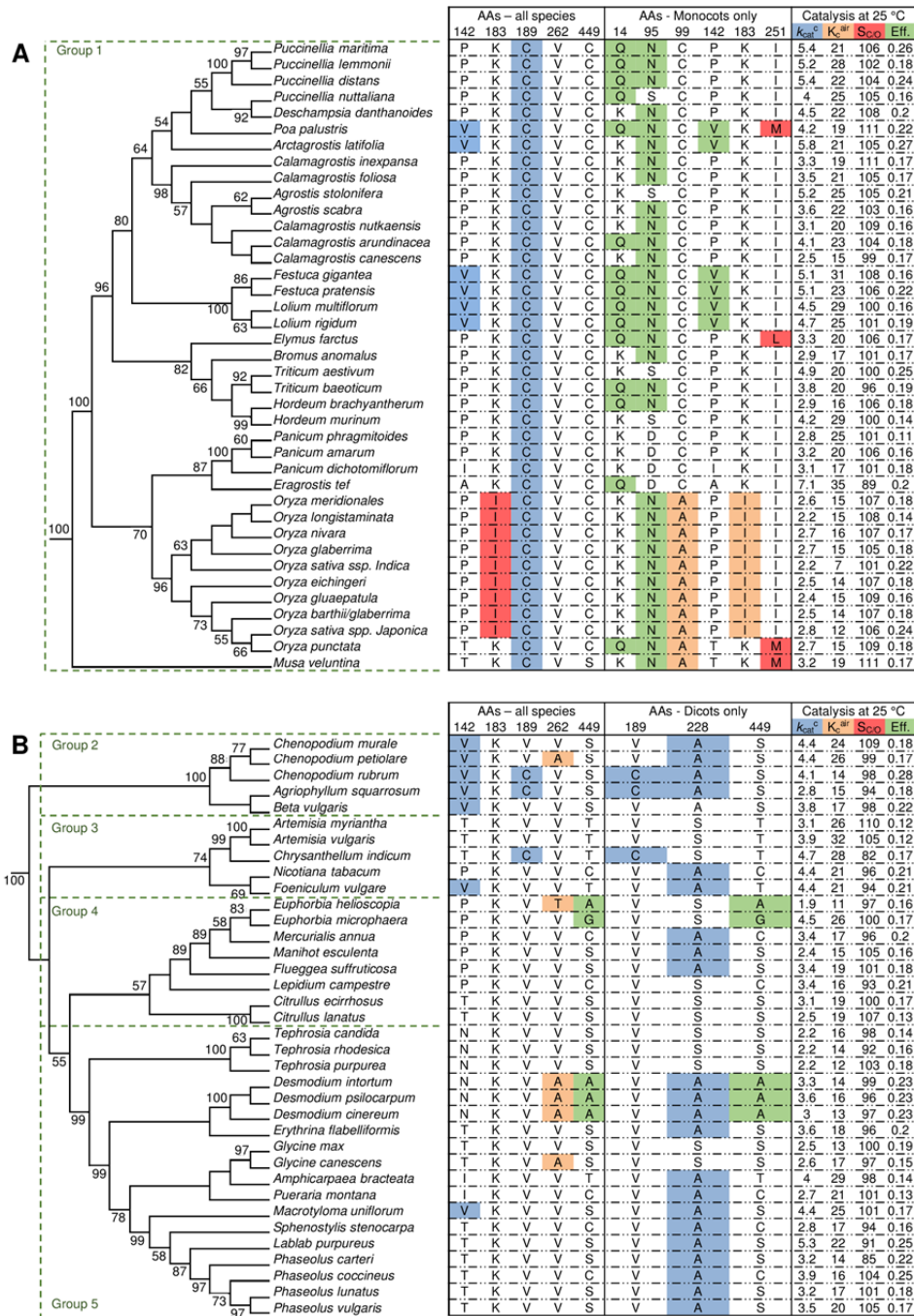


Figure 3. Tree diagram illustrating Rubisco large subunit amino acid positions under positive selection linked to superior Rubisco properties in (A) monocot species, and (B) dicot species. Eff; carboxylation efficiency (k_{cat}^c/K_c^{air}). Colour highlighting indicates amino acid substitutions at residues that are under positive selection along phylogenetic tree branches leading to species with particular catalytic properties (e.g., high k_{cat}^c). 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).

159 during the main part of the growing season. T_{WQ} was negatively correlated with $S_{C/O}$ measured at 20 and
 160 30°C (at 25°C the correlation was not significant; Fig. 4), indicating that Rubisco from species growing in
 161 higher temperature climates had lower $S_{C/O}$. Oxygenation parameters (K_o and V_o) consistently showed a
 162 significant positive correlation with T_{WQ} . Carboxylation efficiency was negatively correlated with T_{WQ} at

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: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

163 20 and 25°C, but the correlation was not significant for measurements at 30°C.

164

165 Temperature response of Rubisco catalysis

166 To examine the consistency of catalytic changes in response to temperature, the 75 species examined were
 167 divided into five natural groups based on their phylogenetic relationships (indicated in Fig 3). A summary

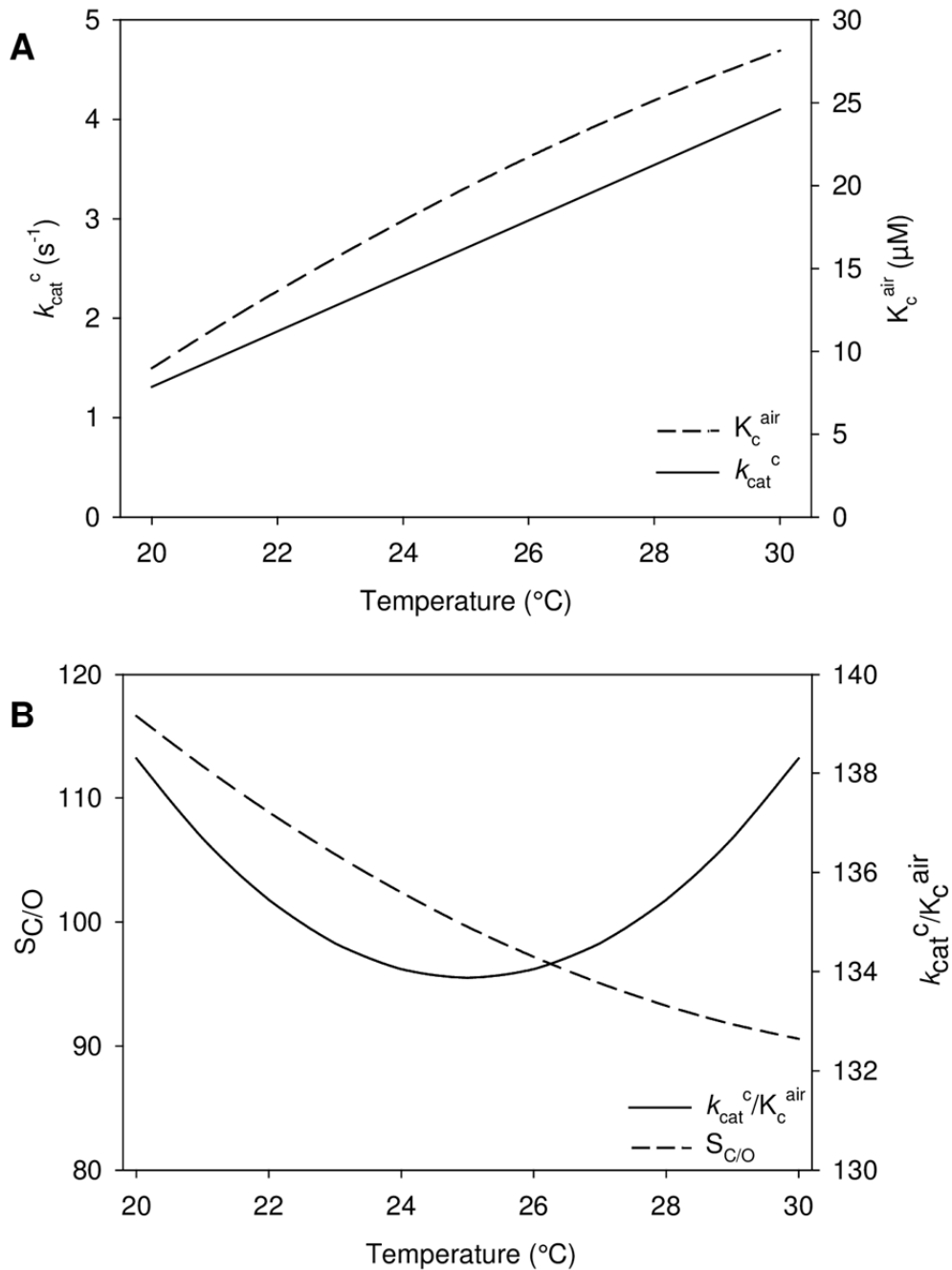


Figure 5. Temperature response of (A) carboxylation rate (k_{cat}^c) and CO₂ affinity in air (K_c^{air}), (B) specificity factor ($S_{C/O}$) and carboxylation efficiency (k_{cat}^c/K_c^{air}) in soybean (*Glycine max*).
 168 of the catalytic properties for each group at each temperature is shown in Table I, and non-linear
 169 regression analysis was used to assess the groups and species variation in temperature response
 170 (Supplemental Fig. S3). There was variation in the temperature response of Rubisco catalysis for the
 171 diverse species and groups analysed, but the trend of the response was consistent. The response of each

172 catalytic property to temperature in soybean (*Glycine max*) is provided as a representative example (Fig.
173 5). Group 3 consisted of a range of dicots, including *N. tabacum* and *Artemisia* spp., and could be fitted
174 with a single model that explained temperature response of k_{cat}° for the whole group (i.e. there was no
175 significant difference in temperature response of k_{cat}° between the species within group 3). For the other
176 groups and individual species, the temperature response of k_{cat}° was similarly explained by a linear model
177 and, while individual species displayed a consistent slope for the model generated, significant variation in
178 the intercept prevented the generation of a single model to explain the entire group. These results show
179 that the relative increase in k_{cat}° with temperature was consistent, despite the significant variation in
180 absolute values within groups.

181 A group level model for K_c^{air} could be fitted to groups 2 and 3, but not groups 1, 4 and 5. Each of
182 the 75 species was modelled with a similar quadratic function; however, only groups 2 and 3 could have
183 all its members statistically explained by a single model. K_c^{air} increased with temperature and the rate of
184 increase was lower above 25 °C, reflected in the representative function shown in Fig. 5A. As mentioned
185 above, $S_{\text{C/O}}$ decreased with temperature. Consistent with previous data, this decrease was non-linear and
186 for each species/group was best described by a quadratic function. The decrease in $S_{\text{C/O}}$ was generally
187 greater between 20-25°C than 25-30°C (Fig. 5B). In group 3, this response was reversed (greater decrease
188 between 25-30°C). Carboxylation efficiency ($k_{\text{cat}}^{\circ}/K_c^{\text{air}}$) was also described by a quadratic model with
189 efficiency being highest at 20 and 30°C, and consistently lower at 25°C. Though the drop in efficiency
190 around 25°C varied between species and groups, the quadratic effect was consistent across the range of
191 species, with variation evident in both the slope and intercept of the functions generated (Supplemental
192 Fig. S4).

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196 **DISCUSSION**

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

198 The present study represents the largest single survey of Rubisco catalysis to date. A large number of
199 studies have previously described Rubisco catalysis (reviewed in Parry et al., 2007; Whitney et al., 2011b;
200 Parry et al., 2013; Carmo-Silva et al., 2015). However, this still represents a very small fraction of known
201 lands plants (approximately 0.2% based on current literature). Unfortunately, many studies have also only
202 partially characterised Rubisco catalysis, with specificity ($S_{C/O}$) in particular lacking from most available
203 datasets (Sharwood and Whitney, 2014). The present study dramatically expands upon our knowledge of
204 Rubisco catalytic variability through full characterisation of 75 plant species, and provides a large
205 comparative dataset to inform future engineering efforts. The results presented here reinforce that, despite
206 the relatively highly conserved nature of the Rubisco large subunit gene *rbcL* (Kapralov and Filatov,
207 2007; Wang et al., 2011), key catalytic parameters vary significantly across diverse plant taxa.
208 Carboxylation rates in particular varied by almost 3-fold at 25°C. Leaf scale modelling predicted that
209 direct replacement strategies using newly characterised Rubiscos could substantially improve maximum
210 photosynthetic capacity, though this will likely require further advances in our ability to test foreign
211 Rubiscos in tobacco based systems (Whitney et al., 2011a). Nevertheless this demonstrates the potential
212 gains in photosynthetic capacity through Rubisco substitution. This dataset characterising a broad range
213 of species at multiple temperatures will also be of use in modelling of photosynthesis at different scales
214 (Smith and Dukes, 2013), and complement *in planta* studies seeking to adapt models of various scales for
215 the increased temperatures expected in many regions in the coming decades (e.g. Bagley et al., 2015).

216

217 **Targeting improvements through mutagenesis**

218 The large subunit of Rubisco, encoded by the chloroplast *rbcL* gene, contains the catalytic sites and is
219 believed to be primarily, though not solely, responsible for the catalytic profile of the holoenzyme
220 (Sharwood et al., 2008). A number of residues were identified that warrant mutagenic testing in model
221 systems, including a number of new candidates not previously highlighted. The residues identified
222 differed dependent on the set of species included in the analysis, demonstrating the need to consider the
223 phylogenetic background of a target Rubisco when determining the potential impact of point mutations. It
224 may also signify the diversity of catalytic solutions found by nature, and the likely difficulty in finding a
225 ‘one size fits all’ approach to targeted improvement of Rubisco. There is also some evidence for a role of
226 the small subunit in explaining some of the catalytic variation found in nature, though further
227 investigation in this area is required (discussed below). Potential unintended effects on assembly could be
228 a factor when mutating residues known to be involved in interactions between the large and small
229 subunits. Careful consideration must also be given to avoiding effects on holoenzyme assembly and

230 compatibility with ancillary proteins or assembly chaperones (Carmo-Silva et al., 2015; Whitney et al.,
231 2015). This presents a promising avenue for future work in model systems, testing these residues either
232 singly or in combination, with previous studies having shown strong potential for modifying Rubisco
233 catalysis with targeted amino acid substitutions (e.g. Whitney et al., 2011b).

234

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

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

248 Higher temperature environments (T_{WQ}) did not consistently correlate with carboxylation
249 parameters across assay temperatures, but did correlate with increasing K_o and V_o . The observed
250 correlations suggest that Rubiscos from warmer climates are less efficient at lower temperatures. Fitting
251 mathematical models to the response of key parameters to measurement temperature resulted primarily in
252 non-linear models, the exception being carboxylation rate (k_{cat}°). The type of model that best explained
253 temperature response of each parameter was consistent across species, though variation in the absolute
254 values for each species largely prevented fitting a single model to the species groupings. In many cases,
255 species within a group had parallel responses. This provides important new insights on the response of
256 Rubisco catalysis to temperature, and its consistency across diverse species, whilst further highlighting
257 the diversity of catalysis. It is important to note that a number of plant groups such as trees and basal
258 angiosperms remain either underrepresented in biochemical datasets, or have only just begun to be
259 surveyed (Galmés et al., 2014b), and provide potential areas where additional valuable information can be
260 gleaned from characterisation. Data is also lacking for crop species, with few represented in the
261 literature, and often with incomplete characterisation. This is an important gap in our knowledge that will
262 be important when targeting improvements to key crops. This study focused on C_3 species, the potential
263 for C_4 Rubiscos to respond differently has received increased interest recently (e.g. Boyd et al., 2015;

264 Perdomo et al., 2015), however there remains a need to characterise more Rubiscos from C₄ species for
265 thermal response.

266

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

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

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

295

296 **CONCLUSION**

297 This study improves our understanding of the variability of Rubisco catalysis present in nature.

298 Interrogation of this large dataset provides new insights as to the consistency of the response of catalysis
299 to temperature across a broad range of species. Analysis of detailed biochemical characterisation
300 alongside sequence information suggests that targeted mutation of key residues and/or replacement of
301 crop Rubisco with superior existing enzymes will aid in efforts to engineer improved carbon assimilation
302 in key crops. This work highlights the importance of characterising the biochemistry of Rubisco at a
303 range of key temperatures alongside sequence information to improve our understanding of the
304 relationship between structure and function of this critical enzyme.

305

306 **MATERIALS AND METHODS**

307 **Plant material**

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

316

317 **Climatic data**

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

327

328 **Rubisco catalytic properties**

329 Rubisco was extracted and its catalytic properties determined essentially as previously described (Prins et
330 al., 2016), with the following alterations: reactions were carried out in 0% and 21% O_2 conditions only,
331 with two technical replicates of each of these concentrations; and protein extracts were activated and

332 assayed immediately after extraction and desalting.

333

334 **Rubisco specificity factor**

335 Rubisco from each genotype was purified essentially as described by Prins et al. (2016), with the
336 exception that the final Sephacryl S-200 filtration step was found to be unnecessary for most of the
337 genotypes in this study. Testing confirmed that excluding this step did not influence the assay results.

338 Rubisco specificity ($S_{C/O}$) was determined using the oxygen electrode method as described by (Parry et
339 al., 1989). For each species, at least four replicate measurements were made at each temperature. Values
340 were normalised to a value for *T. aestivum* at each temperature, as described by Parry et al. (1989).

341

342 **Rubisco content**

343 An aliquot of the soluble protein extracted for measuring catalytic constants was used to determine total
344 Rubisco content by ^{14}C -CABP binding via either the method of Parry et al. (1997) or Whitney et al.
345 (1999). Testing confirmed that using one or the other method did not influence the quantification results.

346

347 ***rbcL* sequencing**

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

358

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

360 DNA sequences of *rbcL* were aligned using MUSCLE (Edgar, 2004). The software MODELTEST 3.7
361 (Posada and Crandall, 1998; Posada and Buckley, 2004) was used to check for the best model before
362 running the phylogenetic analyses using maximum-likelihood inference conducted with RAxML version
363 7.2.6 (Stamatakis, 2006). Rubisco amino acid residues under positive selection associated with particular
364 kinetic traits were identified using codon-based substitution models in comparative analysis of protein-
365 coding DNA sequences within the phylogenetic framework using branch-site tests of positive selection

366 along pre-specified foreground branches in the PAML v.4.7 package (Yang, 2007) as described in
367 (Kapralov et al., 2011, 2012; Galmés et al., 2014b). Branches leading to species with high or low K_c^{air} ,
368 $k_{\text{cat}}^{\text{c}}$, K_o , $k_{\text{cat}}^{\text{o}}$ and $S_{\text{C/O}}$ at 25°C were marked as foreground branches. The Rubisco L-subunit residues are
369 numbered based on the spinach sequence. The location of sites under positive selection was done using
370 Rubisco protein structure from spinach (*Spinacia oleracea* L.) obtained from the RCSB Protein Data
371 Bank (<http://www.rcsb.org>; file 1RCX; Karkehabadi et al., 2003).

372

373 **Phylogenetically Independent Contrasts (PIC)**

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

383

384 **Statistical analyses**

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

392

393 **Supplemental Material**

394 The following supplemental materials are available.

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

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

397 Supplemental Table S3. EMBL accession codes for *rbcL* sequences.

398 Supplemental Table S4. Model parameters used for plotting temperature responses in Figures 5 and S3.

399 Supplemental Figure S1. Rubisco carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_c^{\text{air}}$) at 20, 25 and 30°C.

400 Supplemental Figure S2. Rubisco specificity ($S_{C/O}$) at 20, 25 and 30°C.

401 Supplemental Figure S3. Temperature response of Rubisco catalytic parameters for the five groups.

402

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404 We thank Jess Evans and Andrew Mead (Rothamsted Research) for support with statistical analyses,

405 Alfred Keys (Rothamsted Research) for useful discussions, and numerous colleagues and institutions who

406 provided plant material or seeds of the species studied (listed in materials and methods).

407

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

409 $k_{\text{cat}}^{\text{c}}$, maximum carboxylation rate; $K_{\text{c}}^{\text{air}}$, Michaelis-Menten constant for CO₂ at atmospheric levels of O₂
 410 (21%); $S_{\text{C/O}}$, specificity for CO₂ vs. O₂. For details of the species within each group see Fig. 3. Values are
 411 means ± standard errors of the mean (*n* as indicated).

412

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

413

414 **FIGURE LEGENDS**

415

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

421

422 **Figure 2.** Potential photosynthetic improvement in soybean (*Glycine max*) that would result from
423 replacement of native Rubisco with Rubisco from *Poa palustris* (yellow) or *Puccinellia maritima* (brown)
424 at 25°C. Rates of net CO_2 assimilation (A) were derived from the model of Farquhar *et al.* (1980) as
425 detailed in von Caemmerer (2000), and using *in vitro* measurements of Rubisco catalysis. Modelling
426 assumed: Rubisco content = 30 $\mu\text{mol m}^{-2}$; $R_d = 0.015 \times V_{\text{c,max}}$; $J = 1.75 \times V_{\text{c,max}}$; and $\text{O}_2 = 21\%$.

427

428 **Figure 3.** Tree diagram illustrating Rubisco large subunit amino acid positions under positive selection
429 linked to superior Rubisco properties in (A) monocot species, and (B) dicot species. Eff; carboxylation
430 efficiency ($k_{\text{cat}}^{\text{c}}/K_c^{\text{air}}$). Colour highlighting indicates amino acid substitutions at residues that are
431 under positive selection along phylogenetic tree branches leading to species with particular
432 catalytic properties (e.g., high $k_{\text{cat}}^{\text{c}}$). Dashed green lines indicate species groupings for analysis of
433 temperature response. Group 1, monocots, Poaceae/Musaceae (n=39); Group 2, Amaranthaceae (n=5);
434 Group 3, Asteraceae/Solanaceae (n=5); Group 4, Euphorbiaceae/Curcubitaceae (n=8); Group 5, Fabaceae
435 (n=18).

436

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

440

441 **Figure 5.** Temperature response of (A) carboxylation rate ($k_{\text{cat}}^{\text{c}}$) and CO_2 affinity in air (K_c^{air}), (B)
442 specificity factor ($S_{\text{C/O}}$) and carboxylation efficiency ($k_{\text{cat}}^{\text{c}}/K_c^{\text{air}}$) in soybean (*Glycine max*).

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