Short title: Rubisco catalytic diversity & temperature response

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Surveying Rubisco diversity and temperature response to improve crop photosynthetic efficiency[1]

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

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ABSTRACT

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

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

INTRODUCTION

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

Rubisco is considered to exhibit comparatively poor catalysis, in terms of catalytic rate, specificity, and CO_2 affinity (Tcherkez et al., 2006; Andersson, 2008), leading to the suggestion that even small increases in catalytic efficiency may result in substantial improvements to carbon assimilation across a growing season (Zhu et al., 2004; Parry et al., 2013; Galmés et al., 2014a; Carmo-Silva et al., 2015). If combined with complimentary changes such as optimising other components of the Calvin Benson or photorespiratory cycles (e.g. Raines, 2011; Peterhansel et al., 2013; Simkin et al., 2015), optimised canopy architecture (Drewry et al., 2014), or introducing elements of a carbon concentrating mechanism (Furbank et al., 2009; Lin et al., 2014a; Hanson et al., 2016; Long et al., 2016), Rubisco improvement presents an opportunity to dramatically increase the photosynthetic efficiency of crop plants (McGrath and Long, 2014; Long et al., 2015; Betti et al., 2016). A combination of the available strategies is essential for devising tailored solutions to meet the varied requirements of different crops and the diverse conditions under which they are typically grown around the world.

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

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

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

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

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

RESULTS

Variability in Rubisco catalysis across plant species

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

Carboxylation rates (k_{cat}^{c}) at 25°C ranged from 1.9 s^{-1} in *Euphorbia helioscopia* (Euphorbiaceae) to 7.1 s^{-1} in the C_4-photosynthesis type annual grass *Eragrostis tef* (Poaceae). Affinity for CO_2 was highest in *Oryza sativa* ssp. Indica (K_c = 7 µM at 25°C), and lowest in C_4 grasses included in this study (K_c ~34-37 µM, *E. tef* and *Panicum* spp.). Across the diverse group of species analysed the CO_2/O_2 specificity ($S_{C/O}$) showed a large range of values, from a 25°C high of 111 in the grass *Poa palustris* (Poaceae) to a low of 82 in the C_4 dicot *Chrysanthellum indicum* (Asteraceae). C_3 plants surveyed ranged

in $S_{C/O}$ from 111 to 91. Catalytic values generally agreed with previously reported ranges (e.g. Ishikawa et al., 2011; Galmés et al., 2014b; Occhialini et al., 2015).

Modelling of leaf photosynthesis shows that the direct replacement of native Rubisco in a crop, such as soybean (*Glycine max*), with two high performing monocot Rubiscos would support significant improvements of leaf-level photosynthetic rates at current atmospheric CO_2 levels and high irradiance (Fig. 2). Photosynthesis improvement was particularly evident at low internal CO_2 concentrations when leaf photosynthesis is typically limited by Rubisco activity.

Linking *rbc*L sequence variation with Rubisco biochemical diversity

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

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

Correlations between catalytic parameters at a range of temperatures

Using phylogenetically independent contrast (PIC) analyses, correlation coefficients between catalytic parameters for each measurement temperature were calculated (Fig. 4). The classical trade-off between increasing k_{cat}^{c} and decreasing CO_2 affinity (increased K_c or K_c^{air}) was evident (Tcherkez et al., 2006). However, the significance and strength of this correlation varied at the different measurement temperatures examined. At 20 and 25°C the strength and significance was high ($P \leqslant 0.01$), while at 30°C

there was no significant correlation between increasing k_{cat}^{c} and CO_2 affinity (K_c or K_c^{air}). $S_{C/O}$ correlated positively with k_{cat}^{c}, K_c and K_c^{air}, most significantly at 20 and 25°C, and negatively with carboxylation efficiency at 25°C. The relationship between k_{cat}^{c} and carboxylation efficiency was notably inconsistent across the three measurement temperatures.

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

Temperature response of Rubisco catalysis

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

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 the 75 species was modelled with a similar quadratic function; however, only groups 2 and 3 could have all its members statistically explained by a single model. K_c^{air} increased with temperature and the rate of increase was lower above 25 °C, reflected in the representative function shown in Fig. 5A. As mentioned above, $S_{C/O}$ decreased with temperature. Consistent with previous data, this decrease was non-linear and for each species/group was best described by a quadratic function. The decrease in $S_{C/O}$ was generally

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

DISCUSSION

Significant variation in Rubisco catalysis amongst diverse species

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

Targeting improvements through mutagenesis

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

differed dependent on the set of species included in the analysis, demonstrating the need to consider the phylogenetic background of a target Rubisco when determining the potential impact of point mutations. It may also signify the diversity of catalytic solutions found by nature, and the likely difficulty in finding a 'one size fits all' approach to targeted improvement of Rubisco. There is also some evidence for a role of the small subunit in explaining some of the catalytic variation found in nature, though further investigation in this area is required (discussed below). Potential unintended effects on assembly could be a factor when mutating residues known to be involved in interactions between the large and small subunits. Careful consideration must also be given to avoiding effects on holoenzyme assembly and compatibility with ancillary proteins or assembly chaperones (Carmo-Silva et al., 2015; Whitney et al., 2015). This presents a promising avenue for future work in model systems, testing these residues either singly or in combination, with previous studies having shown strong potential for modifying Rubisco catalysis with targeted amino acid substitutions (e.g. Whitney et al., 2011b).

The effect of temperature on Rubisco catalysis

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

Higher temperature environments (T_{WQ}) did not consistently correlate with carboxylation parameters across assay temperatures, but did correlate with increasing K_o and V_o. The observed correlations suggest that Rubiscos from warmer climates are less efficient at lower temperatures. Fitting mathematical models to the response of key parameters to measurement temperature resulted primarily in non-linear models, the exception being carboxylation rate (k_{cat}^{c}). The type of model that best explained temperature response of each parameter was consistent across species, though variation in the absolute values for each species largely prevented fitting a single model to the species groupings. In many cases, species within a group had parallel responses. This provides important new insights on the response of

Rubisco catalysis to temperature, and its consistency across diverse species, whilst further highlighting the diversity of catalysis. It is important to note that a number of plant groups such as trees and basal angiosperms remain either underrepresented in biochemical datasets, or have only just begun to be surveyed (Galmés et al., 2014b), and provide potential areas where additional valuable information can be gleamed from characterisation. Data is also lacking for crop species, with few represented in the literature, and often with incomplete characterisation. This is an important gap in our knowledge that will be important when targeting improvements to key crops. This study focused on C_3 species, the potential for C_4 Rubiscos to respond differently has received increased interest recently (e.g. Boyd et al., 2015; Perdomo et al., 2015), however there remains a need to characterise more Rubiscos from C_4 species for thermal response.

Tailored solutions are required for optimising crop carbon assimilation

The variation in catalysis found during this study provides important information for future efforts to engineer improved Rubisco in crops via either replacement with a foreign Rubisco (Fig. 2) or point mutations of the endogenous gene (Fig. 3). In C_3 plants, 20-35°C is considered the optimum temperature range for photosynthesis (Blankenship, 2014), and thus the effects of temperature on Rubisco catalysis should be considered so that an appropriate Rubisco suited to the growth environment can be engineered (Galmés et al., 2014a, 2015; Sharwood and Whitney, 2014). The subcellular environment of the crop is also an important factor; it has been suggested that diversity in Rubisco catalysis may have evolved, at least partly, as a consequence of the variability found in the subcellular environment of different plant leaves (Tcherkez et al., 2006; Galmés et al., 2014c). This remains an important area requiring investigation through the use of model systems such as tobacco, and an important consideration for co-engineering improved Rubisco catalysis alongside large anatomical changes, e.g. the conversion of C_3 crops to C_4 photosynthesis (Driever and Kromdijk, 2013). Direct replacement of Rubisco will also likely necessitate co-engineering of ancillary proteins to achieve maximum results, as demonstrated recently through work with the co-chaperone RAF1 (Whitney et al., 2015). The recent introduction of a faster cyanobacterial Rubisco that could sustain higher photosynthetic rates – albeit at high CO_2 concentrations (Lin et al., 2014b; Occhialini et al., 2015) – confirms the feasibility and potential of interspecies Rubisco substitutions.

The interaction of large and small subunits, and the potential of the small subunit to influence catalysis also warrant further investigation. For example, in a recent study of close relatives of wheat, the observed variability in catalysis appears unlikely to be related to differences in *rbc*L, and may be the result of differences in Rubisco small subunit gene (*rbc*S) sequence (Prins et al., 2016). Wheat is known to contain a large *rbc*S family (Spreitzer, 2003), however for many species the number and sequence

diversity of *rbc*S genes is unknown. The possible influence of environmental conditions on Rubisco small subunit composition may also need to be considered (Cavanagh and Kubien, 2013). The introduction of an *rbc*S gene from *Sorghum* into rice showed how the introduction of foreign small subunits can alter catalysis (Ishikawa et al., 2011), and reinforces the need for more information on the variability of the number, sequence and expression of *rbc*S gene-family members from wild species and crops of interest.

CONCLUSION

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

MATERIALS AND METHODS

Plant material

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

Climatic data

Georeferenced co-ordinates for all species were downloaded from the Global Biodiversity Information Facility (GBIF.org; accessed June-July 2015), and climate data (BioClim, worldclim.org/bioclim; Hijmans et al., 2005) obtained using DIVA-GIS (diva-gis.org; Hijmans et al., 2001). Due to the incompleteness of publically available distribution databases (Maldonado et al., 2015), studies on climate niche typically use species mean values instead of climatic limits. This study used mean values of the average temperature across the warmest quarter for each species as a proxy for the main growing season,

when most of the photosynthetic (and hence Rubisco) activity occurs. This value is referred to as T_{WQ} (temperature of the warmest quarter) throughout the text, and values for each species are listed in Supplemental Table S1.

Rubisco catalytic properties

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

Rubisco specificity factor

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

Rubisco content

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

***rbc*L sequencing**

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

355

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

357 DNA sequences of *rbc*L were aligned using MUSCLE (Edgar, 2004). The software MODELTEST 3.7
358 (Posada and Crandall, 1998; Posada and Buckley, 2004) was used to check for the best model before
359 running the phylogenetic analyses using maximum-likelihood inference conducted with RAxML version
360 7.2.6 (Stamatakis, 2006). Rubisco amino acid residues under positive selection associated with particular
361 kinetic traits were identified using codon-based substitution models in comparative analysis of protein-
362 coding DNA sequences within the phylogenetic framework using branch-site tests of positive selection
363 along pre-specified foreground branches in the PAML v.4.7 package (Yang, 2007) as described in
364 (Kapralov et al., 2011, 2012; Galmés et al., 2014b). Branches leading to species with high or low K_c^{air},
365 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
366 numbered based on the spinach sequence. The location of sites under positive selection was done using
367 Rubisco protein structure from spinach (*Spinacia oleracea L.*) obtained from the RCSB Protein Data
368 Bank (http://www.rcsb.org; file 1RCX; Karkehabadi et al., 2003).

369

370 **Phylogenetically Independent Contrasts (PIC)**

371 The Pearson correlation coefficient was calculated between pairwise combinations of the kinetic
372 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
373 within groups of related taxa might reflect phylogenetic signal rather than true cause-effect relationships,
374 because closely related taxa are not necessarily independent data points and could violate the assumption
375 of randomized sampling employed by conventional statistical methods (Felsenstein, 1985). To overcome
376 this issue, tests were performed for the presence of phylogenetic signal in the data, and trait correlations
377 were calculated with phylogenetically independent contrasts using the AOT module of PHYLOCOM
378 (Webb et al., 2008) for the species phylogeny described above. All these tests were considered significant
379 at $P < 0.05$.

380

381 **Statistical analyses**

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

389

390 **Supplemental Material**

391 The following supplemental materials are available.

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

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

394 Supplemental Table S3. EMBL accession codes for *rbc*L sequences.

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

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

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

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

399

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

404

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

406 $k_{cat\text{-}c}^{c}$, maximum carboxylation rate; K_{c}^{air}, Michaelis-Menten constant for CO_2 at atmospheric levels of O_2

407 (21%); $S_{C/O}$, specificity for CO_2 vs. O_2. For details of the species within each group see Fig. 3. Values are

408 means ± standard errors of the mean (*n* as indicated).

409

Group	*n*	$k_{cat\text{-}c}^{c}$ (s^{-1})			K_{c}^{air} (μM)			$S_{c/o}$		
		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

410

411 **FIGURE LEGENDS**

412

413 **Figure 1.** Range of Rubisco (A) carboxylation rate (k_{cat}^{c}), (B) Michaelis-Menten constant for CO_2 (K_c),
414 and (C) specificity factor ($S_{C/O}$) at 20, 25 and 30°C. The range of values previously reported for C_3 plants
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421 at 25°C. Rates of net CO_2 assimilation (A) were derived from the model of Farquhar *et al.* (1980) as
422 detailed in von Caemmerer (2000), and using *in vitro* measurements of Rubisco catalysis. Modelling
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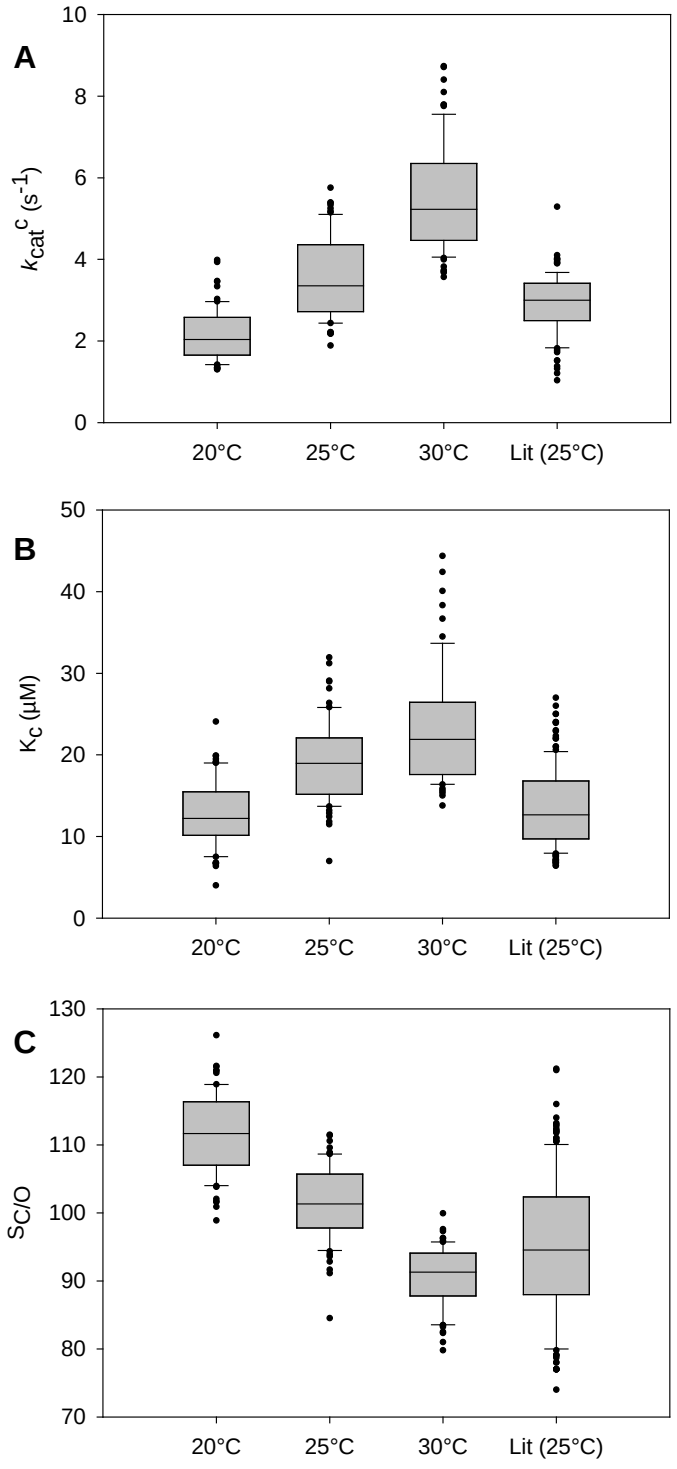

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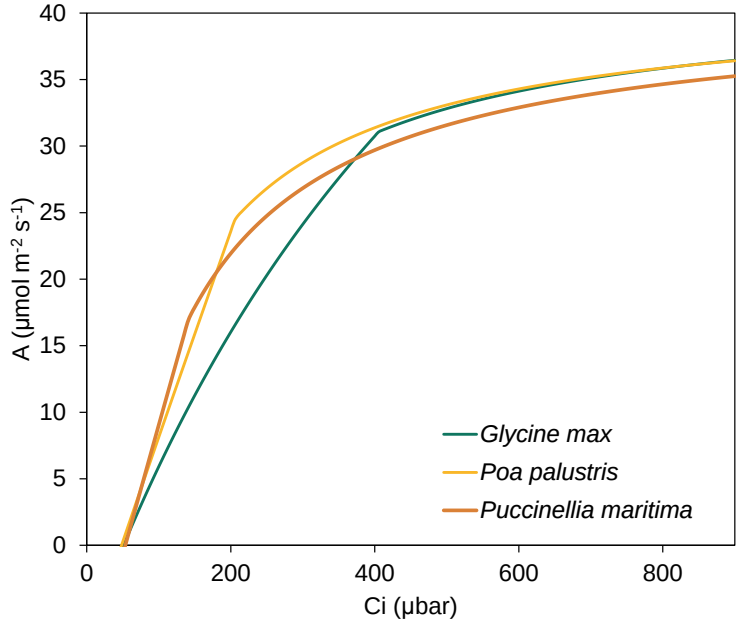

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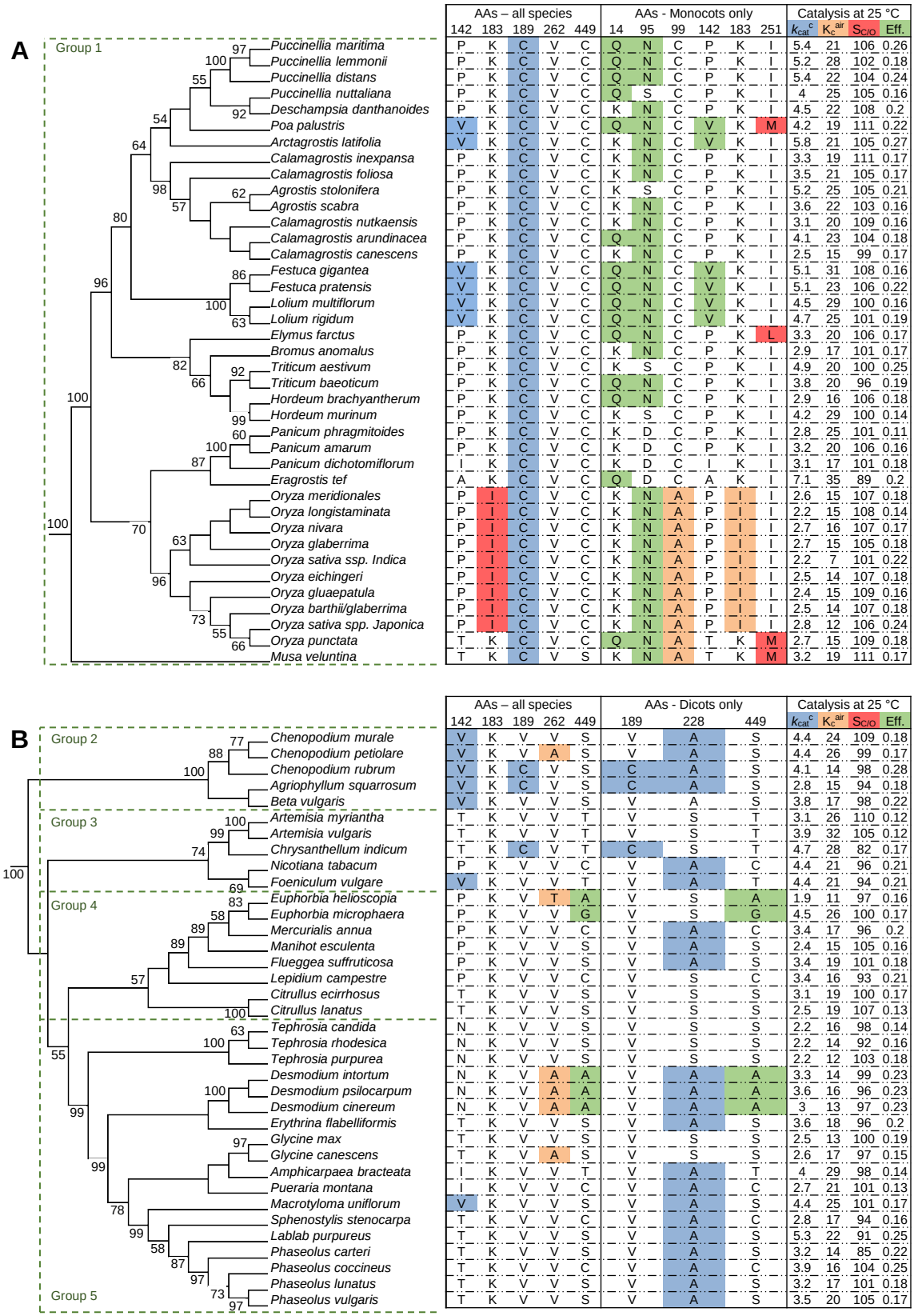


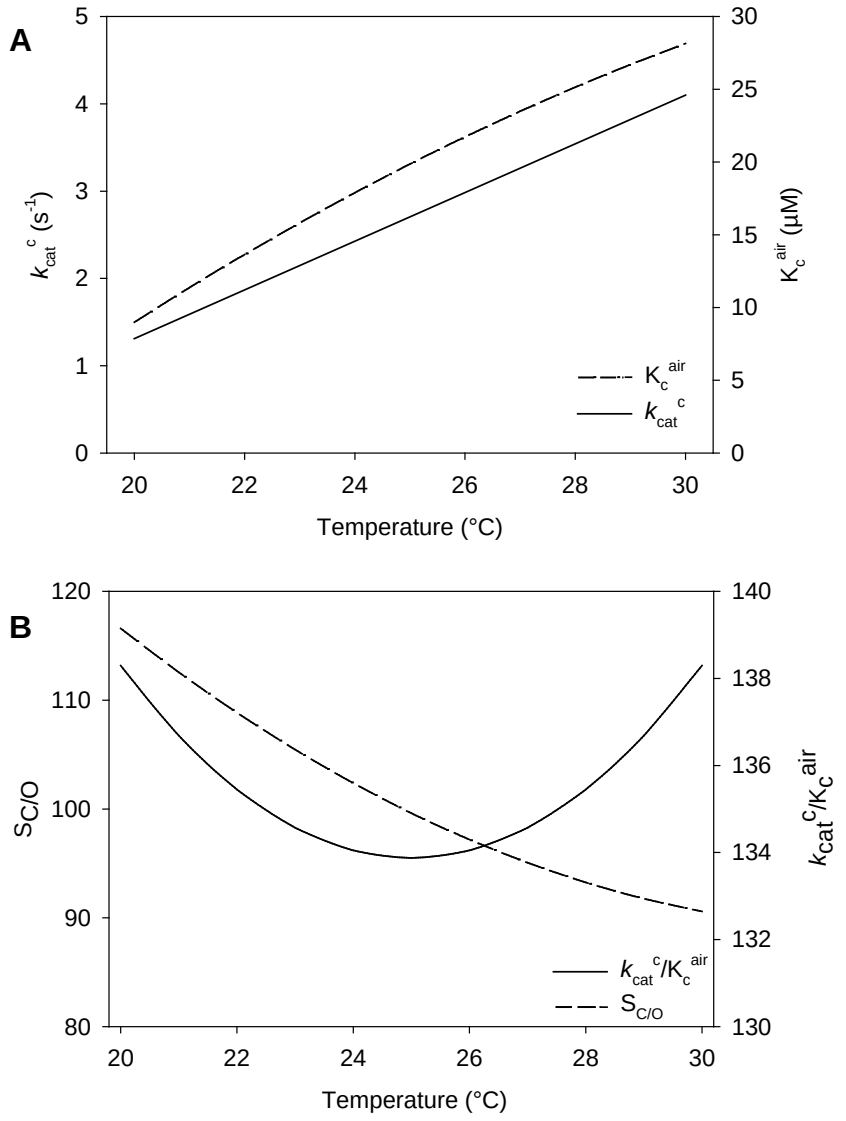
Figure 3. Tree diagram illustrating Rubisco large subunit amino acid positions under positive selection linked to superior Rubisco properties in (A) monocot species, and (B) dicot species. Eff; carboxylation efficiency (k_{cat}^{c}/K_{c}^{air}). Colour highlighting indicates amino acid substitutions 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/Curcubitaceae (n=8); Group 5, Fabaceae (n=18).

A **20°C**	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	k_{cat}^c/K_C^{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_{cat}^c/K_C^{air}							-0.307**

B **25°C**	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	k_{cat}^c/K_C^{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_{cat}^c/K_C^{air}							-0.338**

C **30°C**	K_C	K_C^{air}	K_O	V_O	$S_{C/O}$	k_{cat}^c/K_C^{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_{cat}^c/K_C^{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: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.



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