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Variation in response of C\textsubscript{3} and C\textsubscript{4} Paniceae Rubisco to temperature provides opportunities for improving C\textsubscript{3} photosynthesis

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Abstract:
Enhancing the catalytic properties of the CO$_2$-fixing enzyme Rubisco is a target for improving agricultural crop productivity. Here we reveal high diversity in the kinetic response between 10°C to 37°C by Rubisco from C$_3$- and C$_4$-species within the grass tribe Paniceae. The CO$_2$-fixation rate ($k_{cat}^c$) for Rubisco from the C$_4$-grasses with NADP-malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PCK) photosynthetic pathways was two-fold greater than the $k_{cat}^c$ of Rubisco from NAD-ME species over all temperatures. The decline in the response of CO$_2$/O$_2$ specificity with increasing temperature was slower for PCK and NADP-ME Rubisco – a trait which would be advantageous in the warmer climates they inhabit relative to the NAD-ME grasses. Variation in the temperatures kinetics of Paniceae C$_3$-Rubisco and PCK-Rubisco were modelled to differentially stimulate C$_3$-photosynthesis above and below 25°C under current and elevated CO$_2$. Identified are large subunit amino acid substitutions that could account for the catalytic variation among Paniceae Rubisco. Incompatibilities with Paniceae Rubisco biogenesis in tobacco however hindered their mutagenic testing by chloroplast transformation. Circumventing these bioengineering limitations is critical to tailoring the properties of crop Rubisco to suit future climates.
Concerns about how escalating climate change will influence ecosystems are particularly focused on the consequences to global agricultural productivity where increases are paramount to meet the rising food and biofuel demands. Strategies to improve crop yield by increasing photosynthesis have largely focused on overcoming the functional inadequacies of the CO2-fixing enzyme Rubisco. A competing O2-fixing reaction by Rubisco produces a toxic product whose recycling by photorespiration consumes energy and releases carbon. The frequency of the oxygenation reaction increases with temperature. To evade photorespiration many plants from hot, arid ecosystems have evolved C4 photosynthesis that concentrates CO2 around Rubisco that also facilitates improved plant water, light and nitrogen use. Here we show extensive catalytic variation in Rubisco from Paniceae grasses that align with the biochemistry and environmental origins of the different C4 plant subtypes. We reveal opportunities for enhancing crop photosynthesis under current and future CO2 levels at varied temperatures.

The realization of the dire need to address global food security has heightened the need for new solutions to increase crop yields. Field tests and modelling analyses have highlighted how photosynthetic carbon assimilation underpins the maximal yield potential of crops. This has increased efforts to identify solutions to enhance photosynthetic efficiency and hence plant productivity. Particular attention is being paid to improving the rate at which ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) can fix CO2 (refs 4–8). The complex structure and catalytic chemistry of Rubisco has so far made improving its performance difficult. Diversity screens have identified natural Rubisco variants with catalytic improvements of potential benefit, but most overlook the influence of broad changes in temperature.
In C$_3$-plants Rubisco performance is hampered by slow CO$_2$-fixation rates ($k_{\text{cat}}^C$ ~2-3 s$^{-1}$) and competitive O$_2$-fixation that produces 2-phosphoglycolate, which requires recycling by the energy-consuming and CO$_2$-releasing photorespiratory cycle. This necessitates C$_3$-plants invest up to 50% of their leaf protein (~25% of their nitrogen) into Rubisco to sustain viable CO$_2$ assimilation rates$^{1-3}$. A reduction in the atmospheric CO$_2$-O$_2$ ratio during the Oligocene period (~30 million years ago) heightened plant photorespiration rates, particularly in hot, arid environments$^4$. This led to the convergent evolution of C$_4$ photosynthesis along >65 multiple independent plant lineages$^5$. C$_4$-plants contain a CO$_2$ concentrating mechanism (CCM) that allows Rubisco in the chloroplasts of bundle sheath cells (BSC) to operate under near-saturating CO$_2$ levels. This suppresses O$_2$-fixation and photorespiration. The BSC CCM begins in the adjoining mesophyll cells (MC) where inorganic carbon, as HCO$_3^-$, is fixed to phosphoenolpyruvate (PEP) by PEP carboxylase (PEPC) to form the C$_4$-acid oxaloacetate (OAA). Conversion of OAA to malate (or aspartate) precedes its diffusion into the BSC where it is decarboxylated to elevate CO$_2$ around Rubisco. The three biochemical subtypes of C$_4$-plants correlate to the dominant decarboxylation enzyme: nicotinamide adenine dinucleotide (NAD) phosphate malic enzyme (NADP-ME), NAD malic enzyme (NAD-ME) or phosphoenolpyruvate carboxykinase (PEP-CK)$^6$.$^7$.

An escalating appreciation of the significant kinetic variation among plant, algae and prokaryotic Form I Rubisco has, until recently, paid little consideration to the functional diversity and potential of C$_4$-Rubisco to improve C$_3$-photosynthesis$^8$. Adaptation of C$_4$-Rubisco to elevated BSC CO$_2$ has beneficially increased carboxylation rate ($k_{\text{cat}}^C$) but unfavourably lowered CO$_2$-affinity (i.e. increased $K_m$ for CO$_2$). The increase
in $k_{\text{cat}}^C$ endows $C_4$-plants with accompanying improvements in their nitrogen (less Rubisco
required), water (reduced stomata apertures needed) and energy (reduced photorespiration)
use efficiencies$^9$ – features considered of potential benefit to engineering in $C_3$-plants$^{10}$. What remains unclear is the extent to which variation in the ancestral timing, CCM
biochemistry and biogeographical origin has influenced the kinetic evolution $C_4$-Rubisco,
its response to temperature and it’s potential to benefit $C_3$-photosynthesis without a CCM.

Here we examine the diversity in the temperature response (10°C to 37°C) of
Rubisco catalysis in Paniceae grasses comprising species with $C_3$, $C_3$-$C_4$ intermediate ($C_2$)
and all three $C_4$ biochemical subtypes. We identify significant variation in the kinetic
properties of Paniceae Rubisco which correlates with the photosynthetic physiology and
environmental distribution of each species. We show by modelling how the potential of
Paniceae Rubisco to differentially improve $C_3$-photosynthesis at low and high temperatures
under current and future CO$_2$. Differences in the chaperone requirements of monocot
Rubiscos are revealed that prevent use of chloroplast transformation to validate Paniceae
Rubisco “catalytic switches” using the surrogate model dicot plant tobacco.
Materials and Methods

Plant Seeds and Growth Conditions

Seeds for Panicum antidotale, P. monticola, P. virgatum, P. milliaceum, P. coloratum, P. deustum, P. milioides, P. bisulcatum, Megathyrsus maximus, Urochloa panicoides, U. mosambicensis, Cenchrus ciliaris, Setaria viridis and Steinchisma laxa were obtained from Australian Plant Genetic Resources Information System (QLD, Australia) and Queensland Agricultural Seeds Pty. Ltd., (Toowoomba, Australia) (Table S1) and sown in germination trays containing a common germination mix. Three to four weeks after germination, three seedlings were transplanted into 5L pots containing potting mix and grown in the glass house under natural illumination at 28°C/22°C D/N. Plants were watered regularly with the addition of a commercial of liquid fertilizer (General Purpose, Thrive Professional, Yates, Australia).

Leaf dry matter carbon isotope composition

Leaf dry matter carbon isotope composition was determined to confirm which species use the C₄ photosynthetic pathway. Leaf discs were oven-dried then combusted in a Carlo Erba elemental analyser (Model 1108, Milan, Italy). The emitted CO₂ was analyzed by mass spectrometry (VG Isotech, Manchester, UK) and the δ¹³C was calculated as [(R_{sample} – R_{standard})/ R_{standard}]*1000, where R_{sample} and R_{standard} are the ¹³C/¹²C ratio of the sample and the standard Pee Dee Belemnite (PDB), respectively.

Rubisco catalytic measurements

Rates of ¹⁴CO₂ fixation by fully activated Rubisco were measure at 10 to 37°C using soluble leaf protein extracted from 0.5 to 2.0 cm² of leaf material extracted in 1 mL
extraction buffer as described by Sharwood et al (2008). Preliminary assays as described in Sharwood et al., (2016) were used to confirm the suitability of the extraction process for sustained maximal activity over 30 min at 25°C. The $^{14}$CO$_2$ fixation assays (0.5 mL) were performed in 7-mL septum-capped scintillation vials in reaction buffer (50 mM EPPES-NaOH (pH 8.19 at 25°C), 10 mM MgCl$_2$, 0.4 mM RuBP) containing varying concentrations of NaH$^{14}$CO$_3$ (0–40 μM) and O$_2$ (0–30%) (vol/vol), accurately mixed with nitrogen using Wostoff gas-mixing pumps. The vials were incubated at the appropriate assay temperature for at least 1 hr before adding 20 μL of the soluble leaf protein to initiate the reaction. The assays were terminated with 0.1 mL of 20% (v/v) formic acid after 1 min (for the assays at 25 to 37°C) or 2 min (for the assays at 10 to 20°C). The Rubisco kinetic measurements were performed using two to six biological samples (see Table S2 for detail). Each protein sample was assayed in duplicate following incubation at 25°C for 8 and 12 min. The carboxylation activity varied by <2% between each technical replicate. This confirmed each Rubisco was fully activated after incubating 8 min at 25°C with no detectable loss of activity after incubating a further 4 min. The Rubisco content (determined by $^{14}$C-CABP binding) and integrity of the extracted L$_8$S$_8$ holoenzyme Rubisco was confirmed by non-denaturing PAGE. For each experiment a soluble leaf protein preparation was added to four assays containing the highest [$^{14}$CO$_2$] and 5 nmol of purified RuBP. After reacting to completion (1 to 3 h at different temperatures) they were treated with formic acid, dried and processing for scintillation counting. The measured $^{14}$C cpm in each assay varied by <0.5% and the average value divided by 5 to derive the $^{14}$CO$_2$ specific activity. The values for pH, pK$_1$, pK$_2$ and q (the CO$_2$ solubility at 1 atm) used to calculate CO$_2$ levels in the assays at the different temperatures are provide in Table S5.
The Michaelis-Menten constants ($K_m$) for O$_2$ ($K_O$), for CO$_2$ under nitrogen ($K_C$) or air levels of O$_2$ ($K_{C^{21\%O_2}}$) were determined from the fitted data. The maximal rate of carboxylation ($V_{c}^{\text{max}}$) was extrapolated from the fitted data and the caboxylation rate ($k_{\text{cat}}$) derived by dividing $V_{c}^{\text{max}}$ by the Rubisco-catalytic site content quantified by $[^{14}\text{C}]-2$-CABP binding.

Rubisco CO$_2$/O$_2$ specificity ($S_{C/O}$) was measured using Rubisco rapidly purified by ion exchange then Superdex 200 (GE Life Sciences) size exclusion column chromatography. The assays were equilibrated with 500 ppm CO$_2$ mixed with O$_2$ using Wostoff gas-mixing pumps and $S_{C/O}$ calculated using CO$_2$:O$_2$ solubility ratios of 0.033, 0.035, 0.036, 0.038, 0.039 and 0.041 at assay temperatures of 10, 15, 20, 25, 30 and 35°C, respectively (see Table S5 for gas solubility detail).

**rbcL amplification, sequencing and phylogenetic alignment**

Replica genomic DNA preparations (2 to 4) from each grass species were purified from ~0.5 cm$^2$ leaf discs using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instructions. The full length rbcL coding sequence (including adjoining 5′UTR and 3′UTR sequence) was PCR amplified from each DNA preparation using primers 5′PanrbcL (5′-CTAATCCATATCGAGTAGAC -3′) and 3′PanrbcLDNA (5′-AGAATTACTGCGATTTTCGGAAC -3′). The amplified products varied in size between species (1504 to 1589–bp) but each showed identical sequence for the independent DNA preparations from each species. DNA sequences were translated into protein sequences and aligned using MUSCLE (Edgar, 2004) and the rbcL phylogeny reconstructed using maximum-likelihood inference conducted with RAxML version 7.2.6.
Chloroplast transformation of Panicum rbcL into tobacco

Plasmids pLevPdL and pLevPbL were biolistically transformed into the plastome of the tobacco genotype cmtrL1 as described to derive the transplastomic genotypes tobPdL and tobPbL that, respectively, coded the rbcL genes from P. deustum and P. bisulcatum (in addition to the aadA gene coding spectinomycin resistance) in place of the tobacco rbcL gene. RNA blot, [14C]-2-CABP binding and PAGE analyses of Rubisco expression were performed on independent homoplasmic lines for each genotype as described above with additional experimental detail provided in Figure S7.

Statistical analysis

Statistical analysis was carried out using one-way (species or photosynthetic type/subtype) or two-way analysis of variance, ANOVA (Statistica, StatSoft Inc. OK, USA). Means were grouped using a Post-hoc Tukey test. Detailed description of the temperature response analysis and modelling are provided in the Figure and Table legends for convenience.

Results

Our comprehensive evaluation of Rubisco kinetics within the Paniceae tribe included two C₃, one C₃-C₄ intermediate (signified C₂⁷), four NADP-ME, four PCK and three NAD-ME species (Table S1). Rubisco from tobacco, our model plant for Rubisco engineering and that commonly used in biochemical modeling, was included as a control. The C₃ and C₄ physiologies of each species were confirmed using dry matter carbon isotope ratio (δ¹³C) measurements (Table S1). As expected, the δ¹³C kinetic isotope effect was significantly lower in the C₂ and C₃ species (≈ -28.7‰) relative to the C₄ specie (≈ -13.3 to -14.6‰) (Fig 1a).
The carboxylation properties of Paniceae Rubisco synchronize with C$_4$-subtype.

Substantial variation was found in the Rubisco kinetics measured at 25°C among enzymes from C$_2$/C$_3$-species and each C$_4$-subtype (Table S1). Relative to the carboxylation rates ($k_{\text{cat}}^c$) of the C$_2$/C$_3$ species, the Rubisco $k_{\text{cat}}^c$ was marginally higher in NAD-ME and 2-fold greater in the NADP-ME and PCK species (Fig. 1b). Consistent with the co-dependency of $K_C$ and $k_{\text{cat}}^c$ 14, greater reductions in CO$_2$-affinity (i.e. higher $K_C$’s) were found for Rubisco from NADP-ME and PCK species relative to the NAD-ME and C$_2$/C$_3$ species (Fig 1c). Less variation was observed for the averaged oxygenation rates ($k_{\text{cat}}^o$; Fig. 1d) and O$_2$-affinities ($K_O$; Fig. 1d) among C$_3$ and C$_4$ Rubisco. Nevertheless, the NADP-ME Rubiscos tended to show less sensitivity to O$_2$ inhibition (i.e. a higher $K_O$). This improvement did not, however, improve the specificity for CO$_2$ over O$_2$ ($S_{C/O}$) of NADP-ME Rubisco, which was significantly lower than the more similar $S_{C/O}$ of Rubisco from the C$_3$, NAD-ME and PCK species (Fig 1f).

Analysis of these core catalytic parameters underscored how the strong positive correlation between $k_{\text{cat}}^c$ and $K_C$ shared by plant Rubisco 14-21 extends to Paniceae C$_3$- and C$_4$-Rubisco (Fig 1g). Uniquely, each C$_4$-subtype Rubisco aggregated at a distinctive position along the regression indicative of adaptation to the differences in CCM efficiencies and biogeography among C$_4$-subtypes 22, or reflective of differences in resource partitioning to Rubisco that, in NADP-ME plants for example, correlate with improved N-use efficiency 9. The “subtype-grouping” of the carboxylase kinetics was not evident in the increasingly weaker linear correlations between $k_{\text{cat}}^o$ and $K_O$ (Fig 1h), $k_{\text{cat}}^c$ and $k_{\text{cat}}^o$ (Fig 1i) and $K_C$ with $K_O$ (Fig 1j). Evidently, the coordinated changes in $k_{\text{cat}}^c$ and $K_C$ for each Paniceae C$_4$-subtype are not tightly coupled to changes in oxygenase kinetics. This feature
is common to Rubisco due to differences in the mechanism and energy profiles of the
carboxylation and oxygenation reactions, a property that has facilitated the evolution of
diverse Rubisco kinetics \(^{14,23}\).

**The potential for Paniceae Rubisco to improve \(\text{C}_3\)-photosynthesis at 25°C.**

A recent study of Rubisco kinetic diversity revealed how the enzyme from some \(\text{C}_4\)-
species, such as the increased \(S_{\text{C/O}}\) and carboxylation efficiency under ambient \(\text{O}_2\) (\(k_{\text{cat}}^c / \ K_{\text{C}_{21\%O_2}}\) of *Zea mays* (maize) NADP-ME Rubisco, has the potential to improve \(\text{C}_3\)-
photosynthesis\(^8\). The bi-functionality of Rubisco necessitates consideration of both \(\text{O}_2\) and
\(\text{CO}_2\)-fixing activities when evaluating improvement within \(\text{C}_3\)-photosynthesis\(^{1,24,25}\), and
does not necessarily accord with a higher \(k_{\text{cat}}^c\)\(^{1,8}\). A correlative analysis of these parameters
for Paniceae Rubisco identified a weak relationship between \(k_{\text{cat}}^c\) and \(S_{\text{C/O}}\) \(r^2 = 0.43;\ Fig 2a)\) supporting mounting evidence that the trade-off proposed between these parameters\(^{14,21}\)
shows significant natural divergence\(^{18,26}\). Differences in \(\text{O}_2\) inhibition among the Paniceae
Rubisco (i.e. variable \(K_0\) values, Fig 1e) resulted in \(K_{\text{C}_{21\%O_2}}\) values (quantified as
\(K_{\text{C}}(1+[\text{O}_2]/K_0))\) that showed a weaker co-dependence with \(k_{\text{cat}}^c\) \(r^2 = 0.76;\ Fig 2b)\) relative
to \(K_{\text{C}}\) \(r^2 = 0.88;\ Fig 1g)\). This underscores the inaccuracy of using \(K_{\text{C}}\) measures as a proxy
to interpret the relative \(\text{CO}_2\)-affinity of Rubisco under ambient \(\text{O}_2\) (i.e. \(K_{\text{C}_{21\%O_2}}\)).

The biochemical models of Farquhar et. al., (1980)\(^{24}\) provide a useful tool to
evaluate how the kinetic properties of Rubisco influence carbon assimilation in \(\text{C}_3\)-plants.
These \(\text{C}_3\)-models often use tobacco Rubisco as the reference \(^{1,8,27,28}\). This stems from
tobacco having well characterized Rubisco kinetics, it being the model species for
bioengineering Rubisco by chloroplast and nucleus transformation, and its potential to
support higher rates of photosynthesis at 25°C under low chloroplast \(\text{CO}_2\) pressures (\(C_c\))
than wheat Rubisco\(^8,29\). Figure 2c shows comparable C\(_3\)-modeling using the averaged S\(_{\text{C/O}}\), \(k_{\text{cat c}} / K_{\text{C21\%O2}}\) and \(k_{\text{cat c}}\) values from each Paniceae biochemical subtype (Table S1). Under low \(C_c\) where CO\(_2\)-assimilation rates are carboxylase limited, Rubisco from the NADP-ME and PCK species would support higher rates of photosynthesis than the Paniceae C\(_3\) and NAD-ME and tobacco Rubisco (Fig 2c). Under higher \(C_c\) where photosynthesis becomes limited by light dependent rates of electron transport the lower S\(_{\text{C/O}}\) of the Paniceae C\(_4\)-Rubiscos would support lower rates of CO\(_2\)-assimilation relative to tobacco. In contrast the higher S\(_{\text{C/O}}\) of Paniceae C\(_3\)-Rubisco would enhance their CO\(_2\)-assimilating capacity at \(C_c\)'s above \(\sim 240\) µbar (Fig 2c).

The temperature diversity of Paniceae Rubisco

Most diversity screens of Rubisco kinetics are undertaken at 25\(^\circ\)C and possibly one or two other temperatures\(^18,20,30,31\). More rigorous studies providing kinetics that can be extrapolated over a broad temperature range have primarily focused on Rubisco from C\(_3\)-plants \(^15,19,28,32,33\). In general, the level of kinetic variation has been sufficient to highlight weakness in the customary use of the temperature response for tobacco Rubisco kinetics \(^27\) to reliably model the photosynthetic responses of other species. This weakness is particularly apparent from our high precision temperature response measurements that reveal substantial kinetic diversity among Paniceae Rubisco from NAD-ME, NADP-ME, PCK and C\(_2\)/C\(_3\) groupings (Fig 3). The parameters analyzed were S\(_{\text{C/O}}\), \(k_{\text{cat c}}\) and \(K_{\text{C21\%O2}}\) (averting the need to measure \(K_O\) for C\(_3\)-modeling purposes) at six incremental temperatures between 10 and 37\(^\circ\)C (Fig S1 to S3). The activation energies (\(\Delta H_a\)) for each Rubisco parameter were comparable among the Paniceae species tested within each C\(_3\) and C\(_4\)-subtype grouping (Table S3). This facilitated the derivation of averaged \(\Delta H_a\) and scaling
constant values (c) for each parameter (Fig 3a). Consistent with the highly variable properties of Rubisco from each Paniceae grouping (Fig 1) the $\Delta H_a$ values showed greater variation (Fig 3a) than that reported for Rubisco from differing C$_3$ species$^{18}$ and C$_4$-dicot Flaveria species$^{19}$. This divergence is readily apparent from plots using the averaged $\Delta H_a$ values to extrapolate the temperature response of $k_{\text{cat}}^c$ (Fig 3b), $K_{C^{21\%O_2}}$ (Fig 3c), $k_{\text{cat}}^c / K_{C^{21\%O_2}}$ (Fig 3d) and $S_{C/O}$ (Fig 3e) for each Paniceae Rubisco grouping and tobacco Rubisco (control).

The $k_{\text{cat}}^c$ for each Rubisco showed a biphasic Arrhenius temperature response above and below $\sim 25^\circ C$ (Fig S1). This necessitated the derivation of two $\Delta H_a$ and c measurements for each Rubisco $k_{\text{cat}}^c$ (Fig. 3a) whose modeled temperature responses intersect at $25^\circ C$ (Fig 3b). Importantly, the dual activation energy response of $k_{\text{cat}}^c$ is universal to all temperature response studies of plant Rubisco but mostly not acknowledged$^{15,18-20,28,30-32}$. The basis for the asymmetric response remains uncertain.

At each assay temperature the $k_{\text{cat}}^c$ and $K_{C^{21\%O_2}}$ for each NADP-ME and PCK Rubisco were consistently $\sim 2$-fold higher than Rubisco from P. bisulcatum (C$_3$), P. milioides (C$_2$) and each NAD-ME species (Table S1 and S2). The shared change in $k_{\text{cat}}^c$ with temperature by NAD-ME and C$_2$-C$_3$ Rubisco (Fig 3b) was not evident in the measured $K_{C^{21\%O_2}}$ values that showed a heightened rate of increase with temperature by NAD ME Rubisco (Fig. 3c). The biphasic response of $k_{\text{cat}}^c$ was evident in corresponding measures of carboxylation efficiency ($k_{\text{cat}}^c / K_{C^{21\%O_2}}$) that showed two linear responses that deviated at temperatures above and below $\sim 25^\circ C$ for each Rubisco (Fig 3d). A comparable $k_{\text{cat}}^c / K_c$ temperature dependency is apparent for the Rubisco from Flaveria C$_3$ and C$_4$ species$^{19}$ and Setaria viridis C$_4$-Rubisco$^{15}$. The differential slopes of the linear regression underscores
the significant variation in $k_{\text{cat}}^c$ and $K_C^{21\%O_2}$ between each Paniceae Rubisco grouping (both below and above 25°C) and emphasizes the extrapolative limitations of kinetic surveys examining only a few temperatures. This is particularly relevant for measures of $S_{C/O}$ where the extent of exponential change appears more prevalent with reducing temperature (Fig. 3e).

**The potential for improving C₃-photosynthesis under current and future CO₂ and elevated temperatures**

The temperature response of each Paniceae Rubisco showed varying extents of improvement in $S_{C/O}$ and/or $k_{\text{cat}}^c/K_C^{21\%O_2}$ relative to tobacco Rubisco (Fig S3 and S4). The improvements observed were greater for Rubisco from *P. bisulcatum* (C₃), *Urochloa panicoides* (C₄-PCK) and *P. deustum* (C₄-PCK). When modeled in a C₃-photosynthesis context under varying temperature and chloroplast CO₂ pressures ($C_c$) under saturating illumination (Fig S5) all three Rubiscos differentially improved carbon assimilation relative to tobacco Rubisco (Fig 4). At temperatures below 20°C the simulated photosynthesis rates were limited by electron transport rate at atmospheric CO₂ ($C_a$) levels above those of pre-industrial times ($C_a > 280$ ppm $\approx C_c > 170$ ppm) (Fig S5). Improvements in $S_{C/O}$ were therefore required to enhance photosynthetic rates at low temperature, a kinetic trait afforded by Paniceae C₃/C₂ Rubisco (Fig 3c), in particular *P. bisulcatum* Rubisco (Fig 4 and S3). However, the heightened $S_{C/O}$ sensitivity of *P. bisulcatum* Rubisco to increasing temperature (Fig S3a) caused these improved photosynthetic rates to wane with increasing $C_a$ and temperature (Figs 4 and S5). In contrast, the improved $S_{C/O}$ response to temperature by *P. deustum* Rubisco (Fig S3) and rising $k_{\text{cat}}^c/K_C^{21\%O_2}$ (Fig S4) substantially improved photosynthesis rates at temperatures $>20$°C under current and
future C₄ levels (Fig 4b). This improvement exceeded that simulated for U. panicoides Rubisco whose lower Sₜ_/Oₐ hindered its enhancement potential. The antagonistic advantage of these Paniceae Rubisco to lower (P. bisulcatum) and higher (U. panicoides, P. deustum) temperatures were not apparent from the 25°C kinetic measurements.

**The challenge of identifying catalytic switches in Paniceae Rubisco**

The rbcL gene in the plastome of each Paniceae species were fully sequenced and their amino acid sequences compared (Fig 5a). A phylogenetic analysis revealed the L-subunit sequences branched according to C₃ and C₄-subtype physiology (Fig. S6) except P. monticola (NADP-ME) and M. maximus (PCK) Rubisco that share identical L-subunits but show large catalytic variation (Table S1 and S2). This suggests that Paniceae Rubisco small subunits influence catalysis, a function likely shared by the small (S-) subunits of sorghum³⁴ and wheat³⁵ Rubisco.

While examination of the S-subunit diversity among Paniceae remains to be undertaken, our L-subunit analysis identified Ala 94 and Ala 228 (spinach Rubisco numbering) as exclusive to C₄ Rubisco with Ser 328 and Glu 470 substitutions favored by PCK and NADP-ME Rubisco (Fig 5a). Potential roles for amino acids 94 and 228 in catalysis are unclear. Residue 94 is distal to the catalytic sites in the equatorial region of Rubisco exposed to solvent where it facilitates interactions with Rubisco activase (RCA)³⁶,³⁷. Residue 228 is within the α2 helix also distal to the catalytic site but proximal to residues at the interface of each L-subunit and two S-subunit βA-βB loops (Figure 5b). Ala-228-Ser substitutions influence structural movements in these loops and can influence kinetics via long range effects ³⁸,³⁹. Catalytic roles for Ser-328 and Glu-470 appear more obvious. Amino acid 328 is located at the hinge of loop 6 that closes over the catalytic site
to facilitate intra-molecular interactions that influences both the fixation rate and partiality
for carboxylation or oxygenation. Loop 6 closure involves the L-subunit C-terminus
where amino acid 470 resides (Fig 5b). As a hydrophobic Ala-470 in the Paniceae NAD-
ME and C3 Rubisco, burial of the side chains into the enzyme surface may slow C-terminus
movement. In contrast, Glu/Gln-470 might enhance solvent exposure and increase C-
terminal tail mobility to alter the dynamics of loop 6 closure and stimulate $k_{cat}^c$.

We sought to test the possible role of L-subunit amino acid replacement(s) in
influencing the variability in kinetics and temperature response among Paniceae Rubisco
by tobacco chloroplast transformation. Multiple chloroplast genome (plastome) transformed tobacco lines were made (tob$^{PdL}$ and tob$^{PbL}$) where the tobacco plastome rbcL
gene was replaced with the rbcL gene from P. bisulcatum or P. deustum were generated
(Fig S7a). Each transformed line was unable to survive outside of tissue culture (Fig 5c).
Despite producing ample levels of Panicum rbcL mRNA (Fig S7b), no hybrid L$_8$S$_8$
holoenzyme (comprising Panicum L-subunits and tobacco S-subunits, Fig S7c) or
unassembled Panicum L-subunits (Fig S7d) were detected. This suggests there are
incompatibilities in the biogenesis requirements (translation, folding and/or assembly) of
Rubisco between monocot and dicot species.
Discussion:

As calls for expanding the range of Rubiscos included in catalytic diversity studies increase, so should the range of temperatures examined. Unlike prior C$_3$-focused Rubisco diversity studies, our high resolution catalytic screen revealed variation in the kinetic trajectories of Paniceae Rubisco capable of enhancing C$_3$-photosynthesis at temperatures otherwise missed, or misjudged, from assaying at 25°C and one or two other temperatures.

Our analyses validate the co-evolution of higher $k_{\text{cat}}^c$ and $K_C$ across C$_4$-Rubiscos in response to a CCM$^{4,8,12,16,18,20}$, and unveil the widest variability in temperature kinetics reported for vascular plant Rubisco to date. We uniquely reveal alignment of Rubisco kinetics with CCM biochemistry and Paniceae biogeography. For example, the higher $k_{\text{cat}}^c$ and $K_C$ of the NADP-ME and PCK Paniceae Rubisco correlated with the forecast higher BSC CO$_2$ levels in these C$_4$-subtypes relative to the NAD-ME and the CCM deficient C$_3$/C$_2$ species$^{41}$. The slower decline in S$_{\text{CO}_2}$/O by PCK and NADP-ME Rubisco under increasing temperature (Fig 3e) may reflect their warmer origins relative to the drier and cooler origins of NAD-ME and C$_3$ grasses, respectively$^{42}$. Endeavors to determine whether these correlations extend to other C$_4$-species should take heed of inaccurately extrapolating the response of $k_{\text{cat}}^c$ to temperature using a single Arrhenius fit rather than correctly accounting for its biphasic response that deviates at ~25°C (Fig 3b) – a relationship recognized 40 years ago$^{32}$, but whose mechanistic origin remains an unsolved.

The clustering of carboxylase properties of Paniceae Rubisco according to photosynthetic physiology contrasted with the more variable oxygenase activities (Fig 1i & j) supporting assertions these competing reactions can evolve independently due to differences in the mechanism and energy profile of their multi-step reactions$^{23}$. This
variability engenders natural kinetic diversity which, on the Rubisco superfamily scale, is relatively restricted for C$_3$-Rubisco$^{2,14,17,21,25,29}$ $^{19,31}$. In contrast the broad kinetic diversity among Paniceae Rubisco presents opportunities for enhancing C$_3$-photosynthesis under varying atmospheric CO$_2$ and temperature (Fig S5). In particular P. bisulcatum (C$_3$) and P. deustum (PCK) Rubisco could distinctly improve C$_3$-photosynthetic potential under cooler and warmer temperatures, respectively, relative to the standardized tobacco Rubisco control (Fig 4) $^{#wheat/rice}$. The simulated improvements stemmed from temperature dependent enhancements in S$_{C/O}$ and/or k$_{cat}^{c} / K_C^{21\%O_2}$ (Fig. 3d,e), and not necessarily from high k$_{cat}^{c}$ (as emphasized by$^8$). Our findings suggest that improving C$_3$-crop photosynthesis under warmer future climates may be best served by exploring the Rubisco kinetic diversity of C$_4$-land plants, in particular among PCK and NADP-ME species.

Four L-subunit residues could contribute kinetic diversity among Paniceae Rubisco. These included two amino acids within structural regions whose movements influence Rubisco kinetics: the catalytic loop 6 (residue 328) and C-terminal tail (residue 470). Positive selection of Ala-328-Ser substitutions have been reported for some Limonium haplotypes$^{43}$ and a few C$_3$ and CAM plant species$^{17}$. This suggests the higher k$_{cat}^{c}$ and K$_C$ of Paniceae NADP-ME and PCK Rubisco might arise from the Glu/Gln-470 substitution (Fig 5a). Our attempts to test this by heterologous expression in tobacco chloroplasts proved unsuccessful (Fig 5c). The transformation limitation appears associated with differences in the ancillary protein requirements of Paniceae Rubisco biogenesis (Fig 5C), a constraint also preventing the production of Rubisco from red algae$^{44}$ and seemingly other monocot species$^{45}$ in tobacco chloroplasts.
Our data indicate the S-subunits also likely influence the kinetic diversity among Paniceae Rubisco. A comparable kinetic determining property was postulated for S-subunits in rice and wheat Rubisco\textsuperscript{34,35}. In P. virgatum four RbcS mRNAs are made (Phytozome) whose translated 121-123 amino acid S-subunits vary by 1 to 6 residues. Mutagenic study of the multiple RbcS transcripts produced in Paniceae would be a significant undertaking, but one possibly made easier using modern site specific nucleus gene editing tools that are now available in a variety of crop species\textsuperscript{46}. Clarifying the influence of S-subunits on the temperature kinetics of Rubisco from differing plant origins is critical to developing appropriate L- and/or S-subunit mutagenic technologies for modifying crop Rubisco kinetics to suit future climates.
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Acknowledgements

We thank Asaph Cousins for supplying their S. viridis Rubisco kinetic data for analysis.

This research was funded by the following grants from the Australian Research Council: DE130101760 (RES), DP120101603 (OG, SMW) and CE140100015 (OG, SMW).

Author contributions

RS, OG and SMW designed the study and undertook the experimental work. MVK undertook the phylogenetic analysis and LHG the structural analysis. All authors contributed to drafting the paper.

Competing financial interests

The authors declare no competing financial interests.


**Figure 1.** The diversity in the catalytic properties of Rubisco at 25°C across C_3 and C_4 grasses within Paniceae.

Box plots of comparative (a) leaf dry matter $^{12}$C/$^{13}$C isotopic fractionation ($\delta^{13}$C) and (b to f) in vitro measured Rubisco kinetics from tobacco and Paniceae species with C_2, C_3 and varying C_4 subtypes (NADP ME, NAD ME and PCK). See Table S1 for species list. Median values shown in boxes as vertical line, 95% confidence limits represented by horizontal lines. Letter variation indicates significant differences ($p < 0.05$) between parameters (Table S1). Kinetic properties analyzed include (b) substrate saturated
carboxylation and (d) oxygenation turnover rates ($k_{\text{cat}^c}$, $k_{\text{cat}^o}$), the Michaelis constants ($K_m$)
for (e) CO$_2$ and (f) O$_2$ ($K_C$, $K_O$) and (g) relative specificity for CO$_2$ over O$_2$ ($S_{C/O}$). (g to j)
Pairwise relationships among the kinetic parameters to assess the quality of their linear
correlations (dashed line).

Figure 2

Figure 2. Variation among the Paniceae Rubisco kinetics differentially affect
simulated rates of C$_3$-photosynthesis at 25°C.

Comparison of the relationships between $k_{\text{cat}^c}$ and either (a) $S_{C/O}$ or (b) $K_C$$_{21\%O_2}$, the value
for Kc under ambient O$_2$ (O) calculated as $K_C(1+O/K_O)$ (Table S1). The $r^2$ values show the
quality of their linear correlations (dashed lines). (c) The influence of the averaged
Paniceae C$_3$ and C$_4$ subtype Rubisco kinetics (Table S1) on CO$_2$ assimilation rates (A) at
25°C in a C$_3$-leaf as a function of C$_c$. Lines are modelled (Farquhar et al., (1980)) using the
carboxylase activity limited assimilation equation:
using a CO₂ solubility in H₂O \((s_c)\) of 0.0334 M bar\(^{-1}\), an O of 253 µM, a Rubisco content \((B)\) of 30 µmol catalytic sites.m\(^{-2}\) and a non-photorespiratory CO₂ assimilation rate \((R_d)\) of 1 µmol.m\(^{-2}\).s\(^{-1}\). The light limited CO₂ assimilation rates (to the right of the symbols) were modelled according to the equation:

\[
A = \frac{(C_c \cdot s_c - 0.5 \frac{O}{S_{c/o}}) k_{cat}^c \cdot B}{C_c \cdot s_c + K (1 + \frac{O}{Ko})} - R_d
\]

assuming an electron transport rate \((J)\) of 160 µmol.m\(^{-2}\).s\(^{-1}\). Yellow shading indicates where the modeled CO₂-assimilation rates of C\(_3\), NADP-ME and PCK Paniceae Rubisco exceed that of Rubisco from the model C\(_3\)-plant, tobacco (dotted line).
Figure 3. Divergence in the catalytic properties of Paniceae and tobacco Rubisco in response to temperature.

(a) The heat of activation ($\Delta H_a$) and scaling constant (c) for the kinetic parameters of tobacco Rubisco and the mean (±S.E) values measured for the various Paniceae species with C$_4$ (NAD ME, NADP ME, PCK) or C$_3$ (including the aligning C$_2$) biochemical physiologies (see Table S3). Letters show the statistical ranking using a post hoc Tukey test among the biochemical physiology groupings (different letters indicated differences at
the 5% level, p < 0.05). (b to e) Differences in the temperature response of tobacco (grey dashed line) and the averaged kinetic properties for Rubisco from Paniceae species with varying biochemical physiologies. The lines are derived as described in Figures S1 to S4 using the values listed in panel (a).
**Figure 4.** The potential for improving the thermal response of C₃-photosynthesis.

The benefits of (a) *P. bisulcatum* (C₃) and (b) *P. deustum* (C₄-PCK) Rubisco to the rate of photosynthesis in a C₃-leaf under varying chloroplast CO₂ concentrations (Cₐ) and temperature (see scale). Rate increases are presented as a percentage above that provided by tobacco Rubisco. The data was modelled according to⁴ using the parameters listed in Table S4 and plotted in Fig S4.
Figure 5.

**Figure 5. Approaches to decipher possible catalytic switches in the L-subunit of Paniceae Rubisco.**

(a) Amino acid variation in the L-subunit of each Paniceae Rubisco analysed in this study. A phylogenetic analysis of the L-subunit sequences and their Genbank accession information is provided in Figure S6. (b) Structure of spinach L₈S₈ Rubisco (L-subunits in green, S-subunits grey) viewed from the top (left) and side (middle) showing the relative
locations of $^{94}$Glu on the solvent-exposed Rubisco surface (yellow triangle), $^{228}$Ala in the
$\alpha_2$ helix (orange triangle), $^{328}$Ser at the hinge of loop 6 (purple triangle) and $^{470}$Pro in the
C-terminal tail extension (blue triangle) of one L-subunit. A closer view of a L-subunit pair
(right) with one showing ribbon structural detail and the other showing the positioning of
$^{94}$Glu, $^{228}$Ala, $^{328}$Ser and $^{470}$Pro relative to each other, an N-terminal domain loop, the $\alpha_2$
helix, loop 6, C-terminal tail extension and S-subunit $\beta$A-$\beta$B loops (yellow, orange, purple,
blue and grey, respectively). An active site bound reaction-intermediate analogue 2-CABP
is shown as a ball and stick. (c) Chloroplast transformation of the Rubisco L-subunit genes
from P. bisulcatum (Pbis-rbcL) and P. deustum (Pbis-rbcL) into tobacco was undertaken
to identify the amino acids (catalytic switches) responsible for their differing catalytic
properties. No Rubisco biogenesis was detected in the tob$^{PbL}$ and tob$^{PdL}$ tobacco genotypes
produced. Accordingly these plants could only grow in tissue culture on sucrose containing
media and were highly chlorotic (as shown). Detailed analysis of the transformation,
Rubisco mRNA and protein biochemistry is provided in Figure S7.
Supplemental data

Variation in response of C\textsubscript{3} and C\textsubscript{4} Paniceae Rubisco to temperature provides opportunities for improving C\textsubscript{3} photosynthesis

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Number of Supplemental Figures: 7

Number of Supplemental Tables: 4
Figure S1. Variation in the temperature response of $k_{\text{cat} c}$ among the Paniceae and tobacco Rubisco.

(a) Substrate saturated rates of carboxylation ($k_{\text{cat} c}$) determined using soluble leaf protein extract ($n \geq 3$ biological samples per species) were measured at 10, 15, 20, 25, 30 and 37°C with the expected exponential decrease in $k_{\text{cat} c}$ less evident at the lower assay temperatures for all Rubisco samples, as evident in prior published data {18-20,28,30-32}, including that of Boyd et al., (2015) {15} (orange triangles) for Seteria viridis Rubisco measured by Membrane Inlet Mass Spectrometry (MIMS). Plotted data are listed in Table S2. (b) Evaluation of the data via Arrhenius-style plots (i.e. ln $k_{\text{cat} c}$ vs 1/T) indicated the $k_{\text{cat} c}$ response diverged at around 25°C. Shown are the averaged linear fits to each Arrhenius plot in a biphasic manner with the < 25°C measurements (i.e. at 10, 15, 20, 25°C) separated from the > 25°C measurements (25, 30 and 37°C). The averaged data values were fitted to the following equation

\[ \text{Parameter} = \exp \left[ c - \frac{\Delta H_a}{RT} \right] \]

and the heat of activation ($\Delta H_a$) for $k_{\text{cat} c}$ at both < 25°C and > 25°C was derived from the slope (ln($k_{\text{cat} c}$) = -$\Delta H_a$/R; where R is the molar gas constant, 8.314 J K\(^{-1}\) mol\(^{-1}\)) and the scaling constant (c) from the ordinal intercept. The calculated values are listed in Table S3 and were fitted to the above equation to derive the exponential curves in panel a. For comparison, the fitted lines for tobacco Rubisco $k_{\text{cat} c}$ data are shown as dotted lines in each C\(_4\) Rubisco plot. See Table S3 for statistical analysis.
Figure S2. Variation in the temperature response of CO$_2$ affinity among the Paniceae and tobacco Rubisco.

(a) The Michaelis constant for CO$_2$ measured in the presence of ambient (253 µM) O$_2$ concentration ($K_{C^{21\%O_2}}$) determined from the same assays used to determine $k_{cat}^e$ in Fig S1 ($n \geq 3$ biological samples analyzed per species) varied exponentially over 10 to 37°C. Orange triangles, data for Seteria viridis Rubisco measured by MIMS$^{15}$. Plotted data are listed in Table S2. (b) Arrhenius-style plots of the data with the averaged linear regression fitted as described in Fig S1 to determine the heat of activation ($\Delta H_a$) and the scaling constant (c) values listed in Table S3 and used to derive the exponential curves shown in panel (a). As a scaling comparison, the fitted lines for tobacco Rubisco $K_{C^{21\%O_2}}$ data are shown as dotted lines in each C$_4$ Rubisco plot. See Table S3 for statistical analysis.
Figure S3. Variation in the temperature response of CO₂ over O₂ specificity among the Paniceae and tobacco Rubisco

(a) The influence of temperature on the specificity of CO₂ over O₂ (SC/O) determined using Rubisco purified from at least two biological samples per specie. Orange triangles, data for Seteria viridis Rubisco measured by MIMS¹⁵. Plotted data are listed in Table S2. (b) Arrhenius plots of the data with the averaged linear regression fitted as described in Fig S1 to determine the heat of activation (ΔHₐ) and the scaling constant (c) values for SC/O listed in Table S3 and used to derive the exponential curves shown in panel (a). The fitted lines for tobacco Rubisco SC/O data are shown as dotted lines in each C₄ Rubisco plot as a scaling comparison. See Table S3 for statistical analysis.
Figure S4. Variation in carboxylation efficiency under ambient O$_2$ among the Paniceae and tobacco Rubisco under varying temperature.

The temperature response of carboxylation efficiency ($k_{\text{cat}}$/$K_{C_{21\%O_2}}$) for each Rubisco was calculated by dividing the averaged values of $k_{\text{cat}}$ (FigS1) by its corresponding $K_{C_{21\%O_2}}$ values (Fig S2) for each assay temperature. As shown previously for Flaveria $^{19}$ and Seteria viridis $^{15}$ Rubisco, the carboxylation efficiency of each Paniceae and tobacco Rubisco declined at temperatures below 25°C and showed less variation at temperatures $>$ 25°C. Shown are the linear regressions fitted to the averaged Paniceae data for each biochemical physiology. See Table S3 for statistical analysis.
Figure S5. Effect of Rubisco kinetics on the thermal photosynthetic response.

The effects of Rubisco catalytic properties on the thermal response of leaf photosynthesis \( (A) \) to leaf chloroplastic \( \text{CO}_2 \) concentration \( (C_c) \). The curves were modelled according to Farquhar et al. (1980)\(^{24}\) using equations and parameters shown in Supplementary Table S5. The solid and dashed lines refer to the Rubisco limited \( (A_c) \) and RuBP-regeneration limited \( (A_d) \) assimilation rates, respectively. The circles represent the measured data points.
refer to assimilation rates under current $C_a$ (400 $\mu$bar, white) and that predicted for 2050 (550 $\mu$bar, black). Data for tobacco Rubisco shown in grey in each panel for comparison.
Figure S6. Rubisco L-subunit phylogeny in the Paniceae.

Maximum likelihood phylogeny of Rubisco L-subunit sequences from the fourteen Paniceae species examined in this study relative to the outgrouped Rubisco from Hordeum vulgare (barley) and Triticum aestivum (wheat). ML trees assembled under the Dayhoff model implemented in RAxML v.8 using translated L-subunit sequences from the full length rbcL genes available from the following Genbank accession: P. bisulcatum, (*); S. laxa, (*); P. milioides, (*); P. antidotale, (*); P. monticola, (*); C. ciliaris, (*); S. viridis, (KT289405.1); P. virgatum, (HQ731441.1); P. miliaceum, (KU343177.1); P. coloratum, (*); M. maximus, (*); U. panicoides, (*); U. panicoides, (*); P. deustum, (*); U. mosambicensis, (*); H. vulgare, (KT962228.1) and T. aestivum, (KJ592713.1). *sequences submitted to Genbank, awaiting accession numbers.
Figure S7. Chloroplast transformation of the *P. bisulcatum* (C3) and *P. deustum* (C4-PCK) *rbcL* genes to assess Paniceae Rubisco biogenesis in tobacco.

(a) Comparison of the plastome sequence in wild-type, cmtrL and the plastome transformed tobPbL and tobPdL tobacco genotypes generated in this study. Duplicate tobPbL and tobPdL lines were made by plastome transformation as described 13 by homologous recombination replacement of the cmrbcM gene in the plastome of the cmtrL tobacco genotype with rbcL genes for *P. bisulcatum* or *P. deustum* Rubisco (synthesized to match the tobacco rbcL nucleotide sequence where feasible) and the aadA selectable marker gene (coding resistance to spectinomycin). Numbering represents the flanking plastome sequence in the pLEVPdL and pLEVPbL transforming plasmids. P, 292-bp rbcL promoter/5’UTR; T, 288-bprbcL 3’UTR; T, 112-bp of psbA 3’UTR; t, 147-bp rps16 3’UTR. Position of the 221-bp 5UTR probe 13 and the corresponding rbcL and rbcL-aadA mRNAs (dashed lines) to which it hybridizes are indicated. (b) Total leaf RNA (5µg) extracted from tissue culture grown plant samples was separated on denaturing formaldehyde gels and the EtBr stained RNA visualised (upper panel) before blotting onto Hybond-N nitrocellulose membrane (GE healthcare) as described 11 and probed with the 32P-labelled 5UTR probe (lower panel). The probe correctly hybridised to the wild-type tobacco rbcL mRNA and the rbcL and rbcL-aadA mRNA transcripts in each tobPbL and tobPdL line. (c) Soluble leaf protein from the same leaves analyzed in (b) was processed for measuring Rubisco levels by NdPAGE analysis and 14C-CABP quantification as described 25. While wildtype tobacco LaS8 Rubisco was readily detected by 14C-CABP binding, Coomassie staining and by immunoblot analysis with an antibody to tobacco Rubisco following ndPAGE, these methods detected no hybrid LaS8 Rubisco biogenesis in the tobPbL and tobPdL genotypes (i.e. complexes comprising the introduce Panicum L-subunits and the endogenous, cytosol made tobacco S-subunits). (d) Further inspection of the soluble and total (comprising soluble + insoluble) leaf protein separated by SDS PAGE did not detect any Rubisco L-subunit (~50 kDa) or S-subunit (~14.5
kDa) in either cellular protein fraction of the tob\textsuperscript{PbL} or tob\textsuperscript{PdL} lines by Comassie staining or Rubisco antibody blot analysis. This indicated that even when grown in tissue culture the resource limitations confronting the photosynthetically deplete tob\textsuperscript{PbL} and tob\textsuperscript{PdL} lines precluded the synthesis and/or accumulation of Panicum sp. Rubisco L-subunits. Whether co-expressing their cognate SSu or/and Raf1\textsuperscript{48} can circumvent this biogenesis challenge remains to be tested.
Table S1: Summary of the catalytic parameters of Paniceae and tobacco Rubisco at 25°C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Physiology</th>
<th>δ^{13}C (%)</th>
<th>k_{cat} (s^{-1})</th>
<th>K_c (µM)</th>
<th>K_c^{21% O2} (µM)</th>
<th>k_{cat} (s^{-1})</th>
<th>K_{cat}/K_c (µM)</th>
<th>S_c/O (µM)</th>
<th>k_{cat}/K_c^{21% O2} (µM)</th>
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<td>74.5 ± 0.4</td>
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<td>PCK</td>
<td>-13.3±0.4 b</td>
<td>5.7±0.2 b</td>
<td>18.4±0.3 c</td>
<td>27.1±1.1 b</td>
<td>2.3±0.2 a</td>
<td>4.2±0.3 a</td>
<td>74±3 b</td>
<td>211±10 a</td>
<td>309±10 ab</td>
</tr>
</tbody>
</table>

For each species data are the mean±SE of at least N=3 biological samples assayed in duplicate. One-way ANOVA was undertaken using the photosynthetic type/subtype as the main factor. Symbols show the statistical significance levels (ns = p > 0.05; * = p < 0.05; ** = p < 0.01; ***: p < 0.001), while letters show the ranking of the means using a post hoc Tukey test (different letters indicate statistical differences at the 5% level, p < 0.05). k_{cat}, maximal oxygenation rate calculated from S_c/O = (k_{cat}/K_c)/(k_{cat}/K_O). K_c^{21% O2}, K_c under ambient atmospheric O2 levels (O = 252 µM O2 in air saturated H2O) calculated as K_c(1+O/K_O). n.m, not measured.
Table S2: The catalytic parameters of Paniceae and tobacco Rubisco between 10°C and 37°C.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>NADP</th>
<th>P. deust</th>
<th>M. max</th>
<th>U. panic</th>
<th>PCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.88±0.19</td>
<td>1.09±0.22</td>
<td>1.98±0.13</td>
<td>3.10±0.08</td>
<td>5.45±0.67</td>
</tr>
<tr>
<td>15</td>
<td>1.37±0.22</td>
<td>0.86±0.05</td>
<td>2.84±0.02</td>
<td>2.31±0.01</td>
<td>4.20±0.06</td>
</tr>
<tr>
<td>20</td>
<td>1.98±0.13</td>
<td>2.12±0.06</td>
<td>3.90±0.03</td>
<td>3.02±0.01</td>
<td>3.24±0.24</td>
</tr>
<tr>
<td>25</td>
<td>3.10±0.08</td>
<td>2.10±0.00</td>
<td>2.84±0.02</td>
<td>2.37±0.12</td>
<td>3.70±0.11</td>
</tr>
<tr>
<td>30</td>
<td>5.45±0.67</td>
<td>4.20±0.06</td>
<td>3.24±0.24</td>
<td>3.70±0.11</td>
<td>5.34±0.90</td>
</tr>
<tr>
<td>35</td>
<td>7.6±1.9</td>
<td>6.8±1.0</td>
<td>9.4±1.0</td>
<td>13.1±1.1</td>
<td>19.5±2.0</td>
</tr>
<tr>
<td>37</td>
<td>10.4±1.4</td>
<td>8.8±1.0</td>
<td>7.5±1.0</td>
<td>13.1±1.1</td>
<td>19.6±1.5</td>
</tr>
<tr>
<td>39</td>
<td>13.6±1.2</td>
<td>15.7±2.5</td>
<td>19.1±2.3</td>
<td>19.6±1.5</td>
<td>25.1±6.0</td>
</tr>
<tr>
<td>40</td>
<td>30.8±3.8</td>
<td>20.6±5.3</td>
<td>19.2±1.5</td>
<td>19.6±1.5</td>
<td>25.1±6.0</td>
</tr>
</tbody>
</table>

**C2/C3**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>NADP</th>
<th>P. deust</th>
<th>M. max</th>
<th>U. panic</th>
<th>PCK</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.51±0.02</td>
<td>0.51±0.02</td>
<td>0.51±0.02</td>
<td>0.51±0.02</td>
<td>0.51±0.02</td>
</tr>
<tr>
<td>15</td>
<td>1.00±0.08</td>
<td>1.00±0.08</td>
<td>1.00±0.08</td>
<td>1.00±0.08</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>20</td>
<td>1.40±0.07</td>
<td>1.40±0.07</td>
<td>1.40±0.07</td>
<td>1.40±0.07</td>
<td>1.40±0.07</td>
</tr>
<tr>
<td>25</td>
<td>2.81±0.10</td>
<td>2.81±0.10</td>
<td>2.81±0.10</td>
<td>2.81±0.10</td>
<td>2.81±0.10</td>
</tr>
<tr>
<td>30</td>
<td>5.70±0.21</td>
<td>5.70±0.21</td>
<td>5.70±0.21</td>
<td>5.70±0.21</td>
<td>5.70±0.21</td>
</tr>
<tr>
<td>35</td>
<td>11.8±1.35</td>
<td>11.8±1.35</td>
<td>11.8±1.35</td>
<td>11.8±1.35</td>
<td>11.8±1.35</td>
</tr>
</tbody>
</table>

**K_{cat}^{C2/C3} (s^{-1})**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>NADP</th>
<th>P. deust</th>
<th>M. max</th>
<th>U. panic</th>
<th>PCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.86±0.77</td>
<td>4.86±0.77</td>
<td>4.86±0.77</td>
<td>4.86±0.77</td>
<td>4.86±0.77</td>
</tr>
<tr>
<td>15</td>
<td>10.0±0.21</td>
<td>10.0±0.21</td>
<td>10.0±0.21</td>
<td>10.0±0.21</td>
<td>10.0±0.21</td>
</tr>
<tr>
<td>20</td>
<td>19.2±0.11</td>
<td>19.2±0.11</td>
<td>19.2±0.11</td>
<td>19.2±0.11</td>
<td>19.2±0.11</td>
</tr>
<tr>
<td>25</td>
<td>38.6±0.18</td>
<td>38.6±0.18</td>
<td>38.6±0.18</td>
<td>38.6±0.18</td>
<td>38.6±0.18</td>
</tr>
<tr>
<td>30</td>
<td>66.6±0.22</td>
<td>66.6±0.22</td>
<td>66.6±0.22</td>
<td>66.6±0.22</td>
<td>66.6±0.22</td>
</tr>
<tr>
<td>35</td>
<td>97.2±0.27</td>
<td>97.2±0.27</td>
<td>97.2±0.27</td>
<td>97.2±0.27</td>
<td>97.2±0.27</td>
</tr>
</tbody>
</table>

Rubisco catalysis parameters (average ± SE) measured in duplicate (x2) or triplicate (x3) for each biological sample (N). *Data for S. viridis Rubisco shown in blue italic (N=4 ± S.E.) measured by MIMS 15. The data for each species are plotted in Figures S1 to S4 with the parameter averages (± SE) for tobacco Rubisco and for each Paniceae photosynthetic type/subtype shown in bold and shaded grey.
are plotted in Figures 3. n.m, not measured. The data from this study are statistically analyzed following derivation of the heat of activation ($\Delta H_a$) and scaling constant ($c$) values for each parameter (see Table S3).
Table S3. Heat of activation ($\Delta H_a$) and scaling constant (c) values among Paniceae and tobacco Rubisco kinetic parameters.

<table>
<thead>
<tr>
<th>Species</th>
<th>Physiology</th>
<th>$k_{cat}$ (&gt;25°C) $\pm$ S.D (kJ mol$^{-1}$)</th>
<th>$k_{cat}$ (&lt;25°C) $\pm$ S.D (kJ mol$^{-1}$)</th>
<th>$K_C^{21% \text{O}_2}$ $\pm$ S.D (kJ mol$^{-1}$)</th>
<th>$S_{\text{C/O}}$ $\pm$ S.D (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum</td>
<td>C$_3$</td>
<td>36.4±2.9</td>
<td>15.8±1.2</td>
<td>25.5±0.8</td>
<td>17.9±0.7</td>
</tr>
<tr>
<td>Panicum bisulcatum</td>
<td>C$_3$</td>
<td>32.4±8.5</td>
<td>13.9±3.7</td>
<td>29.7±3.0</td>
<td>15.0±1.8</td>
</tr>
<tr>
<td>Panicum milioides</td>
<td>C$_2$</td>
<td>40.3±0.5</td>
<td>17.0±0.2</td>
<td>28.4±1.8</td>
<td>12.7±1.0</td>
</tr>
<tr>
<td>Panicum monticola</td>
<td>C$_4$</td>
<td>30.6±1.8</td>
<td>14.0±0.8</td>
<td>24.5±1.8</td>
<td>16.9±0.9</td>
</tr>
<tr>
<td>Cenchrus ciliaris</td>
<td>C$_4$</td>
<td>26.3±0.7</td>
<td>12.4±0.3</td>
<td>23.9±1.3</td>
<td>22.1±2.4</td>
</tr>
<tr>
<td>Setaria viridis</td>
<td>#Boyd et al 2015</td>
<td>75.0±1.1</td>
<td>31.9±0.5</td>
<td>47.3±9.4</td>
<td>22.3±1.9</td>
</tr>
<tr>
<td>Panicum virgatum</td>
<td>C$_4$</td>
<td>31.1±2.9</td>
<td>13.7±1.3</td>
<td>27.4±4.5</td>
<td>16.9±2.2</td>
</tr>
<tr>
<td>Panicum milliaceum</td>
<td>C$_2$ (NADP-ME)</td>
<td>28.6±4.0</td>
<td>12.3±1.7</td>
<td>29.6±4.0</td>
<td>16.6±2.7</td>
</tr>
<tr>
<td>Panicum coloratum</td>
<td>C$_3$ (NADP-ME)</td>
<td>23.2±4.5</td>
<td>10.6±2.1</td>
<td>21.3±2.8</td>
<td>11.4±1.5</td>
</tr>
<tr>
<td>Megathyrsus maximus</td>
<td>PCK</td>
<td>24.5±1.2</td>
<td>11.6±0.5</td>
<td>21.9±1.4</td>
<td>12.3±1.7</td>
</tr>
<tr>
<td>Urochloa panicoides</td>
<td>C$_4$</td>
<td>26.9±0.9</td>
<td>12.6±0.4</td>
<td>29.9±3.0</td>
<td>15.2±1.5</td>
</tr>
<tr>
<td>Panicum deustum</td>
<td>C$_3$/C$_2$</td>
<td>27.8±1.7</td>
<td>12.8±0.8</td>
<td>25.6±0.5</td>
<td>14.9±0.9</td>
</tr>
</tbody>
</table>

Values of $\Delta H_a$ and c were determined from measures of $k_{cat}$ ($\text{Fig S1}$), $K_C^{21\% \text{O}_2}$ ($\text{Fig S2}$) and $S_{\text{C/O}}$ ($\text{Fig S4}$) made at 10, 15, 20, 25, 30 and 35 (or 37)°C (see Table S2 for data) and fitted to the Arrhenius-type equation

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

where R the molar gas constant (8.314 J K$^{-1}$ mol$^{-1}$) and T the assay temperature (K). n.m, not measured. For each species data are the mean ± SE of at least N=3 biological samples assayed in duplicate. One-way ANOVA was undertaken using the photosynthetic type/subtype as the main factor. Symbols show the statistical significance levels (ns = $p > 0.05$; * = $p < 0.05$), while letters show the ranking of the means using a post hoc
Tukey test (different letters indicate statistical differences at the 5% level, $p < 0.05$). Comparative data for S. viridis Rubisco from Boyd et al., (2015) measured by MIMS.
Table S4. Summary of parameters used in the modelling plots shown in Figure 4 and Figure S5.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>Parameters</td>
</tr>
<tr>
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<tr>
<td>15</td>
</tr>
<tr>
<td>20</td>
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<tr>
<td>25</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>37</td>
</tr>
<tr>
<td>Reference</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>$k^\text{cat}.$ (s⁻¹)</td>
</tr>
<tr>
<td>$K^\text{cat}$ (µM)</td>
</tr>
<tr>
<td>$S_C^\text{CO}$ (M M⁻¹)</td>
</tr>
<tr>
<td>$J_{\text{max}}/V_{\text{max}}$</td>
</tr>
<tr>
<td>Panicum bisulcatum</td>
</tr>
<tr>
<td>$k^\text{cat}.$ (s⁻¹)</td>
</tr>
<tr>
<td>$K^\text{cat}$ (µM)</td>
</tr>
<tr>
<td>$S_C^\text{CO}$ (M M⁻¹)</td>
</tr>
<tr>
<td>Panicum deustum</td>
</tr>
<tr>
<td>$k^\text{cat}.$ (s⁻¹)</td>
</tr>
<tr>
<td>$K^\text{cat}$ (µM)</td>
</tr>
<tr>
<td>$S_C^\text{CO}$ (M M⁻¹)</td>
</tr>
<tr>
<td>Urochloa panicoides</td>
</tr>
<tr>
<td>$k^\text{cat}.$ (s⁻¹)</td>
</tr>
<tr>
<td>$K^\text{cat}$ (µM)</td>
</tr>
<tr>
<td>$S_C^\text{CO}$ (M M⁻¹)</td>
</tr>
<tr>
<td>Common parameters</td>
</tr>
<tr>
<td>$g_m$ (mol m² s⁻¹ bar⁻¹)</td>
</tr>
<tr>
<td>TPU (µmol m² s⁻¹)</td>
</tr>
<tr>
<td>$R_d$ (µmol m² s⁻¹)</td>
</tr>
<tr>
<td>$s_c$ (M bar⁻¹)</td>
</tr>
<tr>
<td>$s_o$ (M bar⁻¹)</td>
</tr>
<tr>
<td>$J_{\text{max}}$ (µmol m² s⁻¹)</td>
</tr>
<tr>
<td>Rubisco sites (µmol s⁻¹)</td>
</tr>
</tbody>
</table>

Photosynthesis rate, $A$, was calculated as $A = \min (A_c, A_j, A_t)$, where $A_c$, $A_j$ and $A_t$ are the CO₂-limited ($A_c$), light-limited ($A_j$) and the triose phosphate utilisation (TPU)-limited ($A_t$) assimilation rates, respectively. Their expressions are defined as:

$$ A_c = \frac{m_{\text{cat}} c_c (c_c s_c - 0.50 c_c / s_c / \theta)}{(c_c s_c + K_{\text{air}})} - R_d $$
\[ A_j = \frac{(c_c s_c - 0.5 c_c / S_c / o) I_{max}}{4(c_c s_c + c_c / S_c / o)} - R_d \); and

\[ A_t = 3TPU - R_d \].

The model parameters used are: \( m = \) amount of leaf Rubisco set at 30 \( \mu \)mol active sites \( \text{m}^{-2} \); \( k_{cat}^c (\text{s}^{-1}) = \) Rubisco carboxylation rate; \( K_{C_{\text{air}}} \) (\( \mu \)M) = Michaelis-Menten constant of Rubisco for \( \text{CO}_2 \) and \( S_{C/O} = \text{CO}_2/\text{O}_2 \) specificity of Rubisco. The maximal RuBP carboxylation-limited assimilation rate, \( V_{c_{max}} = m \cdot k_{cat}^c \). The maximal RuBP regeneration-limited assimilation rate, \( J_{max} (\mu \text{mol m}^{-2} \text{s}^{-1}) \) is set to equal 1.7\( V_{c_{max}} \) for tobacco at 25°C; its values at other temperatures were calculated using the thermal dependence from Bernacchi et al (2003) 

\[ J_{max}(T) = J_{max_{25}} \cdot e^{\left(\frac{-H_a}{R(T_{273}+T)}\right)} \], where \( c = 17.7 \) and \( \Delta H_a = 43.9 \) (in kJ mol\(^{-1}\)).

The values at 25°C for TPU (11.4 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and mitochondrial respiration, \( R_d \) (1 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and their thermal dependence were adapted from Sharkey et al (2007) 

\[ R_d(T) = R_{d_{25}} \cdot e^{\left(\frac{-H_a}{R(T_{273}+T)}\right)}, \] where \( c = 18.72 \) and \( \Delta H_a = 46.4 \) (in kJ mol\(^{-1}\))

\[ TPU(T) = TPU_{25} \left[ \frac{e^{\left(\frac{-H_a}{R(T_{273}+T)}\right)}}{1+e^{\left(\frac{-H_a}{R(T_{273}+T)}\right)}} \right], \] where \( c = 21.46 \) and \( \Delta H_a = 53.1, \Delta H_d = 201.8 \) and 0.65 (in kJ mol\(^{-1}\)).
C_c and O_c are the CO_2 and O_2 concentrations in the chloroplast, respectively. Gas concentrations in the liquid phase were calculated using the solubility constants for CO_2 (s_c = 0.0334 M bar^{-1}) and O_2 (s_o = 0.00126 M bar^{-1}) at 25°C. Their thermal dependence was determined according to Henry’s law using the following expressions (https://en.wikipedia.org/wiki/Henry's_law):

\[
s_c(T) = s_{c_{25}} \cdot 2400. e^{\left(\frac{1}{273+T} - \frac{1}{298}\right)} \quad \text{and} \\

s_o(T) = s_{o_{25}} \cdot 1700. e^{\left(\frac{1}{273+T} - \frac{1}{298}\right)}
\]

Intercellular CO_2 concentration, C_i was calculated using a constant C_i/C_a ratio of 0.70^{49}. C_c was calculated as C_c = C_i - A/g_m, where g_m is the mesophyll conductance to CO_2 transfer. Tobacco g_m at 25°C (0.57 mol m^{-2} s^{-1} bar^{-1}) and its thermal dependence were taken from von Caemmerer and Evans (2015)^{49}.
Table S5. Parameters used to calculate CO₂ concentrations in \(^{14}\text{CO}_2\)-fixation assays at the varying temperatures.

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T); assay temperature</td>
<td>(283, \text{K} ) (288, \text{K} ) (293, \text{K} ) (298, \text{K} ) (303, \text{K} ) (310, \text{K} )</td>
</tr>
<tr>
<td>(q); CO₂ solubility at 1 atm ((\text{Mol.L}^{-1} \cdot \text{atm}^{-1}))</td>
<td>(0.0524) (0.0455) (0.0382) (0.0329) (0.0289) (0.0240)</td>
</tr>
<tr>
<td>(R); universal gas constant ((\text{L.atm.K}^{-1} \cdot \text{mol}^{-1}))</td>
<td>0.082057</td>
</tr>
<tr>
<td>(pK_1)</td>
<td>6.362 (6.327) (6.280) (6.251) (6.226) (6.202)</td>
</tr>
<tr>
<td>(pK_2)</td>
<td>10.499 (10.431) (10.377) (10.329) (10.290) (10.238)</td>
</tr>
<tr>
<td>*(\text{pH})</td>
<td>8.27 (8.24) (8.21) (8.16) (8.11) (8.03)</td>
</tr>
</tbody>
</table>

The values were fitted to the Henderson-Hasselbalch derived equation

\[
[\text{CO}_2] = \frac{\left(C_t\right)}{1 + \frac{V}{vqRT} + 10^{(pH-pK_1)} + 10^{(2pH-pK_1-pK_2)}}
\]

\(V/v\): ratio of reaction vial headspace (\(V\)) to assay volume (\(v\)).

*example pH variation for 50 mM EPPES-NaOH buffer adjusted to pH 8.16 at 25°C; the 0.26 pH variation has <1% effect on tobacco Rubisco carboxylase activity.
References


Sage, R. F. Variation in the $k_{cat}$ of Rubisco in C\textsubscript{3} and C\textsubscript{4} plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot 53, 609-620 (2002).


