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The Coevolution of RuBisCO, Photorespiration, and Carbon Concentrating Mechanisms in Higher Plants

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Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) is the carbon-fixing enzyme present in most photosynthetic organisms, converting CO_2 into organic matter. Globally, photosynthetic efficiency in terrestrial plants has become increasingly challenged in recent decades due to a rapid increase in atmospheric CO_2 and associated changes toward warmer and dryer environments. Well adapted for these new climatic conditions, the C_4 photosynthetic pathway utilizes carbon concentrating mechanisms to increase CO_2 concentrations surrounding RuBisCO, suppressing photorespiration from the oxygenase catalyzed reaction with O_2. The energy efficiency of C_3 photosynthesis, from which the C_4 pathway evolved, is thought to rely critically on an uninterrupted supply of chloroplast CO_2. Part of the homeostatic mechanism that maintains this constancy of supply involves the CO_2 produced as a byproduct of photorespiration in a negative feedback loop. Analyzing the database of RuBisCO kinetic parameters, we suggest that in genera (*Flaveria* and *Panicum*) for which both C_3 and C_4 examples are available, the C_4 pathway evolved only from C_3 ancestors possessing much lower than the average carboxylase specificity relative to that of the oxygenase reaction ($S_{C/O} = S_C/S_O$), and hence, the higher CO_2 levels required for development of the photorespiratory CO_2 pump (C_2 photosynthesis) essential in the initial stages of C_4 evolution, while in the later stage (final optimization phase in the *Flaveria* model) increased CO_2 turnover may have occurred, which would have been supported by the higher CO_2 levels. Otherwise, C_4 RuBisCO kinetic traits remain little changed from the ancestral C_3 species. At the opposite end of the spectrum, C_3 plants (from *Limonium*) with higher than average $S_{C/O}$, which may be associated with the ability of increased CO_2, relative to O_2, affinity to offset reduced photorespiration and chloroplast CO_2 levels, can tolerate high stress environments. It is suggested that, instead of inherently constrained by its kinetic mechanism, RuBisCO possesses the extensive kinetic plasticity necessary for adaptation to changes in photorespiration that occur in the homeostatic regulation of CO_2 supply under a broad range of abiotic environmental conditions.

Keywords: ribulose-1,5-bisphosphate carboxylase/oxygenase, photorespiration, carbon concentrating mechanism, photosynthesis, evolution, homeostasis, climate change

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INTRODUCTION

What makes Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) kinetic parameters the way they are? This question has now persisted for several decades, without a definitive explanation. Although RuBisCO is the principal carbon-fixing enzyme in the biosphere, its product turnover rate (circa $3\,s^{-1}$ per active site for plants) may be considered, if not particularly slow, rather unexceptional (Bar-Even et al., 2011). The primary substrate (CO_2) must also compete for binding to the RuBP in the RuBisCO active site with the more abundant O_2 in the atmosphere leading to photorespiration, consuming additional energy and compromising the process of photosynthesis. The combined effects of increasing population and anthropogenic climate change have motivated efforts to enhance carbon fixation in plants for increasing both agricultural crop yield and carbon sequestration generally (Niinemets et al., 2017; Andralojc et al., 2018; Erb and Zarzycki, 2018; Fernie et al., 2020; Lawson and Flexus, 2020). Although the possibility of enhancing photosynthesis by improving RuBisCO kinetic traits has been given due consideration (Whitney et al., 2011; Sharwood et al., 2016; Gomez-Fernandez et al., 2018; Wilson et al., 2018; Zhou and Whitney, 2019; Davidi et al., 2020; Lin et al., 2020; Bouvier et al., 2021), a conclusive picture of RuBisCO's molecular mechanism (Cleland et al., 1998; Tcherkez, 2013, 2015; Cummins et al., 2018b, 2019b; Kannappan et al., 2019; Bathellier et al., 2020; Cummins and Gready, 2020) and a general consensus understanding of the observed tradeoffs between RuBisCO's kinetic parameters remains elusive, despite having been analyzed in varying ways with the objective of gaining insights into the possible connection between evolutionary and biochemical (or catalytic) constraints (Bouvier et al., 2021). The earliest of these studies, (Tcherkez et al., 2006; Savir et al., 2010) based on general mechanistic assumptions and limited data samples, concluded that variations in the elementary rate constants must be tightly constrained by the limitations inherent in RuBisCOs kinetic mechanism, resulting in an enzyme which provides only limited scope for further optimization (Tcherkez et al., 2006; Tcherkez, 2013, 2015), while later studies of more extensive data sets have challenged this view, revealing greater flexibility (Cummins et al., 2018a, 2019a; Flamholz et al., 2019). Other studies have examined the coevolution of RuBisCO kinetics and carbon concentrating mechanism (CCMs) (Goudet et al., 2020; Iñiguez et al., 2020).

Various forms of CCMs have occurred independently and at different times in a wide range of photosynthetic organisms from diverse environments, directing the evolution of RuBisCO kinetics (Iñiguez et al., 2020). In higher plants, the vast majority follow the C_3 photosynthetic pathway initiated by CO_2 fixing to the bound form of activated RuBP substrate (Cleland et al., 1998) which proceeds through hydrolysis to break down into two molecules of the 3-carbon compound 3-phosphoglyceric acid (3PGA). The C_4 pathway, although present in only about 8,000 species (Sage, 2016), including some important agricultural crops (maize and sorghum), accounts for about 25% of terrestrial photosynthesis. In C_4 photosynthesis, CO_2 is initially converted by carbonic anhydrase (CA) to bicarbonate which is fixed by phosphoenolpyruvate carboxylase into oxaloacetate. Oxaloacetate is converted into malate (4-carbon compound) or aspartate for diffusion into the bundle sheath cells where they are decarboxylated in high concentrations and the released CO_2 is then, as in C_3 photosynthesis, fixed into 3PGA by RuBisCO. By increasing CO_2 supplies and thereby suppressing photorespiration, plant CCMs began evolving from C_3 species, probably in the early Oligocene (circa 30 ma) in response to decreasing CO_2 levels, as an efficient way of increasing photosynthesis in the more challenging environmental conditions (Sage, 2004). The evolutionary success of C_4 photosynthesis and the observation of increased carbon assimilation in some C_3 crops under elevated CO_2 (Xu et al., 2016; Thompson et al., 2017) have stimulated research into the possibility of incorporating CCMs into C_3 plant species via genetic modification (McGrath and Long, 2014; Jurić et al., 2019; Kubis and Bar-Even, 2019; Atkinson et al., 2020).

Selective abiotic forces have determined the evolution of C_4 photosynthesis over a number of distinct phases (Sage et al., 2018). C_2 photosynthesis, a crucial step in the early stages of this evolution (Sage et al., 2012; Bräutigam and Gowik, 2016), is characterized by the formation of a photorespiratory CO_2 pump that utilizes the two-carbon compound, glycine, to transport and concentrate the photorespiratory CO_2 into the bundle sheath cells where it can be re-fixed by RuBisCO. The processes involved in elevating levels of photorespiration relative to photosynthesis in C_3 plants are, therefore, a fundamentally important selection factor initiating the evolution to C_4 photosynthesis (Sage et al., 2018). This ratio can be modelled using the rate, or velocity, v, of the oxygenase relative to the carboxylase catalyzed reaction, given by (Laing et al., 1974; Jordan and Ogren, 1984)

$$v_{O/C} = \frac{v_O}{v_C} = \frac{O}{S_{C/O}C} \tag{1}$$

where O and C are, respectively, the O_2 and CO_2 concentrations at carboxylation sites, and $S_{C/O}$ is the specificity of the carboxylase relative to the oxygenase reaction which can be expressed in various algebraic forms as

$$S_{C/O} = \frac{S_C}{S_O} = \frac{V_{C/O}}{K_{C/O}} = \frac{V_C}{V_O}\frac{K_O}{K_C} = V_{C/O}K_{O/C} \tag{2}$$

In an obvious notation, the RuBisCO kinetic parameters V and K denote the maximum catalytic (turnover) rates (V_{max} or k_{cat}) and Michaelis constant (K_M), respectively. A recent data compilation contains RuBisCO kinetic parameters (at 25° C) from over 300 species (Flamholz et al., 2019), with more than 50% derived from higher plants. Of these plant species, the ubiquitous C_3 plants constitute by far the largest sample, followed by around 40 examples of C_4 plants, while samples of C_3-C_4 intermediate (extant plants exhibiting C_2 photosynthesis on the pathway to C_4) and some C_4-like plants from *Flaveria*, a genus adopted as a model for the evolutionary pathway of C_4 photosynthesis (McKown et al., 2005; Kapralov et al., 2011; Sage et al., 2013; Schulze et al., 2013; Mallmann et al., 2014), and other miscellaneous plants, including examples of

CCM-containing plants that follow the crassulacean acid metabolism pathway, are fewer in number.

The reduction of atmospheric CO_2 levels and associated increase in O_2 would have undoubtedly enforced adaptation of plants to increased photorespiration over geological time. Increasing temperature also increases $v_{O/C}$ (Jordan and Ogren, 1984) by decreasing both $S_{C/O}$ and the ratio of substrate concentrations, C/O, and is an important environmental factor driving the evolution of the C_4 pathway (Sage et al., 2018). However, while photorespiration is not particularly advantageous to C_3 plants due to net loss of carbon, it is also important to recognize that a significant amount of photorespiratory CO_2 can feed into chloroplasts (Sage and Sage, 2009; Tholen and Zhu, 2011; Busch et al., 2013), increasing the potential for the recapture of carbon for photosynthesis and thus facilitating evolution along the C_4 pathway. This creates a negative feedback loop that mitigates the photorespiratory response and thus limiting the increases in $v_{O/C}$. Moreover, the combined action of CA (releasing CO_2 from bicarbonate) and photorespiration have been postulated to form the basis of a homeostatic mechanism that ensures a stable supply of CO_2 to RuBisCO, essential for the energy efficient maintenance of photosynthesis (Riazunnisa et al., 2006; Igamberdiev and Roussel, 2012; Igamberdiev, 2015).

While the importance of abiotic environmental conditions leading to carbon restriction (reduced atmospheric CO_2, higher temperatures, and lack of water) in driving evolution of the photorespiratory CO_2 pump is well understood (Sage et al., 2018), the role of RuBisCO kinetic variability, which underpins $S_{C/O}$, warrants further critical investigation (Sage, 2013; Sage et al., 2018). In the present study, we have attempted to delineate possible coevolutionary relationships between RuBisCO, photorespiration, and CCMs by analyzing RuBisCO kinetic parameter data for higher plant species derived from the most recent compilation (Flamholz et al., 2019). In two genera, *Flaveria* and *Panicum*, the results suggest that in addition to abiotic conditions that increase photorespiration by lowering $S_{C/O}$, much lower than average $S_{C/O}$ in the C_3 populations could also be a critically important precondition in C_4 evolution. In contrast, C_3 plants (e.g., *Limonium*) that have adapted to extreme abiotic environments are typically characterized by higher than average $S_{C/O}$ (Galmés et al., 2005), which compensates for the lower levels of photorespiratory CO_2 (Equation 1) through higher CO_2 affinity.

STATISTICAL METHODS

The data sets for our analysis were accessed from the compilation by Flamholz et al. (2019). Where there are multiple entries per species in this database, these were averaged prior to statistical analysis. The complete list of species and associated kinetic parameters used in the analysis is provided in the **Supplementary Material** (**Supplementary Tables S1** and **S2**). It is worth noting here that correlations between these kinetic parameters have been recently analyzed using Phylogenetic Generalized Least Squares (PGLS) as opposed to standard least-squares regression (Bouvier et al., 2021). Standard regression analysis assumes independence of the residuals, which may not necessarily be true when looking for correlations between traits in evolutionary biology as those taxa with a more common ancestor (more related) would exhibit similar traits and hence dependent residuals. PGLS methods are often used to account for such dependencies (for a general overview of PGLS methodology, see Mundry, 2014). The PGLS study by Bouvier et al., (2021) suggests that correlations between RuBisCO kinetic parameters are over estimated by standard regression; i.e., a significant phylogenetic signal is present. Nevertheless, the covariance between K_C and V_C appears not affected, which is perhaps not surprising given the interdependence between K and V (Briggs and Haldane, 1925). In view of this underlying covariance present in the enzyme kinetics, which is quite distinct from phylogenetic and catalytic constraints, standard linear least-squares regression analysis was carried out on the total C_3 (excluding *Limonium*), C_3 *Limonium* collected from high stress (high temperature and water restricted) habitat (Galmés et al., 2014), *Flaveria* sample and total C_4 samples. The vast majority of grass species are split fairly evenly between the closely related Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae (PACMAD) and Bamboos, Oryzoideae, and Pooideae (BOP) sister clades. Among grasses, C_4 photosynthesis has evolved from C_3 only in PACMAD species (for an overview, see Christin et al., 2013). As both clades are relatively well represented in the kinetic data, correlations are also examined separately for *Oryza*, *Aegilops*, *Puccinellia* (all BOP), and *Panicum* (PACMAD) species.

The estimation of effect sizes (Cumming, 2012) of primary interest in our analysis is both the differences between mean kinetic parameters and, in particular, the differences between coefficients obtained from linear least-squares regression. As by definition K_M is expressed as an explicit function of V_{max} (Briggs and Haldane, 1925), they are not independent variables. The Michaelis constant for the carboxylase and oxygenase reactions can be written in the general linear in V_{max} form (Cummins et al., 2018a),

$$K_M = mV_{max} + b \tag{3}$$

The coefficient of the K_M intercept and coefficient of V_{max} obtained by linear regression of the data can be interpreted as the sample mean values of b and m, respectively, which are functions of the rate constants for the elementary steps in the kinetic mechanism (Cummins et al., 2018a). Prior linear regression analysis of the explicit dependence of K_M on V_{max} indicates that K_M is to some extent dependent on the value of b, which is a function of the dissociation rates (among other rate constants) of the CO_2 and O_2 gas substrates (Cummins et al., 2018a, 2019a).

The margin of error in the difference between two means is estimated using confidence intervals (CIs), calculated from the standard errors of the means (SE). For two independent samples of size n_1 and n_2 with means M_1 and M_2 (e.g., regression coefficients for C_3 and C_4 samples), the combined SE for the difference in the means (ΔM) can be obtained by quadrature, giving the CI as follows:

$$\mathrm{CI} = z\sqrt{\left(\mathrm{SE}_1\right)^2 + \left(\mathrm{SE}_2\right)^2} \tag{4}$$

Alternatively, quadrature may be used with fractional errors to express differences as a percentage, as

$$\mathrm{CI} = z\frac{\mathrm{M}_2}{\mathrm{M}_1}\sqrt{\left(\frac{\mathrm{SE}_1}{\mathrm{M}_1}\right)^2 + \left(\frac{\mathrm{SE}_2}{\mathrm{M}_2}\right)^2} \tag{5}$$

In Equations (4) and (5), Student's inverse cumulative distribution function (t^{-1}) is given by

$$z = t^{-1}\left(\alpha, n_1 + n_2 - 2\right) \tag{6}$$

The CIs can be readily interpreted in terms of p values. If $|\Delta \mathrm{M}| >$ CI, the difference may be considered "statistically significant" (null hypothesis may be rejected) at $\alpha = p$. We consider that only very small p values (at best, $p < 0.01$) should provide a reliable foundation for rejecting a null hypothesis (Cumming, 2012). The statistical analysis was generated using the Real Statistics in Excel software package (Zaiontz, 2020).

RESULTS

Mean values of the RuBisCO kinetic parameters are given in **Table 1** for the total samples of C_3 (excluding *Limonium*), C_3 *Limonium* and C_4 plants. Since K_C differences increase in proportion to the V_C (probably by around 40% of mean C_3 values), carboxylase specificity ($S_C = V_C/K_C$) does not make a significant contribution to differences in mean $S_{C/O}$. The difference in $S_{C/O}$ between C_3 and C_4 is therefore determined largely by S_O, primarily through the increase maximum turnover rate for the oxygenase reaction, V_O. The transition from C_3 to C_4 plants is accompanied by an estimated 10–25% decrease in mean $S_{C/O}$, due overwhelmingly to changes in the kinetics of the oxygenase reaction alone. Overall, the results in **Table 1** are similar to those in the PGLS study (Bouvier et al., 2021); in C_4 species, $S_{C/O}$ is lower than in C_3, and both V_C and K_C are higher than in C_3 species. The sample of C_3 *Limonium* species differs from the main C_3 sample in both higher S_C and S_O means, determined largely by decreases in K_C and K_O. The resulting mean $S_{C/O}$ in *Limonium* is 15% higher than the mean of the C_3 sample.

Regression coefficients and their standard errors are given in **Table 2** for the C_3 (excluding *Limonium*), C_3 *Limonium*, and C_4 samples. Scatter plots of the data and lines of best fit with R^2 are shown in **Figure 1** for correlations between parameters from the same reaction (carboxylase or oxygenase), and in **Figure 2** for correlations between carboxylase and oxygenase parameters. Results obtained for carboxylase (**Figure 1A**) indicate differences between carboxylation parameters in C_3, C_3 *Limonium*, and C_4 plant RuBisCOs. On average, b is negligible (when compared to the product, mV_{max}) in the C_3 plant sample. For the carboxylase reaction, the gradients (m) and intercept (b) of the lines of best fit decrease and increase, respectively, from C_3 to C_3 *Limonium*, to C_4. Also of note are the lower R^2 values (higher variance) for the C_3 and C_4 samples as compared to C_3 *Limonium* sample.

The actual trend lines for S vs. V_{max} (**Figures 1B,D**) can be calculated by expressing the specificity (V_{max}/K_M) in the general hyperbolic in V_{max} form

$$S = \frac{V_{max}}{mV_{max} + b} \tag{7}$$

and substituting m and b with the corresponding K_M vs. V_{max} regression coefficients given in **Table 2**. S increases from zero with increasing V_{max}, reaching the asymptotic limit value m^{-1} when $mV_{max} >> b$. It is also clearly apparent that the linear equation obtained from regression of the *Limonium* data (with higher R^2) is tangential to the predicted curves in the vicinity of the data points. That the coefficient corresponding to m^{-1} should approximate the mean value of S is easily verified by comparing the results in **Tables 1** and **2**, justifying the underlying assumptions of the regression analysis. The rate at which this limit is reached depends on $m.b^{-1}$ (verifiable from the corresponding coefficients in **Table 2**). For carboxylase, we find that the rate of increase in S with respect to increasing V_{max} is relatively fast in the sample of C_3 plants, while significantly slower for C_3 *Limonium*,

TABLE 1 | Sample sizes (n) and mean values of RuBisCO kinetic parameters: maximum turnover rates, V_{max} (s^{-1}), Michaelis constants, K_M (μM), and specificities, $S = V_{max}/K_M$ ($s^{-1}.mM^{-1}$) and relative specificities $S_{C/O}$ with standard errors in the means (SE) for C_3, C_3 *Limonium*, and C_4 plants.

	C_3[a]			C_3 *Limonium*			C_4			C_4–C_3[b]	
	n	**Mean**	**SE**	***n***	**Mean**	**SE**	***n***	**Mean**	**SE**	**Mean**	**%C_3**
V_C	126	3.22	0.08	17	2.76	0.13	26	4.42	0.23	1.27***	40.3 ± 16.0
K_C	124	16.0	0.5	17	8.61	0.23	30	21.0	1.4	5.9***	39.4 ± 19.9
S_C	116	213	6	17	320	9	25	241	19	14	6.1 ± 17.7
V_O	94	1.01	0.03	14	1.09	0.05	21	1.46	0.13	0.43**	42.2 ± 25.5
K_O	108	497	14	17	380	12	25	512	54	31	6.5 ± 22.6
S_O	106	2.03	0.05	14	2.92	0.13	25	3.02	0.26	0.88***	41.2 ± 24.6
$S_{C/O}$	126	97.8	0.8	14	112	1	31	81.0	1.5	−18.1***	−18.3 ± 6.2

*Differences in the means (C_4–C_3) are also expressed as percentage of C_3 values (%C_3) with 95% confidence ($\alpha = 0.05$) intervals. **$p < 0.01$; ***$p < 0.001$.*

[a]*Excluding C_3 Limonium.*

[b]*Including C_3 Limonium.*

TABLE 2 | Results of linear regression analysis for C_3 (excluding *Limonium*), C_3 *Limonium*, and C_4 plants.

		C_3		C_3 *Limonium*		C_4		C_4-C_3	C_4-*Lim.*	C_3-*Lim.*
Figure		**coeff.**	**SE**	**coeff.**	**SE**	**coeff.**	**SE**	**coeff.**	**coeff.**	**coeff.**
1A	K_C (0)	1.31	1.37	4.67***	0.76	19.2**	6.2	17.9**	14.6*	−3.4*
	V_C	4.58***	0.41	1.42***	0.27	0.36	1.35	−4.22*	−1.06	3.2***
1B	K_O (0)	269***	42	243**	71	457*	171	188	214	26.3
	V_O	242***	41	123	64	60	109	−182	−63	119
1C	S_C (0)	203***	21	154***	31	57	66	−146*	−97	49
	V_C	3.1	6.3	60.1***	10.9	41.4**	14.3	38.2*	−18.8	−57.0***
1D	S_O (0)	1.03***	0.16	1.17	0.58	1.19	0.62	0.16	0.02	−0.14
	V_O	0.97***	0.15	1.61**	0.52	1.25**	0.65	0.17	−0.35	−0.63
2A	V_O (0)	0.58***	0.12	0.33	0.17	0.37	0.41	−0.21	0.04	0.25
	V_C	0.13***	0.03	0.27***	0.06	0.25*	0.09	0.12	−0.02	−0.14
2B	K_O (0)	248***	36	283*	116	30	125	−218	−253	−35
	K_C	14.9***	2.0	11.3	13.4	23.7***	5.8	8.8	12.3	3.6
2C	$S_{C/O}$ (0)	94.1***	2.3	131***	5	91.4***	5.5	−2.8	−39.7***	−36.9***
	V_C	1.72*	0.70	−6.87**	1.71	−2.31	1.21	−4.02**	4.56*	8.58***
2D	S_C (0)	39.6***	4.7	74.4***	16.5	15.3	8.0	−24.3**	−59.2**	−34.9*
	S_O	79.2***	2.2	85.6***	5.6	74.7***	2.4	−4.4	−10.9	−6.5
2E	$S_{C/O}$ (0)	90***	2	127***	11	78***	5	12*	−49***	−37***
	V_C/V_O	2.82***	0.61	−6.11	4.10	1.27	1.52	−1.55	7.38	8.93*
2F	K_C/K_O (0)[a]	3.99***	0.79	2.82	2.77	−3.03	2.11	−1.17	11.7**	5.85*
	V_C/V_O[a]	8.8***	0.2	11.3***	0.8	10.1***	0.8	2.50**	2.83*	1.17
2G	$S_{C/O}$ (0)	108***	3	89.6***	7.5	81.6***	4.5	−26.7***	−8.1	18.6*
	K_O/K_C	−0.27***	0.08	0.50	0.17	0.04	0.16	0.32	−0.46	−0.77***

*The coefficients of the independent variable (e.g., V_C in **Figure 1A**) and intercept on the dependent variable axis (0) correspond to the sample mean values of m and b, respectively, in Equation 3, and are dependent on details of the enzyme's kinetic mechanism. *p <0.05; **p <0.01; ***p <0.001. [a]×10³.*

and in C_4 plants, the limiting value is reached very slowly. Unlike carboxylase, the regression coefficients obtained for the oxygenase reaction (**Figure 1C**) indicate little difference between the C_3, C_3 *Limonium*, and C_4 plant groupings. In contrast to the C_3 sample which shows practically no correlation between S_C and V_C (**Figure 1B**), all groups exhibit positive correlations for S_O vs. V_O (**Figure 1D**).

Varying degrees of correlation are evident between carboxylase and oxygenase kinetic parameters (**Figure 2**). Positive correlations are observed in both V_O vs. V_C (**Figure 2A**) and K_O vs. K_C (**Figure 2B**), although the differences between C_3, C_3 *Limonium* and C_4 are not significant. Nevertheless, differences between the three groups do become apparent in many of the other correlations. In particular, very strong ($R^2 > 0.9$) S_C vs. S_O correlations (**Figure 2D**) clearly distinguish the three groups. The different forms in Equation (2) suggest similarly strong linear correlations should also be obtained for V_C/V_O vs. K_C/K_O (**Figure 2F**). Inverting the linear equations obtained from the V_C/V_O vs. K_C/K_O regression provide hyperbolic-like functions which accurately predict the reciprocal V_C/V_O vs. K_O/K_C plots (**Figure 2H**) for each of the three group samples. Other correlations, $S_{C/O}$ vs. V_C, $S_{C/O}$ vs. $V_{C/O}$, and $S_{C/O}$ vs. $K_{O/C}$, relevant for the discussion and interpretation of Equation (2) are also shown in **Figure 2**.

As shown in **Figure 1**, the correlation coefficients obtained from the linear regression of K_M vs. V_{max} in **Table 2** can be used to predict the correlations in S vs. V_{max}. The relatively high level of correlation in the *Limonium* data suggests that an accurate estimation of the curve for S_C vs. S_O should be obtainable using parametric equations derived from S_C vs. V_C and S_O vs. V_C. Equations for S_C vs. V_C and S_O vs. V_O are already defined (**Figures 1B,D**), and an equation (**Figure 3C**) for V_O in terms of V_C derived from nonlinear regression of V_O vs. V_C (**Figure 2A**) can be substituted for V_O in the equation for S_O vs. V_O, yielding the desired equation for S_O vs. V_C (**Figure 3B**). In each case, the linear equations obtained from regression of data in **Figure 3** are quite clearly tangential to the predicted curves in the vicinity of the data points. Moreover, the predicted curve for S_C vs. S_O (**Figure 3A**) is in fact very nearly linear.

For each genus, the near-linear trend predicted in **Figure 3A** suggests performing the linear regression for S_C vs. S_O with the intercept fixed at zero (**Figure 4A**). Further, the gradient (regression coefficient) of the regression line in this way quite accurately predicts the sample $S_{C/O}$ means for each of the genera (**Figure 4B**), which again validates our basic assertion that the coefficients of the linear regression should correspond to sample means (Cummins et al., 2018a, 2019a). Examples of genera with data for more than a few species are rather limited. In addition to *Limonium*, for which there is a reasonably sized sample (**Table 1**), C_3 plant data are available for numbers of BOP species from *Oryza*, *Aegilops*, and *Puccinellia*. Both C_3 and C_4 plant data are available for *Panicum*, while *Flaveria* is the only genus for which there are parameters for C_3, C_4, and transitional (C_3-C_4 and C_4-like) species (**Supplementary Tables S1** and **S2**). The results in **Figure 4A** reveal a high level ($R^2 > 0.90$) of S_C vs. S_O correlation between species within the various genera, irrespective of photosynthetic (C_3 or C_4) pathway. The distribution of $S_{C/O}$ in these genera is

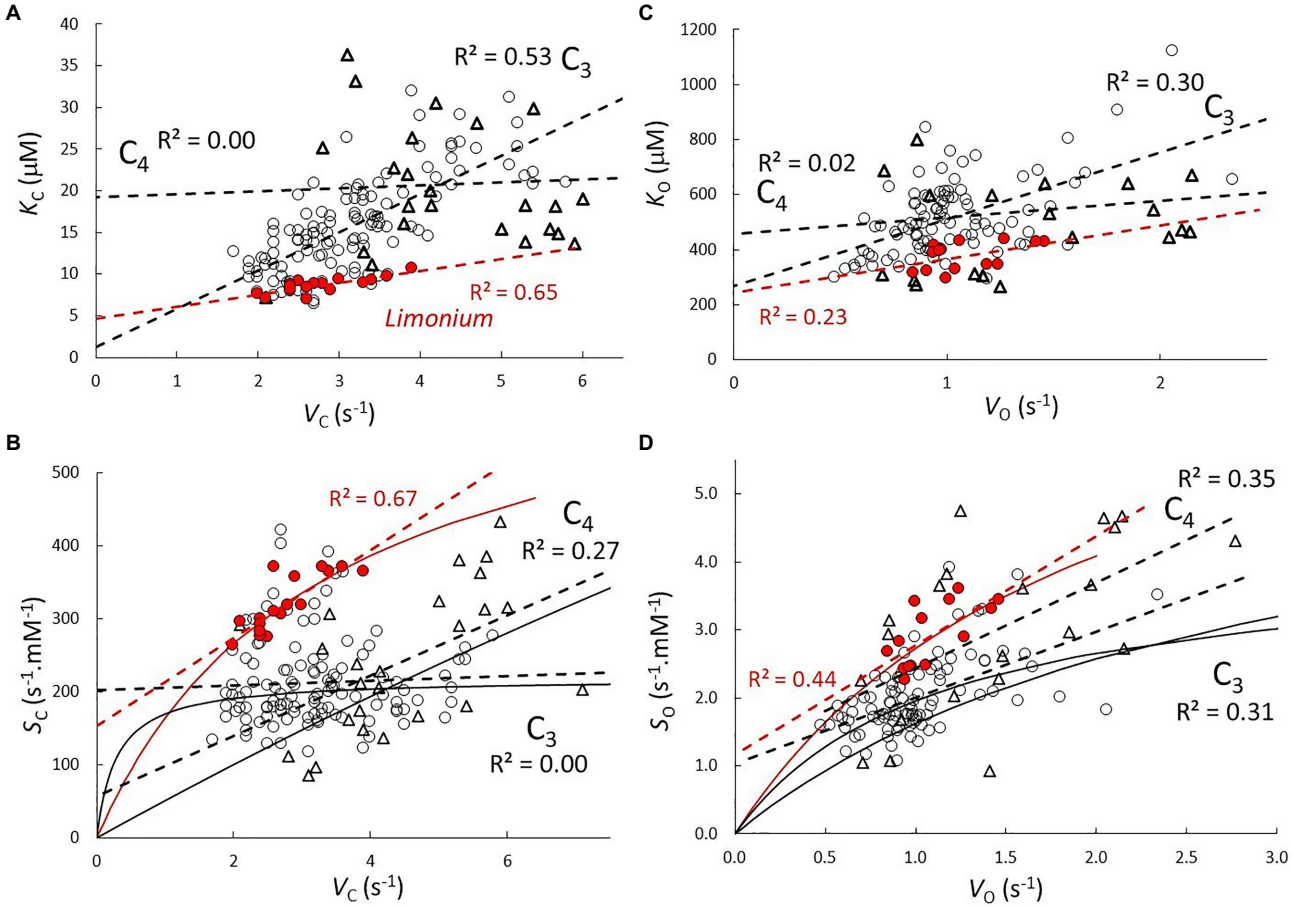

FIGURE 1 | Correlations between parameters V_{max}, K_M, and S from **(A,B)** carboxylase and **(C,D)** oxygenase reactions. Scatter plots and lines (dash) of best fit with R^2 for C_3 (o), C_3 *Limonium* (highlighted in red), and C_4 (Δ) plant RuBisCO parameters. Solid lines in (**B**; S_C vs. V_C) and (**D**; S_O vs. V_O) are predictions of the actual curves (Equation 7) based on coefficients (**Table 2**) obtained from regression of data in **(A,C)**, respectively.

illustrated more clearly in **Figure 4B**. For the vast majority of *Panicum* and *Flaveria* species (both C_3 and C_4), the $S_{C/O}$ is more than one SD below the median of the total C_3 sample.

There are some other correlations of note that distinguish the various genera (**Figure 5**). Significant $S_{C/O}$ vs. V_C correlations are obtained for both *Limonium* and *Flaveria* (5A), but only for *Limonium* in S_O vs. V_C (5B). In **Figure 5C**, increased $S_{C/O}$ in *Limonium*, *Oryza*, *Aegilops*, and *Puccinellia* (exclusively C_3) species correlates with increased CO_2 affinity ($K_{O/C}$), while for *Panicum* and *Flaveria* (predominantly C_4 species), increased $K_{O/C}$ has little tendency to increase $S_{C/O}$, which appears to be true generally for species that have evolved C_4 photosynthesis (**Figure 2G**).

DISCUSSION

Photorespiration in the Evolution of RuBisCO and C_4 Photosynthesis

The evolutionary pathway to C_4 photosynthesis necessitates extensive structural, biochemical, and genetic modifications in the ancestral C_3 plants (Gowik et al., 2011). Considering the current understanding of C_4 evolution has been achieved through a broad multidisciplinary approach (Sage et al., 2018), it is noteworthy that the numbers of published RuBisCO kinetic studies over the last decade have shown a steep decline (Hanson, 2016). C_4 photosynthesis is a prime example of convergent evolution (Blount et al., 2018), having arisen on at least 66 occasions over the past 30 ma (Sage et al., 2011), producing many thousands of species spread globally over many diverse plant families (Sage, 2016). Despite being the most extensively studied enzyme, at least in terms of kinetics (Jeske et al., 2019), compilations of the C_4 RuBisCO kinetic parameters (Flamholz et al., 2019) barely scratch the surface of the total global C_4, and for that matter, C_3 populations.

Despite the large gaps in the available data, what exactly can be understood in relation to the coevolution of RuBisCO kinetics and C_4 photosynthesis? **Table 1** shows clear differences between sample means of most C_3 and C_4 kinetic parameters. However, it seems that the fundamental question is whether, or to what extent, these differences arise from adaptation over time along the evolutionary C_4 pathway, or they are mostly

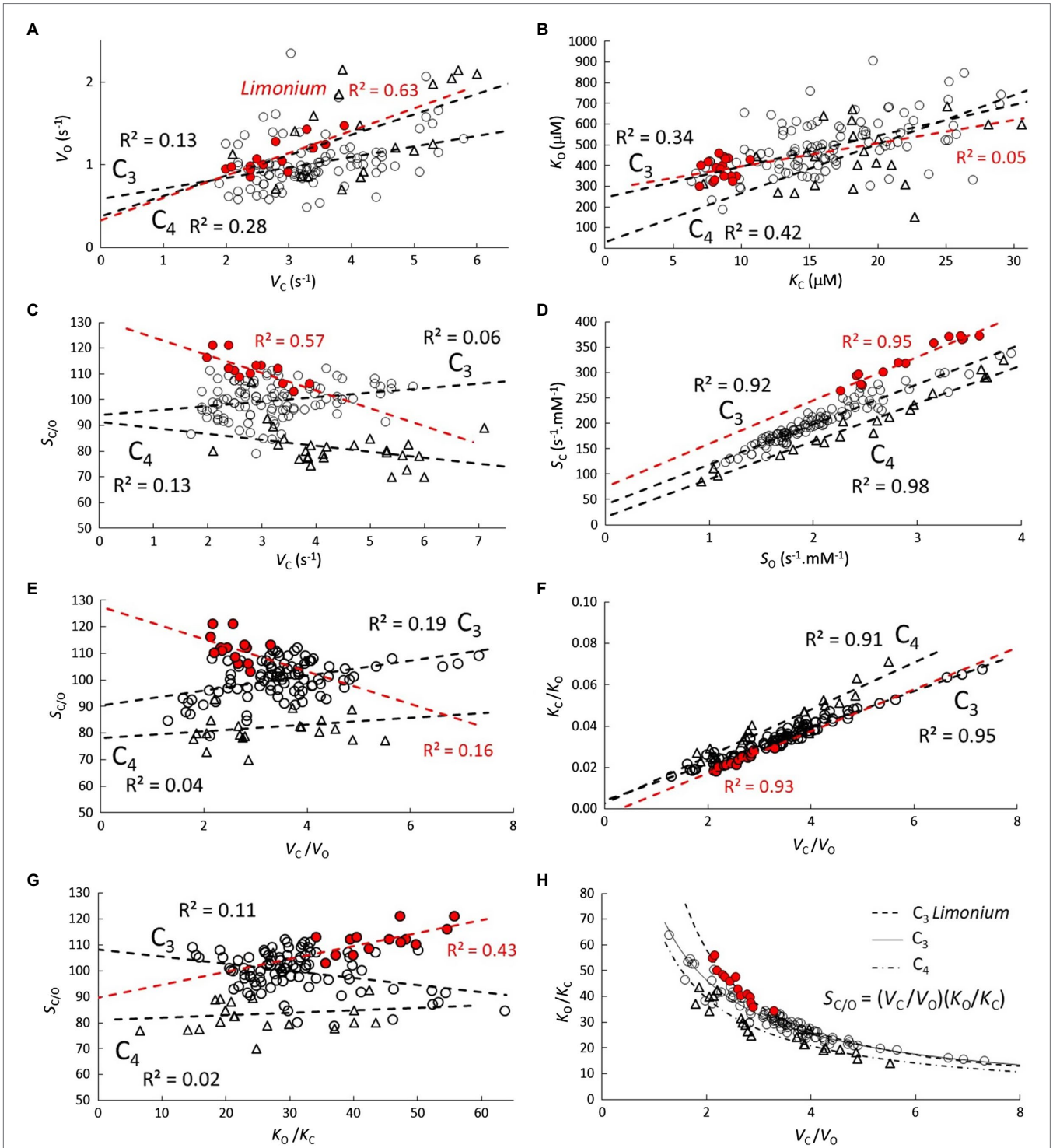

FIGURE 2 | Correlations **(A-H)** between carboxylase and oxygenase kinetic parameters. Scatter plots and lines of best fit with R^2 for C_3 (o), C_3 genus *Limonium* (highlighted in red), and C_4 (Δ) plant RuBisCO parameters.

traits inherited, with minimal change, from the ancestral C_3 species? Comparing sample means can only provide the answer if the evolving C_4 plants were randomly selected from the broader C_3 population. The evidence suggests this may not be the case, as both *Flaveria* and *Panicun* C_3 species exhibit $S_{C/O}$ values much lower than the C_3 average (**Figure 4B**), which could well be an advantage in the early-stage evolution of C_4 plants, as the first stages of C_4 evolution involve establishment

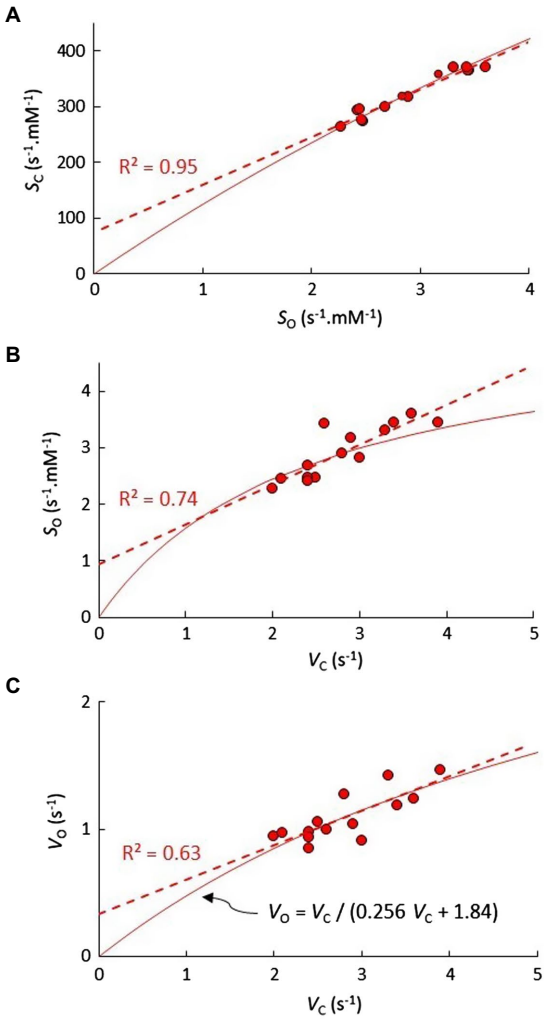

FIGURE 3 | The predicted trend (solid line) in **(A)** S_C vs. S_O for genus C_3 *Limonium*, determined using the set of parametric equations describing the trends in S_C vs. V_C (**Figure 1B**) and **(B)** S_O vs. V_C. In **(B)**, the solid trend line for S_O vs. V_C is determined by substituting **(C)** the hyperbolic equation obtained from the nonlinear least squares fit of the V_O vs. V_C, data for V_O in the equation for the predicted S_O vs. V_O curve (**Figure 1D**).

of the photorespiratory CO_2 pump (C_2 photosynthesis). Given this initial requirement for photorespiratory CO_2, it would not be unexpected to find positive selection of C_3 species with low $S_{C/O}$.

While C_4 evolution may have followed a number of different pathways (Schüssler et al., 2017), in the *Flaveria* model, C_2 photosynthesis is associated with intermediate C_3-C_4 species, transitioning to C_4-like in the final "optimization" stages (Sage et al., 2018); phylogenetic analysis of C_3, transitional and C_4 species in *Flaveria* (Kubien et al., 2008; Kapralov et al., 2011) reveals correlations with variation in kinetic parameters. While not so apparent in the combined C_3 and C_4 samples (**Figure 2C**), the expected negative $S_{C/O}$ vs. V_C trend (Tcherkez et al., 2006) found in *Flaveria* (Kubien et al., 2008) is reproduced in **Figure 5A**. One interpretation of this result is that increased CO_2 and decreased O_2 levels favor selection of RuBisCO with lower S_C and higher V_C, with little change in oxygenase kinetics. This appears to be supported in **Figure 5B** which reveals that the trend is determined exclusively by decreasing S_C (increasing K_C), as S_O vs. V_C in *Flavaria* shows no correlation, so that by inference the observed reduction of $S_{C/O}$ with increasing V_C in *Flaveria* must arise from S_C alone. However, as we explain below, these trends in the adaptation of RuBisCO would most likely have arisen much later in the evolution of C_4, well after the establishment of a functional CCM.

There is no difference between the C_3 and C_4 mean S_C to suggest there is adaptation toward decreased carboxylation (K_C) in favor of speed (V_C); increased V_C alone causes the increase in K_C, maintaining the stability in S_C (**Table 1**). Rather, the difference between C_3 and C_4 mean $S_{C/O}$ stems from S_O alone, predominately through higher oxygenase turnover (V_O). If sampling is restricted to the C_3 species with $S_{C/O}$ less than one SD below the median (**Figure 4B**), the resulting mean values of V_O (1.43 s^{-1}) and K_O (489 μM) are comparable to the corresponding values obtained for the C_4 sample. The C_3 species with lower $S_{C/O}$ and C_4 species exhibit very similar oxygenase traits. These similarities suggest that the C_4 plants sampled (*Panicum* and *Flaveria*) may have evolved from C_3 with $S_{C/O}$ well below the mean of the total C_3 population. Notwithstanding the other preconditions (Gowik et al., 2011), if this restriction extends more generally to the *Poaceae* and *Asteraceae* families, it alone would have significantly limited the numbers of C_4 species that could have evolved. The increased mean V_C observed in the C_4 sample is consistent with adaptation in response to increased supply of CO_2 to the enzyme following development of the CCM, decreasing the selection pressure to optimize the oxygenase reaction (V_O, K_O) and carboxylation (K_C) traits.

Photorespiration and the Homeostatic Maintenance of Chloroplast CO_2 Levels in C_3 Photosynthesis

Although the mitigation of photorespiration is seen as a pathway for improving crop yields, it is well recognized that under some conditions it is likely essential for healthy plant growth (Betti et al., 2016). Photorespiration can protect photosynthesis from light damage and help maintain cellular redox balance as well as plant immune responses (Voss et al., 2013). While the current evidence is largely circumstantial (Ratcliffe, 2018), another study suggests that photorespiration may not waste as much energy a first thought and enhances nitrate assimilation (Bloom and Lancaster, 2018). The scavenging of photorespiratory CO_2 in plant cells helps maintain chloroplast *C* levels in C_3 photosynthesis (Sage and Sage, 2009; Tholen and Zhu, 2011; Busch et al., 2013). The flow of chloroplast CO_2 should be sufficient to occupy all available RuBisCO sites (Igamberdiev, 2015). When ambient CO_2 decreases to lower than normal levels (as under extreme climatic conditions), CA may be unable to produce enough CO_2 from the reservoir of bicarbonate to fuel RuBisCO, resulting in the underutilization of the energy produced by

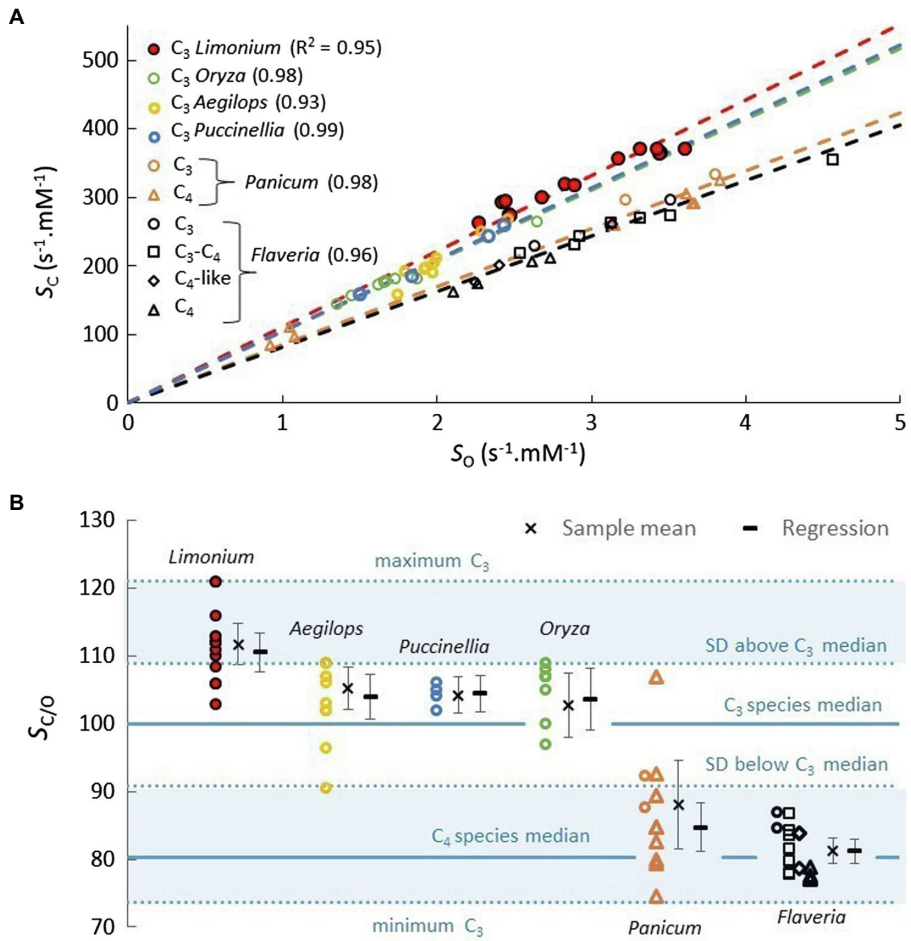

FIGURE 4 | (A) Plots of S_C vs. S_O for genera with lines of best fit passing through $S_C = 0$. **(B)** Diagram illustrating the variance of $S_{C/O}$. The sample means are well approximated by the gradients (regression coefficient) of the trend lines in **(A)**. The error bars correspond to 95% ($\alpha = 0.05$) confidence intervals. The lower shaded area, less than one SD below the median, represents around 10% of the total C_3 species sample, with a mean $S_{C/O}$ of 83.4 ($V_C = 2.85\,s^{-1}$, $K_C = 12.1\,\mu M$, $S_C = 245\,s^{-1}.mM^{-1}$, $V_O = 1.43\,s^{-1}$, $K_O = 489\,\mu M$, $S_O = 2.85\,s^{-1}.mM^{-1}$).

the light reactions, but this can be mitigated by the supply of photorespiratory CO_2 (Igamberdiev, 2015). Moreover, the efficient operation of C_3 photosynthesis may require that fluctuations in *O* and *C* be contained within certain limits (Roussel and Igamberdiev, 2011), which we expect would then tend to limit $v_{O/C}$ (Equation 1). Based on these considerations, we might posit that $v_{O/C}$ should also be maintained within certain limits. The availability of photorespiratory CO_2 to chloroplasts supports a homeostatic mechanism that helps renormalizes $v_{O/C}$ and CO_2 levels by negative feedback of photorespiratory CO_2 into chloroplasts in response to decreasing *C* levels.

Apart from substrate concentrations (*C* and *O*), the other factor that determines $v_{O/C}$ is $S_{C/O}$, which must then also be somehow constrained to keep $v_{O/C}$ within certain limits. Loosely correlated changes in S_C and S_O, which may result in an increase in one and a decrease in the other, have the potential to produce much larger variations in $S_{C/O}$ than are observed (**Figure 2D**). The strong ($R^2 > 0.90$) positive correlations between S_C and S_O produce tightly constrained $S_{C/O}$ variability (**Figure 2D**), particularly within C_3 genera (**Figure 4A**). An increase in S_C is accompanied by a proportionate increase in S_O, facilitating the containment of $v_{O/C}$ within the limits required for efficient photosynthesis. Such correlations between kinetic traits have usually been attributed to constraints inherent in RuBisCO's catalytic mechanism (Tcherkez et al., 2006; Tcherkez, 2013, 2015; Flamholz et al., 2019), which we consider in detail below. However, another recent study suggests phylogenetic, rather than catalytic (or mechanistic), constraints have largely determine RuBisCO adaptation (Bouvier et al., 2021).

RuBisCO Mechanistic Constraints

A fundamental understanding of correlations between RuBisCO parameters requires consideration of its kinetic mechanism (**Figure 6**), together with a knowledge of the functional dependence of V_{max}, *m* and *b* in Equation (3) on the rate constants (k_i) for the elementary steps in both carboxylase

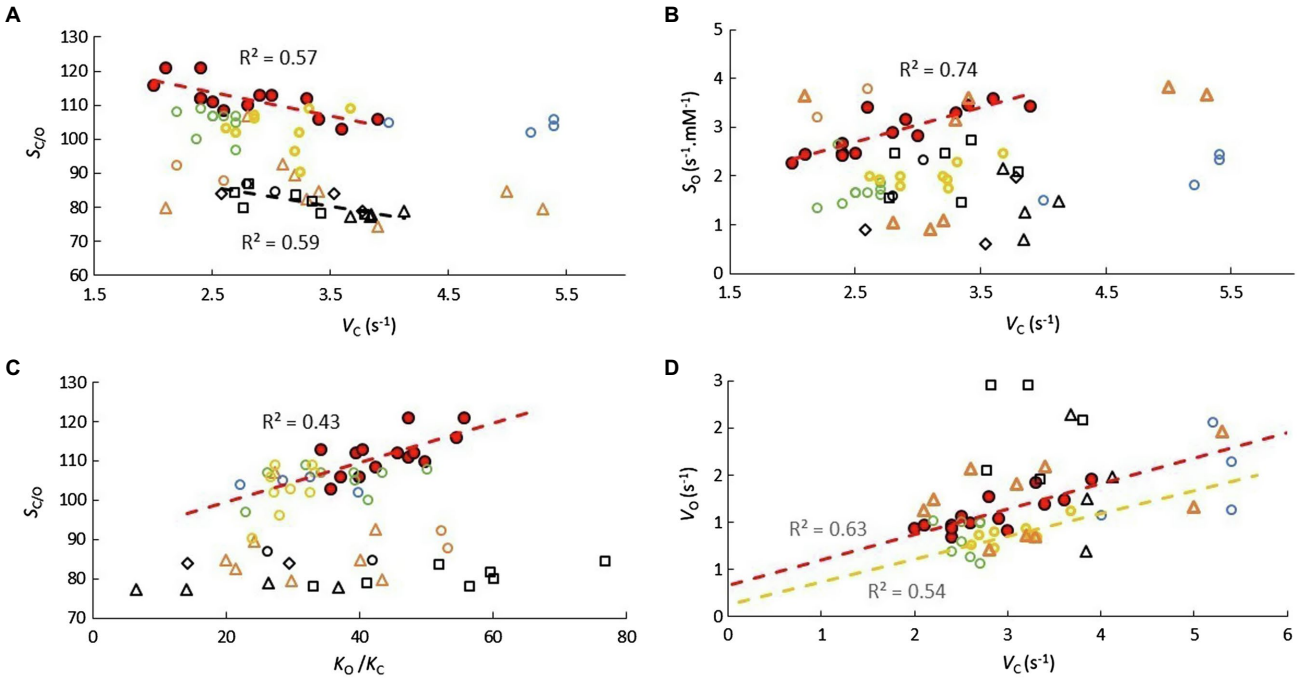

FIGURE 5 | Scatter plots of **(A)** $S_{C/O}$ vs. V_C, **(B)** S_O vs. V_C, **(C)** $S_{C/O}$ vs. K_O/K_C, and **(D)** V_O vs. V_C for various genera. Description of symbols used is the same as in **Figure 4**.

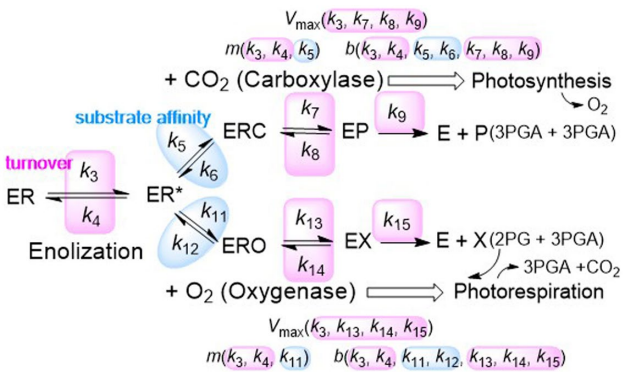

FIGURE 6 | The kinetic mechanism of RuBisCO. RuBisCO processes the three substrates, ribulose-1,5-bisphosphate (RuBP), and CO_2 or O_2, the complete reactions taking place over several stages. RuBP (R) binds first, forming a complex (ER) with the activated form of the enzyme (E), followed by enolization of RuBP (ER*) which facilitates binding with the CO_2 (C) or O_2 (O) molecule to R* forming the ERC or ERO enzyme-substrate complexes. The 6-carbon compound formed by the addition of carbon dioxide to RuBP breaks apart forming a product complex (EP) which dissociates into two molecules of the 3-carbon compound (P), 3-phosphoglyceric acid (3PGA). Oxygenation proceeds through analogous steps except that the dissociation products (X) are one 3PGA molecule and one of 2-phospho-glycolate (2PG) to be recycled into 3PGA by photorespiration, producing CO_2 which can be made available for photosynthesis. The rate constants can be associated with turnover of product or affinity of CO_2 or O_2 substrate molecules for RuBP. The functional dependence of V_{max}, m and b in K_M (Equation 3) on the elementary rate constants is indicated for each of the reactions. A derivation of the actual kinetic equations is given in the **Supplementary Material**.

and oxygenase reactions. As indicated in **Figure 6**, V_{max}, m and b share a number of k_i associated with product turnover, while only m and b depend on the k_i that determine substrate affinity. Correlations between kinetic traits arising out of RuBisCOs mechanism must derive from correlations between the k_i. However, the k_i are unknown quantities, difficult if not impossible to determine empirically with any certainty, and without simplifying assumptions (Tcherkez et al., 2006), complexity of the functional relationships hinders efforts to uncover such correlations (for details of the actual kinetic equations, see Cummins et al., 2018a). Nevertheless, several important observations can be made based on the results of the present analysis.

The absence of correlation between S_C and V_C in the C_3 sample (**Figure 1B**) demonstrates that the k_i on which V_C depends are not correlated to that associated with CO_2 affinity for enolized RuBP (k_5). When $mV_C >> b$, S_C converges asymptotically to the mean value for the sample. Compared to C_3 *Limonium* and C_4 plants, S_C converges very quickly to this mean value for the C_3 sample (**Table 1**), and so the dependence of K_C on b, which depends on the decarboxylation rate (k_6), may be neglected. This would suggest a lesser significance of k_6 in C_3 species, resolving to some extent the apparent differences of opinion on the issue of decarboxylation (Tcherkez et al., 2018; Cummins et al., 2019a). Nevertheless, the results suggest significant ($mV_{max} \approx b$) decarboxylation in both C_3 *Limonium* and C_4 samples (**Figure 1A**) and deoxygenation (k_{12}, i.e., breakdown of the Michaelis complex by dissociation of O_2) in all three (**Figure 1C**).

Correlations between carboxylase and oxygenase reactions are not precluded. The carboxylase and oxygenase reactions are preceded by enolization of the bound RuBP required for activation of CO_2 or O_2 binding, which has been shown to co-limit V_{max} (Tcherkez et al., 2013). As a consequence of this co-limitation, the forward rate constant (k_3) for the enolization of RuBP is a common factor of V_C and V_O, which effectively couples the two reaction rates. However, the actual correlation observed between V_C and V_O is overall weak (**Figure 2A**) and variable between genera (**Figure 5D**), with only *Limonium* and *Aegilops* (both C_3) exhibiting a moderate level of correlation. Consequently, these correlations are more likely due to adaptation, rather than by a tradeoff enforce by RuBisCO's mechanism. In *Limonium*, the linear correlation between V_C and V_O maintains a more or less constant ratio $V_{C/O}$ (in the range 2–3; **Figure 2E**), limiting variation in $S_{C/O}$, and hence photorespiration, to mainly the ratio of K_Ms, i.e., $K_{O/C}$ (**Figure 2G**).

In contrast, the positive correlations between S_C and S_O seem to establish a manifest constraint between carboxylase and oxygenase (**Figures 2D, 4A**) kinetic parameters. The correlation becomes stronger (as measured by R^2) the more closely related the species; R^2 values tend to be somewhat higher within genera (**Figure 4A**), than within the general C_3, C_4, or total plant sample (**Figure 2D**). The positive correlation seems to be preserved in mutants when the changes in kinetic parameters are relatively small (Genkov et al., 2010), while breaks for mutations that cause large perturbations to the kinetics, sometimes resulting in decreased S_C and increased S_O, i.e., increasing photorespiration (Whitney et al., 1999). This tends to suggest some level of correlation between carboxylase and oxygenase k_i, most likely with those associated with substrate affinity (**Figure 6**). Both substrates present similar electrostatic potentials to the RuBisCO active site (Kannappan and Gready, 2008), so that the binding of CO_2 to enolized RuBP induces a redistribution of charge similar to that induced by O_2 binding (Cummins et al., 2018b; Kannappan et al., 2019; Bathellier et al., 2020; Cummins and Gready, 2020) which will then interact similarly with the external (to the active site) electrostatic field, which is thought to be the primary driver in enzyme catalysis (Warshel et al., 2006; Fried and Boxer, 2017). Moreover, evolutionary changes in this electrostatic field have been linked to RuBisCO substrate specificity (Poudel et al., 2020) This electrostatic field would change slightly with sequence variation outside the highly conserved active site to produce the small free energy changes required to maintain the correlation between S_C and S_O when mutations occur. However, other types of biophysical constraints may limit the fitness of some mutations (Studer et al., 2014; Duraõ et al., 2015), and while superior traits are still being discovered (Davidi et al., 2020), the practical limits of RuBisCO's kinetic variability remain unclear.

Coevolution of RuBisCO, Photorespiration, and CCMs in Plants.

The effect of abiotic environmental stress on RuBisCO in C_3 plants may be succinctly rationalized in terms of the ratios of V_{max}, $V_{C/O} = V_C/V_O$, and K_Ms, $K_{O/C} = K_O/K_C$ ($V_{C/O}$ vs. $K_{O/C}$ in **Figure 2H**). Both the carboxylase and oxygenase reactions produce 3PGA (**Figure 6**), although RuBisCO processes the primary substrate (CO_2) more efficiently than O_2 due to superior kinetic traits (V_{max}, K_M) and the fact that one of the oxygenase products (2PG) has to be reprocessed into 3PGA by photorespiration, albeit costing a certain amount of additional energy and carbon. On the other hand, the additional CO_2 produced as a byproduct in photorespiration may be reutilized in photosynthesis if captured by chloroplasts. Thus $V_{C/O}$ seems to strike a practical balance between photosynthesis and photorespiration as a measure of 3PGA production efficiency; the higher $V_{C/O}$ the more energy efficiently 3PGA is produced. According to the familiar form of the classical Michaelis-Menten (MM) equation (Michaelis and Menten, 1913; Briggs and Haldane, 1925; Michaelis et al., 2011),

$$v = \frac{V_{max}[S]}{K_M + [S]} \tag{8}$$

when the reaction rate (v) reaches half of V_{max}, K_M is equivalent to the concentration of substrate, [S]. Consequently, for a given V_{max}, a substrate with a lower value of K_M saturates the enzyme with a smaller concentration of substrate. Actually (*in vivo*), the rate of carbon assimilation in plants typically deviates from the classical MM curve (Equation 8) reaching only about 50% of V_C (Laisk, 1985; Laisk and Oja, 1998; Ruuska et al., 1998) due to some other limiting factors (Igamberdiev, 2015). To reach saturation, lower values of K_C require less CO_2, and higher values of K_O more O_2. In terms of the RuBisCO kinetic mechanism (**Figure 6**), lower K_C can be best achieved by increasing CO_2 affinity (k_5/k_6), and higher K_O by decreasing O_2 affinity (k_{11}/k_{12}) for the enolizied form of RuBP.

The curvature of the predicted trend lines in **Figure 7** is largely determined by the explicit linear dependence of K_M on V_{max} (Equation 3); increasing $V_{C/O}$ produces the monotonic decrease in $K_{O/C}$. There is no obvious explanation for the divergence of $K_{O/C}$, and hence $S_{C/O}$, curves between C_3 *Limonium* and the mainstream C_3 species at lower $V_{C/O}$ as arising from mechanistic constrains imposed by the enzyme. Alternatively, it is posited that the observed trends arise from adaptation of RuBisCO in response to changes in chloroplast carbon (C) levels according to the prevailing environmental conditions. Under more temperate conditions (applicable to most of the C_3 sample), in species with less than the mean $V_{C/O}$ of about three (**Table 1**), C can be supplemented by increased photorespiration (decreasing $S_{C/O}$). Increasing $S_{C/O}$ reduces photorespiration and hence the maintenance of C, requiring increased $K_{O/C}$ (higher CO_2 relative to O_2 affinity) to maintain sustainable levels of photosynthesis under high stress at the lower end of $V_{C/O}$. In fact, a unique study provides some empirical evidence that those (*Limonium*) species with higher $S_{C/O}$ are associated with reduced C (Galmés et al., 2014).

As $V_{C/O}$ increases from its mean toward the maximum value, the requirement for additional carbon appears to diminish; photorespiratory CO_2 declines with a drift toward increased $S_{C/O}$.

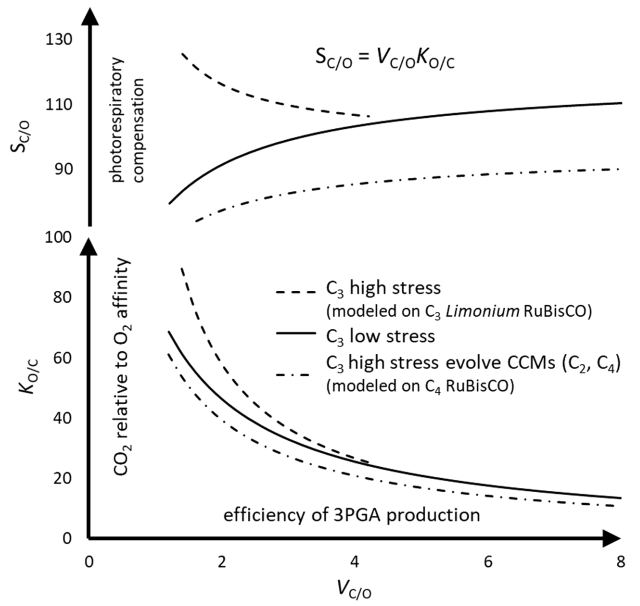

FIGURE 7 | The effect of abiotic environmental stress on RuBisCO kinetics in C_3 plants. The trend lines (**Figure 2H**) in relative specificity, $S_{C/O}$, are obtained by the product of $V_{C/O} = V_C/V_O$, a measure of 3PGA product turnover efficiency, and $K_{O/C} = K_O/K_C$, a measure of CO_2 relative to O_2 affinity for enolizied RuBP. The efficiency of C_3 photosynthesis also relies on the constant supply of CO_2 to RuBisCO, which photorespiration can help maintain. Abiotic stress factors increase the photorespiration relative to photosynthesis (Equation 1). Most C_3 species, with more or less average $S_{C/O}$, are situated in usually low stress (or temperate) habitats; however, some level of photorespiratory CO_2 may be necessary to maintain photosynthesis during short periods of increased stress (Igamberdiev, 2015). Some C_3 plants can leverage off the additional photorespiratory CO_2 produced by their much lower than average $S_{C/O}$ in high stress environments (C_2 photosynthesis). Some of these C_2 plants may go on to evolve fully developed carbon concentrating mechanisms (C_4 photosynthesis). Alternatively, the much higher than average $S_{C/O}$ in other C_3 plants, while mitigating photorespiration, may compensate for the carbon restriction (reduced levels of chloroplast CO_2) associated with high stress environments by gains in CO_2 relative to O_2 affinity.

Thus, there is a positive correlation between $V_{C/O}$ and photorespiratory *C*, which is also clearly apparent in C_4 evolution where the establishment of increased *C* levels (by the CCM) is followed by optimization of V_C. Increased throughput of product can only be maintained by increased supply of CO_2 substrate, necessitating the adaptation of RuBisCO kinetic traits. Most of the C_3 sample is tightly clustered about the mean V_C or $V_{C/O}$ (**Figures 2C,E**). If $V_{C/O}$ falls much below the mean, *C* requires supplementation depending on environmental conditions, either by increased photorespiration (low stress) or by increased CO_2 relative to O_2 affinity (high stress), necessitating RuBisCO accommodate an expansive range of $S_{C/O}$. As discussed, C_4 plants have likely evolved only from C_3 with below average $S_{C/O}$, and this appears to be supported by the parallel trends in C_3 and C_4 illustrated in **Figure 7**. C_3 species with the minimal values of $S_{C/O}$ (lower values for $K_{O/C}$) produce additional photorespiratory CO_2 under high stress conditions, as required for the evolution of C_2 and C_4 photosynthesis. The CCM maintains consistently higher *C* levels, regardless of stress factors, reducing the pressure on RuBisCO to adapt, so that its kinetics have remained, except perhaps for a tendency toward higher V_C, relatively unchanged by evolution.

CONCLUSION

Analysis of the RuBisCO kinetic data presented here suggests that the evolution of kinetic parameters in higher plants, rather than being highly constrained or subjected to tradeoffs imposed by the enzyme's kinetic mechanism, has adapted to variations in photorespiration as part the homeostatic maintenance of a constant CO_2 supply to the enzyme under disparate environmental conditions. The positive correlations observed between S_C and S_O, particularly between phylogenetically related species, reflect similarities in the physical binding properties of the two substrates CO_2 and O_2 to RuBP, which serve to contain $S_{C/O}$ within the limits required for maintaining balance between photosynthesis and photorespiration in the regulation of carbon flux when mutations occur. Significantly, the limitation on product turnover (V_C) is the extent to which RuBisCO kinetics can adapt to the availability of carbon. Apparent tradeoffs between turnover and specificity are not symptomatic of an inefficient enzyme, but reflects the necessary adaptation of a flexible one to the changing levels of accessible CO_2 as a consequence of changes in abiotic environmental conditions.

Over the past 30 ma, the evolution of C_4 photosynthesis has dramatically reduced photorespiration in a relatively small number (a few percent) of plant species by maintaining high levels of chloroplast CO_2, although somewhat paradoxically high levels of photorespiration were instrumental at the beginning the evolutionary process. The C_4 RuBisCOs in the *Panicum* and *Flaveria* samples do not exhibit the "average" C_3 kinetics, but inherit traits largely unchanged from a small proportion of the ancestral C_3 population (i.e., those with much lower than the mean $S_{C/O}$ and, therefore, increased photorespiration). Nevertheless, differences in leaf anatomy and biochemistry indicate that a C_4 plant is not simply a C_3 with an attached CCM, and prodigious efforts to artificially introduce CCMs into commercial C_3 crops are ongoing but have yet to bear fruit. Recent modeling suggests that achieving C_2 photosynthesis in rice may be a more realistic goal (Bellasio and Farquhar, 2019).

While this research continues, however, are there any prospects for reengineering C_3 RuBisCOs with the objective of improving its kinetic traits? Despite decades of research, while some progress has been made through directed evolution, a demonstrably better RuBisCO for agriculture also remains elusive (Zhou and Whitney, 2019). What is meant by a "better" RuBisCO needs to be carefully defined, and the traits to be improved should at least, if not necessarily improve yield under the prevailing settings, increase the fitness of a species to survive the expected increases in the frequency and severity of hot and dry weather events over the coming decades. In this regard, C_4 plants demonstrate the importance of a secure supply of carbon under such climatic conditions, and the evolution of increases in product turnover (V_C), although

perhaps modest compared to V_C found in other taxa (Davidi et al., 2020), may well only have been maintained by the increased availability of carbon provided by the CCM. C_3 plants with higher $S_{C/O}$ suffer from the concomitant reduction in the contributions to chloroplast CO_2 levels that would otherwise had been made by photorespiration. In these cases, increasing the affinity of CO_2 relative to O_2 for RuBP makes more efficient uptake of the meager supply of carbon under high stress environmental conditions. In the absence of a functional CCM, the reengineering of RuBisCOs with increased $S_{C/O}$, if possible, may help to futureproof C_3 crops in a rapidly changing climate.

DATA AVAILABILITY STATEMENT

Publicly available data sets were analyzed in this study. This data can be found at: https://pubs.acs.org/doi/abs/10.1021/acs.biochem.9b00237/supplfile/bi9b00237si005.xlsx.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.662425/full#supplementary-material, www.real-statistics.com, www.c4rice.com

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