Temperature Responses of C_4 Photosynthesis: Biochemical Analysis of Rubisco, Phospho*enol*pyruvate Carboxylase, and Carbonic Anhydrase in *Setaria viridis*[1][OPEN]

Ryan A. Boyd, Anthony Gandin[2], and Asaph B. Cousins*

School of Biological Sciences, Molecular Plant Sciences, Washington State University, Pullman, Washington 99164–4236

ORCID IDs: 0000-0003-4009-7700 (R.A.B.); 0000-0001-7184-5113 (A.G.).

The photosynthetic assimilation of CO_2 in C_4 plants is potentially limited by the enzymatic rates of Rubisco, phospho*enol*pyruvate carboxylase (PEPc), and carbonic anhydrase (CA). Therefore, the activity and kinetic properties of these enzymes are needed to accurately parameterize C_4 biochemical models of leaf CO_2 exchange in response to changes in CO_2 availability and temperature. There are currently no published temperature responses of both Rubisco carboxylation and oxygenation kinetics from a C_4 plant, nor are there known measurements of the temperature dependency of the PEPc Michaelis-Menten constant for its substrate HCO_3^-, and there is little information on the temperature response of plant CA activity. Here, we used membrane inlet mass spectrometry to measure the temperature responses of Rubisco carboxylation and oxygenation kinetics, PEPc carboxylation kinetics, and the activity and first-order rate constant for the CA hydration reaction from 10°C to 40°C using crude leaf extracts from the C_4 plant *Setaria viridis*. The temperature dependencies of Rubisco, PEPc, and CA kinetic parameters are provided. These findings describe a new method for the investigation of PEPc kinetics, suggest an HCO_3^- limitation imposed by CA, and show similarities between the Rubisco temperature responses of previously measured C_3 species and the C_4 plant *S. viridis*.

Biochemical models of photosynthesis are often used to predict the effect of environmental conditions on net rates of leaf CO_2 assimilation (Farquhar et al., 1980; von Caemmerer, 2000, 2013; Walker et al., 2013). With climate change, there is increased interest in modeling and understanding the effects of changes in temperature and CO_2 concentration on photosynthesis. The biochemical models of photosynthesis are primarily driven by the kinetic properties of the enzyme Rubisco, the primary carboxylating enzyme of the C_3 photosynthetic pathway, catalyzing the reaction of ribulose-1,5-bisphosphate (RuBP) with either CO_2 or oxygen. However, the CO_2-concentrating mechanism in C_4 photosynthesis utilizes carbonic anhydrase (CA) to help maintain the chemical equilibrium of CO_2 with HCO_3^- and phospho*enol*pyruvate carboxylase (PEPc) to catalyze the carboxylation of phospho*enol*pyruvate (PEP) with HCO_3^-. These reactions ultimately provide the elevated levels of CO_2 to the compartmentalized Rubisco (Edwards and Walker, 1983). In C_4 plants, it has been demonstrated that PEPc, Rubisco, and CA can limit rates of CO_2 assimilation and influence the efficiency of the CO_2-concentrating mechanism (von Caemmerer, 2000; von Caemmerer et al., 2004; Studer et al., 2014). Therefore, accurate modeling of leaf photosynthesis in C_4 plants in response to future climatic conditions will require temperature parameterizations of Rubisco, PEPc, and CA kinetics from C_4 species.

Modeling C_4 photosynthesis relies on the parameterization of both PEPc and Rubisco kinetics, making it more complex than for C_3 photosynthesis (Berry and Farquhar, 1978; von Caemmerer, 2000). However, the activity of CA is not included in these models, as it is assumed to be nonlimiting under most conditions (Berry and Farquhar, 1978; von Caemmerer, 2000). This assumption is implemented by modeling PEPc kinetics as a function of CO_2 partial pressure (pCO_2) and not HCO_3^- concentration, assuming CO_2 and HCO_3^- are in chemical equilibrium. However, there are questions regarding the

[1] This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences, Department of Energy (grant no. DE–FG02–09ER16062), by the National Science Foundation (Major Research Instrumentation grant no. 0923562), and by the Seattle chapter of the Achievement Rewards for College Scientists Foundation (fellowship to R.A.B.).

[2] Present address: Université de Lorraine, Faculté des Sciences et Techniques, Ecologie et Ecophysiologie Forestières, F54506 Vandoeuvre Les Nancy, France.

* Address correspondence to acousins@wsu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Asaph B. Cousins (acousins@wsu.edu).

A.B.C. proposed the original concept and design for the project; R.A.B. performed most of the experiments and data analysis; A.G. performed the carbonic anhydrase temperature measurements; R.A.B. wrote the article with the contributions of all the authors; A.B.C. supervised and complemented the writing.

[OPEN] Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.15.00586

amount of CA activity needed to sustain rates of C_4 photosynthesis and if CO_2 and HCO_3^- are in equilibrium (von Caemmerer et al., 2004; Studer et al., 2014).

The most common steady-state biochemical models of photosynthesis are derived from the Michaelis-Menten models of enzyme activity (von Caemmerer, 2000), which are driven by the V_{max} and the K_m. Both of these parameters need to be further described by their temperature responses to be used to model photosynthesis in response to temperature. However, the temperature response of plant CA activity has not been completed above 17°C, and there is no known measured temperature response of K_m HCO_3^- for PEPc (K_P). Alternatively, Rubisco has been well studied, and there are consistent differences in kinetic values between C_3 and C_4 species at 25°C (von Caemmerer and Quick, 2000; Kubien et al., 2008), but the temperature responses, including both carboxylation and oxygenation reactions, have only been performed in C_3 species (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013).

Here, we present the temperature dependency of Rubisco carboxylation and oxygenation reactions, PEPc kinetics for HCO_3^-, and CA hydration from 10°C to 40°C from the C_4 species *Setaria viridis* (succession no., A-010) measured using membrane inlet mass spectrometry. Generally, the 25°C values of the Rubisco parameters were similar to previous measurements of C_4 species. The temperature response of the maximum rate of Rubisco carboxylation (V_{cmax}) was high compared with most previous measurements from both C_3 and C_4 species, and the temperature response of the K_m for oxygenation (K_O) was low compared with most previously measured species. Taken together, the modeled temperature responses of Rubisco activity in *S. viridis* were similar to the previously reported temperature responses of some C_3 species. Additionally, the temperature response of the maximum rate of PEPc carboxylation (V_{pmax}) was similar to previous measurements. However, the temperature response of K_P was lower than what has been predicted (Chen et al., 1994). For CA, deactivation of the hydration activity was observed above 25°C. Additionally, models of CA and PEPc show that CA activity limits HCO_3^- availability to PEPc above 15°C, suggesting that CA limits PEP carboxylation rates in *S. viridis* when compared with the assumption that CO_2 and HCO_3^- are in full chemical equilibrium.

RESULTS

Rubisco Temperature Response

The V_{cmax} and maximum rate of Rubisco oxygenation (V_{omax}), the K_m for carboxylation (K_C) and K_O, and the specificity of the enzyme for CO_2 over oxygen ($S_{C/O}$) were measured simultaneously using membrane inlet mass spectrometry from 10°C to 40°C on crude leaf extracts of *S. viridis* (Table I). The maximum turnover rates for carboxylation (k_{catCO2}) and oxygenation (k_{catO2}) were determined at 25°C using combined spectrophotometry and radiolabeled binding of the enzyme. At 25°C, the values of k_{catCO2}, k_{catO2}, K_C, and K_O for *S. viridis* are within ranges previously measured for other C_4 species (Yeoh et al., 1980; Sage and Seemann, 1993; Kubien et al., 2008). At 25°C, the k_{catCO2} was 5.44 ± 0.44 mol CO_2 mol^{-1} site s^{-1}, and the K_C and K_O were 94.7 ± 15.1 Pa and 28.9 ± 5.4 kPa, respectively. The measured $S_{C/O}$ in *S. viridis* at 25°C was 1,610 ± 66 Pa Pa^{-1}. The ratio of the maximum carboxylation rate to the maximum oxygenation rate (V_{cmax}/V_{omax}) at 25°C was 5.54 ± 0.73, and the ratio of the K_m for oxygenation to carboxylation (K_O/K_C) had a 25°C value of 0.31 ± 0.04 kPa Pa^{-1}.

The V_{cmax} increased exponentially from 10°C to 40°C, with an energy of activation (E_a) of 78.0 ± 4.1 kJ mol^{-1} (Fig. 1). The temperature response of V_{omax} was lower compared with V_{cmax}, with an E_a equal to 55.3 ± 6.2 kJ mol^{-1} (Fig. 1). The K_C and K_O had E_a values of 64.2 ± 4.5 and 10.5 ± 4.8 kJ mol^{-1}, respectively (Fig. 1). The

Table I. *Measured values at 25°C, modeled values at 25°C (k_{25}), and E_a using the equation parameter = $k_{25}exp(E_a(T_k - 298.15)/(298.15RT_k))$ for all measured temperature responses ± SE*

For parameters k_{CA} and V_{pmax}, a deactivation was included using the model parameter = $k_{25}\exp(E_a(T_k - 298.15)/(298.15RT_k))(1 + \exp((298.15\Delta S - H_d)/(298.15R)))/(1 + \exp((T_k\Delta S - H_d)/(T_kR)))$, where T_k is the temperature in Kelvin, R is the molar gas constant, ΔS is the entropy factor, and H_d is the heat of deactivation.

Parameter	Units	Measured at 25°C	k_{25}	E_a	ΔS	H_d
				kJ mol^{-1}	*kJ mol^{-1} K^{-1}*	*kJ mol^{-1}*
k_{CA}	μmol CO_2 m^{-2} s^{-1} Pa^{-1}	124 ± 6				
	Normalized to 1 at 25°C	1.0 ± 0.02	1.03 ± 0.05	40.9 ± 70.7	0.21 ± 0.19	64.5 ± 50.9
k_h	s^{-1}	0.039 ± 0.000	0.038 ± 0.000	95.0 ± 1.0	–	–
V_{pmax}	μmol HCO_3^- m^{-2} s^{-1}	450 ± 16				
	Normalized to 1 at 25°C	1	1.01 ± 0.07	94.8 ± 40.8	0.25 ± 0.12	73.3 ± 39.6
K_P	Pa CO_2	16.0 ± 1.3	13.9 ± 1.0	36.3 ± 2.4	–	–
	μM HCO_3^-	62.8 ± 5.0	60.5 ± 2.4	27.2 ± 2.8	–	–
k_{catCO2}	mol CO_2 mol^{-1} site s^{-1}	5.44 ± 0.44				
V_{cmax}	Normalized to 1 at 25°C	0.96 ± 0.04	0.89 ± 0.05	78.0 ± 4.1	–	–
V_{omax}/V_{cmax} at 25°C		0.18 ± 0.03	0.16 ± 0.01	55.3 ± 6.2	–	–
K_C	Pa of CO_2	94.7 ± 15.1	121 ± 7	64.2 ± 4.5	–	–
K_O	kPa of oxygen	28.9 ± 5.4	29.2 ± 1.9	10.5 ± 4.8	–	–
$S_{C/O}$	Pa Pa^{-1}	1,610 ± 66	1,310 ± 52	−31.1 ± 2.9	–	–

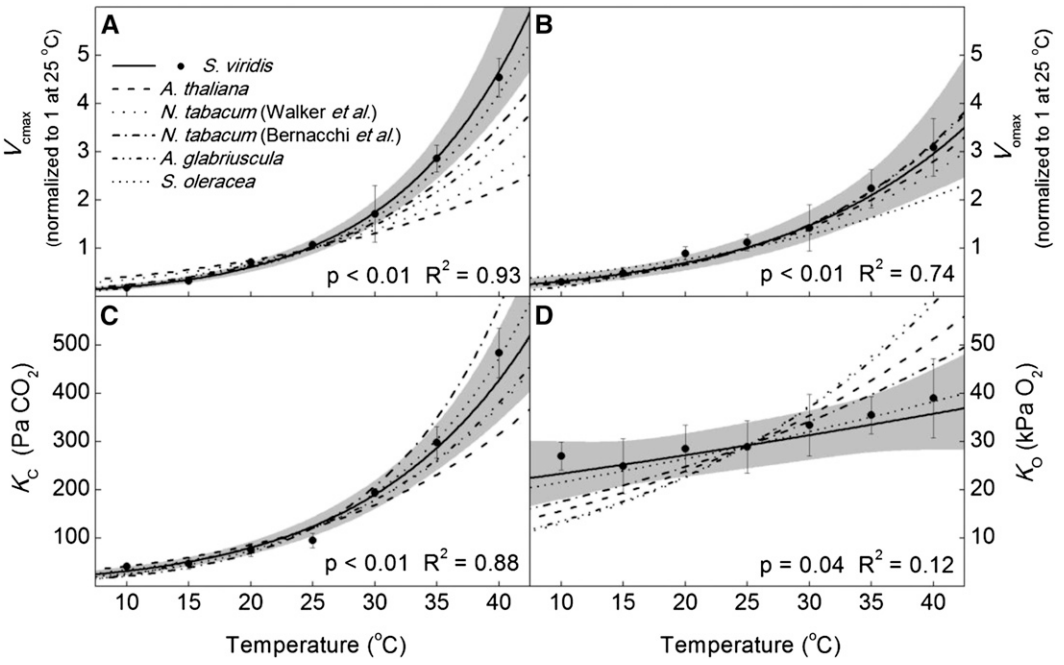

Figure 1. Temperature responses of Rubisco kinetic parameters in *S. viridis* compared with corresponding values from the literature normalized to *S. viridis* at 25°C. The V_{cmax} (A), V_{omax} (B), K_C (C), and K_O (D) were determined using membrane inlet mass spectrometry on crude leaf extracts of *S. viridis* at pH 7.7 (at 25°C). The solid lines are the modeled temperature responses from this report (Table I), with the 95% confidence intervals shaded in gray. Dashed lines are temperature responses from previous reports normalized to *S. viridis* at 25°C (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). Black circles are the means of four technical replicates ± SE. *P* values refer to the significance of the temperature response from zero, and adjusted R^2 values describe the amount of variation in the measured parameter explained by the model.

V_{cmax}/V_{omax} increased from 10°C to 40°C (Fig. 2), but the K_O/K_C decreased with temperature (Fig. 2). The ratio of the catalytic rate of carboxylation to the K_C and the catalytic rate of oxygenation to K_O increased with temperature (Fig. 2). The $S_{C/O}$ decreased with temperature from 10°C to 40°C, with an E_a value of -31.1 ± 2.9 kJ mol^{-1} (Fig. 3).

Rates of Rubisco carboxylation (v_c) and oxygenation (v_o) were modeled at pCO_2 of 400 Pa and oxygen partial pressure (pO_2) of 35 kPa in response to temperature for kinetic parameters from *S. viridis* and previously published values normalized to *S. viridis* values at 25°C (Fig. 4). The temperature response of v_c in *S. viridis* (Fig. 4) was similar to those measured in vitro in *Atriplex glabriuscula* (Badger and Collatz, 1977) and *Spinacea oleracea* (Jordan and Ogren, 1984) but larger than those measured in vivo Arabidopsis (*Arabidopsis thaliana*; Walker et al., 2013) and *Nicotiana tabacum* (Bernacchi et al., 2001, 2002; Walker et al., 2013). The temperature response of v_o in *S. viridis* was similar to previous measurements (Fig. 4). Additionally, the temperature dependency of *S. viridis* was compared with non-normalized temperature responses of v_c and v_o from previous measurements (Supplemental Fig. S1). Under the low pCO_2 (25 Pa) and ambient pO_2 (21 kPa) expected in mesophyll of a C_3 leaf, v_c was lower in *S. viridis* (Supplemental Fig. S1) compared with the C_3 species (Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013), with the exception of *A. glabriuscula* (Badger and Collatz, 1977). The *S. viridis* v_o response (Supplemental Fig. S1) was similar in all reports with the exception of *A. glabriuscula* (Badger and Collatz, 1977), which had a lower value above 35°C. At high pCO_2, the predicted rates of v_c for *S. viridis* (Supplemental Fig. S1) were not different from those for *S. oleracea* (Jordan and Ogren, 1984), *N. tabacum* (Bernacchi et al., 2001, 2002), and, below 25°C, Arabidopsis (Walker et al., 2013). At high pCO_2, *S. viridis* had a higher predicted v_o (Supplemental Fig. S1) at all temperatures compared with the C_3 species (Badger and Collatz, 1977; Jordan and Ogren, 1984; Walker et al., 2013), with the exception of *N. tabacum* (Bernacchi et al., 2001, 2002) above 25°C.

PEPc Temperature Response

The kinetic parameters of PEPc were measured using membrane inlet mass spectrometry on crude leaf extracts of *S. viridis* from 10°C to 40°C (Table I; Supplemental Fig. S2). The V_{pmax} at 25°C was 450 ± 16 μmol m^{-2} s^{-1} when measured on freshly collected leaf tissue. The V_{pmax} increased with temperature from 10°C to 40°C, with an apparent deactivation at 35°C and 40°C (Fig. 5), noted by a deviation from linearity when plotted as a log transformation (transformation not shown). The measured K_P was 60.2 μM HCO_3^- at 25°C. The K_P was calculated as 16 ± 1.3 Pa CO_2 assuming a

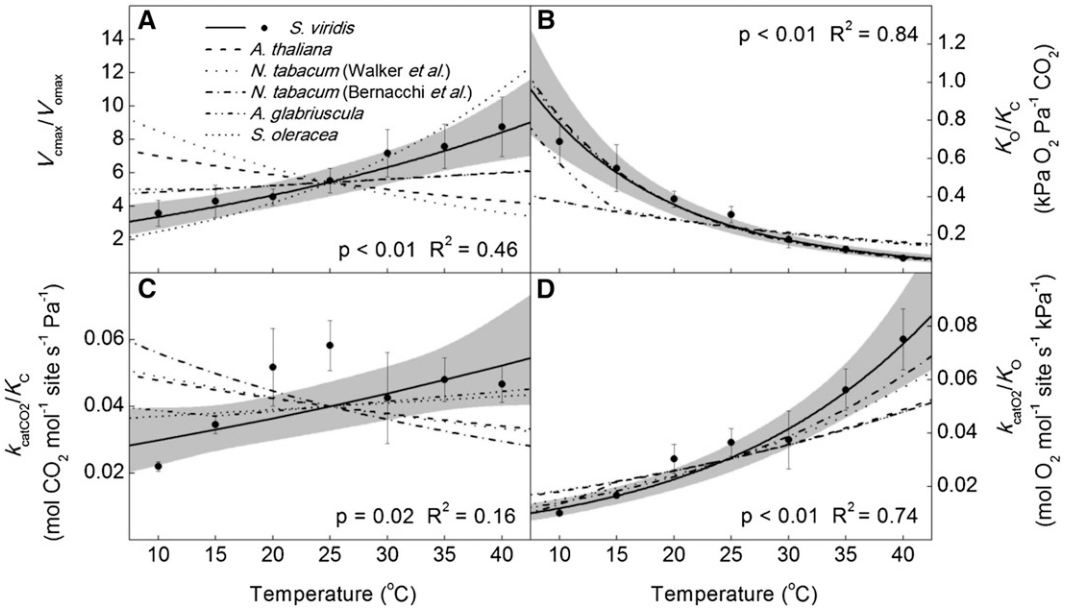

Figure 2. Temperature responses of the ratios of Rubisco parameters in *S. viridis* compared with corresponding values from the literature normalized to *S. viridis* at 25°C. V_{cmax}/V_{omax} (A), K_O/K_C (B), k_{catCO2}/K_C (C), and k_{catO2}/K_O (D) were calculated from data presented in Figure 1. The solid lines are the modeled temperature responses from this report, with the 95% confidence intervals in gray. Dashed lines are temperature responses from previous reports normalized to *S. viridis* at 25°C (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). Black circles are the means of four technical replicates ± SE. *P* values refer to the significance of the temperature response from zero, and adjusted R^2 values describe the amount of variation in the measured parameter explained by the model.

pH of 7.2 and a dissociation constant (pKa) of 6.12 appropriate for the mesophyll cytosol at 25°C (Jenkins et al., 1989). Values of K_P increased exponentially from 10°C to 40°C, with an E_a of 36.3 ± 2.4 kJ mol^{-1}, when modeled in pCO_2 assuming full equilibrium with HCO_3^- (Fig. 5). The temperature dependency of leaf PEPc activity (v_p) modeled at a mesophyll cytosol pCO_2 of 12 Pa at all temperatures increased with temperature but plateaued between 35°C and 40°C (Fig. 5).

CA Temperature Response

The rate constant for CA hydration activity (k_{CA}) was determined from crude leaf extracts using membrane inlet mass spectrometry to measure rates of ^{18}O exchange between labeled $^{13}C^{18}O_2$ and $H_2{}^{16}O$ (Table I; Supplemental Fig. S3). At 25°C, freshly collected leaf tissue of *S. viridis* had a k_{CA} of 124 ± 6 μmol CO_2 m^{-2} s^{-1} Pa^{-1}. The k_{CA} increased from 10°C to 30°C but plateaued from 30°C to 40°C (Fig. 6). When CA activity was modeled for an expected mesophyll pCO_2 of 12 Pa, the hydration rate (v_h) was 1,488 μmol m^{-2} s^{-1} at 25°C (Fig. 6). The temperature change in v_h was identical to k_{CA} because the mesophyll pCO_2 was assumed to be 12 Pa at all temperatures. Alternatively, the temperature response of the uncatalyzed rate of CO_2 hydration in the leaf increased exponentially with temperature and is described by the rate constant, having an E_a of 95 ± 1.0 kJ mol^{-1} (Fig. 6). The E_a for the uncatalyzed rate is much higher than that for the catalyzed rate; however, the uncatalyzed rate is at least 10^5 times lower from 10°C to 40°C (Fig. 6).

The supply of HCO_3^- provided to PEPc by the hydration activity of CA was investigated using the model of Hatch and Burnell (1990) and the temperature responses reported here (Fig. 7). At 12 Pa CO_2 and pH 7.2, CA activity limits PEPc carboxylation above 15°C by more than 5% compared with assuming full chemical equilibrium between CO_2 and HCO_3^- (Fig. 7). Additionally, the modeled HCO_3^- concentration is lower at all temperatures compared with the concentration assuming HCO_3^- is in full equilibrium with CO_2 (Fig. 7). The modeled activity of CA relative to PEPc activity (v_p/v_h) increased with temperature and is lower when v_p is modeled based on HCO_3^- availability provided by CA activity compared with assuming CO_2 and HCO_3^- are in chemical equilibrium (Fig. 7). It should be noted that the modeled limitation of CA to PEPc depends on temperature, pH, and ionic strength, as all these conditions influence the ratio of CO_2 to HCO_3^-.

DISCUSSION

Rubisco Temperature Response

The C_4 biochemical model of photosynthesis uses both carboxylase and oxygenase parameters of Rubisco (von Caemmerer, 2000); therefore, it is important to

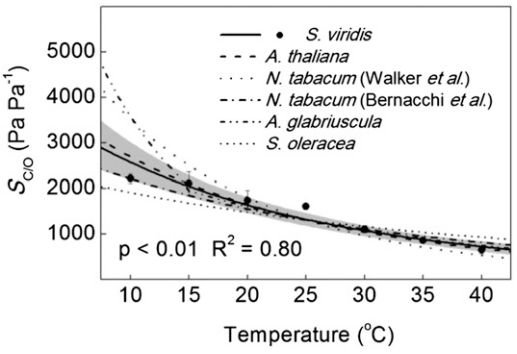

Figure 3. Temperature responses of $S_{C/O}$ compared with corresponding values from the literature normalized to *S. viridis* at 25°C. Black circles represent the averages of four technical replicates ± SE of *S. viridis* measured on crude leaf extracts using membrane inlet mass spectrometry. The solid line is the modeled temperature response from this report (Table I), with the 95% confidence interval in gray. Dashed lines are temperature responses from previous reports normalized to *S. viridis* at 25°C (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). The *P* value refers to the significance of the temperature response from zero, and the adjusted R^2 value describes the amount of variation in the measured parameter explained by the model.

know the temperature dependency of both reactions in order to model C_4 photosynthesis in response to temperature. Rubisco kinetic parameters have been measured in numerous C_3 and C_4 species at 25°C (Yeoh et al., 1980; Sage and Seemann, 1993; Kubien et al., 2008; Galmés et al., 2014); however, there are few studies that measure both Rubisco carboxylation and oxygenation parameters from C_4 species (Kubien et al., 2008; Cousins et al., 2010). The *S. viridis* 25°C values of Rubisco parameters are similar to previous measurements of C_4 species. Notably, the K_C value is at the higher end of measured C_4 species but similar to *Setaria italica* (Jordan and Ogren, 1983) and lower than *Setaria geniculata* (Yeoh et al., 1980). The $S_{C/O}$ was low compared with *Flaveria* C_4 spp. and maize (*Zea mays*; Kubien et al., 2008; Cousins et al., 2010) but larger than a previous measure of *S. italica* (Jordan and Ogren, 1983). However, the values reported here fit the tenuous understanding that C_4 species have higher K_C and k_{catCO2} values and lower $S_{C/O}$ values compared with C_3 species (Yeoh et al., 1980; Sage, 2002; Kubien et al., 2008; Savir et al., 2010; Whitney et al., 2011).

Currently, there are no known temperature dependencies of the full suite of Rubisco kinetics for a C_4 enzyme. However, for C_3 plants, there are two in vitro temperature responses of both carboxylase and oxygenase parameters (*A. glabriuscula* and *S. oleracea*) and three in vivo measurements from Arabidopsis and *N. tabacum* (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). These C_3 temperature responses are typically applied to C_4 Rubisco 25°C values, with the assumption that the temperature response is similar between these two photosynthetic functional types (Berry and Farquhar, 1978). However, this assumption has not been tested for the Rubisco parameters used in the C_4 model of photosynthesis, and the previous comparisons of C_3 and C_4 Rubisco temperature responses have been limited to k_{catCO2} and $S_{C/O}$ (Björkman and Pearcy, 1970; Jordan and Ogren, 1984; Sage, 2002; Galmés et al., 2015) and a recent investigation comparing k_{catCO2}, K_C, and $S_{C/O}$ between C_3, C_4, and intermediate species within the *Flaveria* lineage (Perdomo et al., 2015). However, these studies lack comparisons of the oxygenation parameters k_{catO2} and K_O needed to accurately predict the temperature response of carboxylation and oxygenation. Here, we report the temperature response of complete C_4 Rubisco kinetics from 10°C to 40°C. In general, the temperature responses of Rubisco parameters in *S. viridis* were similar to reports from previous C_3 species; however, variation exists in how individual kinetic parameters change with temperature in relation to one another.

For example, $S_{C/O}$ has been reported to decrease with temperature, but the reason for the decrease has been debated (Badger and Andrews, 1974; Jordan and Ogren, 1984; Walker et al., 2013). However, there are no consistent trends in the literature on the temperature response of V_{cmax}/V_{omax}, suggesting that the temperature

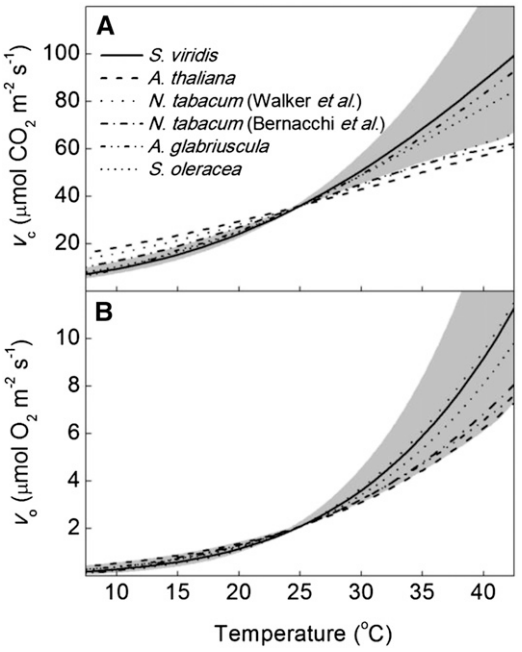

Figure 4. Temperature responses of v_c (A) and v_o (B) modeled at pCO_2 and pO_2 expected at the site of Rubisco carboxylation in a C_4 species (400 Pa of CO_2 and 35 kPa of oxygen) compared with corresponding values from the literature normalized to *S. viridis* at 25°C. The solid lines are the modeled temperature responses of *S. viridis* from this report, with the 95% confidence intervals in gray. Dashed lines are temperature responses from previous reports normalized to *S. viridis* at 25°C (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). A V_{cmax} of 60 μmol m^{-2} s^{-1} was assumed.

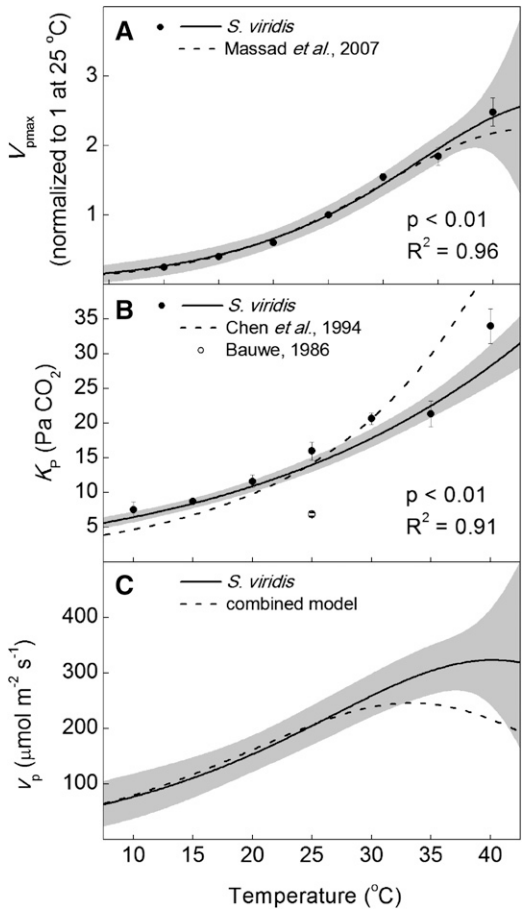

Figure 5. Temperature response of PEPc parameters in *S. viridis* compared with corresponding temperature responses from the literature normalized to *S. viridis* at 25°C. The V_{pmax} (A), K_P (B), and v_p (C) were measured on crude leaf extracts with membrane inlet mass spectrometry. Calculations of K_P assumed a constant leaf pH of 7.2 and a pKa appropriate for an ionic strength or 0.1 M (Jenkins et al., 1989), with a temperature dependency as described by Harned and Bonner (1945). The model of v_p used a measured V_{pmax} of 450 μmol m^{-2} s^{-1} at 25°C and a constant mesophyll pCO_2 of 12 Pa. The solid lines are the modeled temperature responses from this report (Table I), with 95% confidence intervals in gray. Dotted lines are the temperature responses for V_{pmax} for maize (Massad et al., 2007) and for K_P (Chen et al., 1994) normalized to *S. viridis* at 25°C. Black circles are the means of three biological replicates ± SE, and the white circle in B is the K_P reported previously for maize ± SE (Bauwe, 1986). *P* values refer to the significance of the temperature response from zero, and adjusted R^2 values describe the amount of variation in the measured parameter explained by the model.

responses of V_{cmax} and V_{omax} are nearly identical (Badger and Andrews, 1974; Jordan and Ogren, 1984; Bernacchi et al., 2001; Walker et al., 2013). Additionally, there appears to be very little V_{cmax}/K_C temperature dependency (Fig. 2). The decrease of K_O/K_C and increase of V_{omax}/K_O with temperature are consistent between studies (Fig. 2; Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013). Taken together, it is likely that the insensitivity of K_O to temperature drives the decrease in specificity in Rubisco, because between species and measurement methods, the temperature responses of V_{cmax}, V_{omax}, and K_C are similar and are higher compared with K_O (Badger and Collatz, 1977; Jordan and Ogren, 1984; Bernacchi et al., 2001, 2002; Walker et al., 2013).

For comparing the predicted v_c and v_o, the values were plotted in units of mol CO_2 mol^{-1} site s^{-1}, so that a comparison of the predicted tradeoff of k_{catCO2} and K_C between C_3 and C_4 species could be analyzed with temperature. Because no k_{catCO2} values in these units are

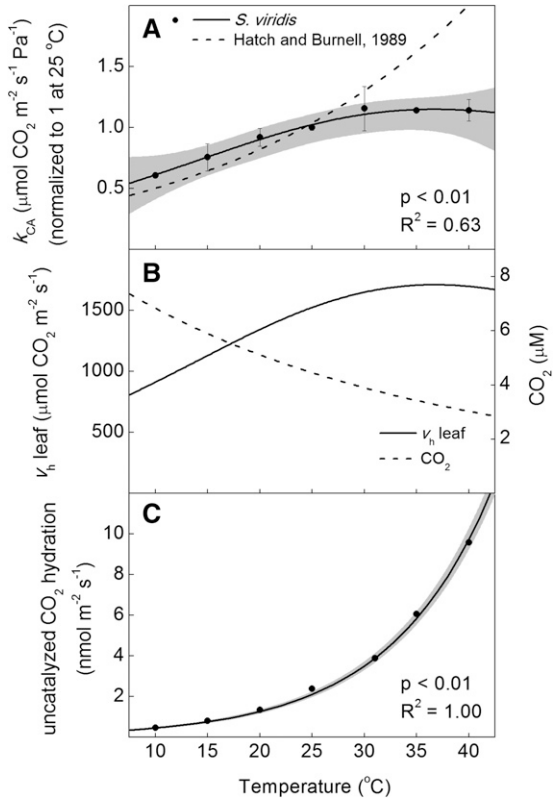

Figure 6. Temperature responses of CA parameters in *S. viridis*. A, The k_{CA} was measured on crude leaf extracts using membrane inlet mass spectrometry and is compared with a previously published maximum hydration rate from maize (Hatch and Burnell, 1990) normalized at 25°C. B, The k_{CA} from *S. viridis* was used to calculate leaf CA activity (v_h; solid line) assuming a constant 12 Pa of CO_2 above the liquid phase and the temperature-dependent change in dissolved CO_2 (dashed line). C, Temperature responses of the uncatalyzed CO_2 v_h for the mesophyll cytosol calculated assuming a cytosol volume of 30 μL mg^{-1} chlorophyll (Badger and Price, 1994) and 200 mg chlorophyll m^{-2} leaf tissue at a constant 12 Pa of CO_2. Black circles represent average values of three biological replicates, each with three technical replicates per temperature, ± SE (n = 3). The solid lines represent models fit to measured data (Table I), with the 95% confidence intervals shown in gray. Calculations of the catalyzed and uncatalyzed v_h account for changes in pH and CO_2 that occurred when measuring at different temperatures (Supplemental Fig. S1); however, it was assumed that the pH of the mesophyll cytosol was buffered at 7.2. *P* values refer to the significance of the temperature response from zero, and adjusted R^2 values describe the amount of variation in the measured parameter explained by the model.

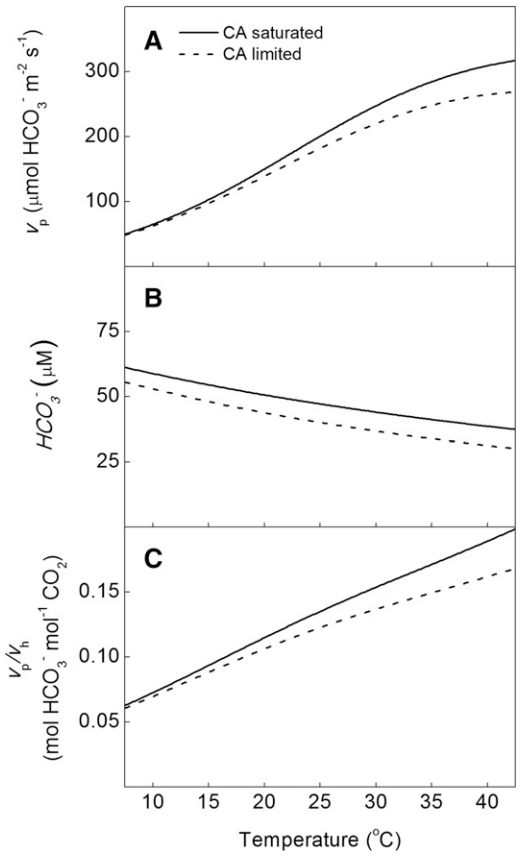

Figure 7. Effects of CA activity on PEPc activity with temperature. A, v_p modeled assuming chemical equilibrium (CA saturated) and calculated based on CA activity (CA limited). B, Concentration of HCO_3^- at equilibrium and calculated based on CA activity. C, v_p/v_h calculated assuming equilibrium and based on CA activity. Calculations assume a constant leaf pH of 7.2 and a 25°C hydration as well as dehydration rate constants described by Jenkins et al. (1989), with the temperature responses for the rate constants described by Sanyal and Maren (1981) applied. The model used a measured V_{pmax} of 450 μmol m^{-2} s^{-1}, a measured k_{CA} of 124 μmol m^{-2} s^{-1} Pa^{-1} at 25°C, and a constant mesophyll pCO_2 of 12 Pa at all temperatures.

available for three of the previous four temperature response publications, the in vitro value of 3.3 mol CO_2 mol^{-1} site s^{-1} measured by Walker et al. (2013) for both Arabidopsis and *N. tabacum* was applied to all species except *S. viridis*. At low pCO_2, the previously reported C_3 Rubisco has higher v_c compared with the *S. viridis* enzyme, with the exception of *A. glabriuscula*, because the K_C for *A. glabriuscula* at 25°C is 3 times larger than for other C_3 enzymes included in this analysis. However, at high pCO_2, the *S. viridis* Rubisco has a slightly higher v_c compared with the C_3 enzymes. The minimal difference of v_c observed between C_3 and *S. viridis* Rubisco at high pCO_2 is likely the result of the high K_C measured in *S. viridis*.

The absolute values of kinetic parameters measured at 25°C for *S. viridis* are similar to previously published values from C_4 plants, which generally differ from C_3 parameters (Yeoh et al., 1980; von Caemmerer and Quick, 2000; Kubien et al., 2008; Savir et al., 2010; Whitney et al., 2011). However, there is less support in the literature for differences in the temperature response between C_3 and C_4 Rubisco (Jordan and Ogren, 1984; Sage, 2002; Galmés et al., 2015; Perdomo et al., 2015). The Rubisco temperature responses of *S. viridis* presented here are similar to the available temperature responses of C_3 species, suggesting a generally conserved variation in kinetic parameters. While the temperature response of Rubisco is becoming increasingly well studied, less work has focused on PEPc kinetics, especially the temperature response of K_P.

PEPc Temperature Response

The temperature response of V_{pmax} presented here (Fig. 5) was similar to measurements from other species (Buchanan-Bollig et al., 1984; Wu and Wedding, 1987; Chen et al., 1994; Massad et al., 2007), which showed a change in the temperature response above 25°C. While this change in the temperature response has been observed previously, the magnitude varies between studies and species (Buchanan-Bollig et al., 1984; Wu and Wedding, 1987; Chen et al., 1994; Massad et al., 2007). It is unclear if there are differences in the temperature response of V_{pmax} between species, because there are not sufficient comparisons made within a single study.

The K_P is important for modeling the response of PEPc to changes in HCO_3^- availability. However, the temperature response of K_P has been left out of models of C_4 photosynthesis (Berry and Farquhar, 1978; von Caemmerer, 2000) or a predicted temperature response has been used (Chen et al., 1994; Massad et al., 2007). This is in contrast to the incorporation of measured Rubisco temperature responses that have been used in the C_4 model (Berry and Farquhar, 1978). The temperature response of K_P measured here was lower than a predicted temperature response (Fig. 5; Chen et al., 1994); however, it should be noted that the temperature response from Chen et al. (1994) was not actually measured but selected to have a 2.1-fold change for every 10°C (Q_{10}). Because the temperature dependency of V_{pmax} is similar between this study and Massad et al. (2007), the effect of differences in K_P temperature response can be seen on modeled v_p in Figure 5, where there is an 80 μmol m^{-2} s^{-1} increase in the optimum occurring 5°C higher. This shift in modeled v_p suggests that the use of the measured K_P presented here is important for estimates of V_{pmax} values based on leaf gas exchange.

CA Temperature Response

CA activity is considered necessary for C_4 photosynthesis because it provides PEPc with HCO_3^- by facilitating the hydration of dissolved CO_2 in the mesophyll cytosol (von Caemmerer et al., 2004).

Additionally, the v_p/v_h can influence the isotopic CO_2 exchange in C_4 plants (Farquhar, 1983). However, the activity of CA is not included in models of C_4 photosynthesis and is generally thought to have little effect on $^{13}CO_2$ isotope exchange, because v_p/v_h is assumed to be low (Berry and Farquhar, 1978; Farquhar, 1983; von Caemmerer, 2000). However, in some C_4 grass species, leaf activity (leaf v_h) is reported to be just sufficient to maintain rates of C_4 photosynthesis at 25°C (Hatch and Burnell, 1990; Cousins et al., 2008). Therefore, under conditions that limit leaf CO_2 availability, the rate of leaf v_h may restrict C_4 photosynthesis and influence leaf CO_2 isotope exchange. This limitation may be particularly important at temperatures higher than 25°C; however, the temperature response of leaf v_h is poorly understood.

The measured 25°C values of leaf v_h and the first-order k_{CA} in *S. viridis* are 4-fold higher than previously published values for other C_4 grasses (Cousins et al., 2008). Published CA activity varies widely between studies, species, tissue collection methods, and growth conditions (Hatch and Burnell, 1990; Gillon and Yakir, 2001; Affek et al., 2006; Cousins et al., 2008). This variation is poorly understood; however, it raises questions regarding CA responses to changes in growth conditions, particularly those that limit CO_2 availability to the leaf such as drought and temperature.

The measured temperature response of k_{CA} from 10°C to 25°C was similar to the 1.9-fold change for every 10°C (Q_{10}) measured from 0°C to 17°C and intermediate to two isozymes of human CA measured from 0°C to 37°C, but it deviated at 30°C and above (Fig. 6A; Sanyal and Maren, 1981; Burnell and Hatch, 1988). The CA temperature response reported here plateaued between 25°C and 40°C, suggesting a deactivation of CA activity in *S. viridis* at temperatures above 25°C (Fig. 1). This apparent deactivation was not observed in previous studies (Sanyal and Maren, 1981; Burnell and Hatch, 1988).

As expected for a chemical reaction, the uncatalyzed rate of CO_2 hydration increases exponentially (Fig. 1). The E_a reported here for the uncatalyzed rate constant of CO_2 hydration at pH 8 was larger than that measured previously for pH 7.2 (Sanyal and Maren, 1981). Following the assumptions of Badger and Price (1994) regarding the volume of the mesophyll cytoplasm per unit of leaf area, the amount of the uncatalyzed CO_2 hydration estimated in the mesophyll cytoplasm was 10^5 times lower than the leaf v_h. The importance of the uncatalyzed rate when modeling isotopic discrimination is unclear and should be investigated further, but the estimated uncatalyzed rates presented here are insufficient to support rates of photosynthesis at any temperature (Fig. 6).

Using the steady-state model of Hatch and Burnell (1990), HCO_3^- concentration and v_p were modeled using the temperature responses of k_{CA}, V_{pmax}, and K_P measured for *S. viridis* assuming a constant mesophyll pCO_2 of 12 Pa. This model was compared with the assumption of current gas-exchange models that assume HCO_3^- is at chemical equilibrium with CO_2 in the mesophyll cytosol (Fig. 7). The results show that CA limits v_p by more than 5% above 15°C when compared with the full equilibrium model due to reduced HCO_3^- availability to PEPc (Fig. 7). These results are contradictory to the assumptions of gas-exchange models, which assume that CA activity is high enough to maintain full chemical equilibrium between CO_2 and HCO_3^-, and may have important implications for comparing models and measurements of C_4 photosynthesis. For example, in wild-type maize plants presented by Studer et al. (2014), there is an 80% reduction in in vivo compared with in vitro V_{pmax}. The in vivo value is calculated from the initial slope of net CO_2 assimilation against intercellular CO_2 concentration (A-C_i curve); therefore, the lower V_{pmax} calculated from an A-C_i curve is possibly driven by inaccurate estimates of the conductance of CO_2 from the intercellular air space to the mesophyll cytosol (g_m) and the proposed disequilibrium between CO_2 and HCO_3^- due to limiting CA activity. Both of these factors would lower the initial slope of an A-C_i curve and, therefore, in vivo estimates of $V_{pmax.}$

The CA limitation in wild-type maize is initially hard to reconcile with evidence from CA mutants showing little change in net CO_2 assimilation, except for homozygous mutants at subambient CO_2 (Studer et al., 2014). However, using the model of Hatch and Burnell (1990) combined with the PEPc-limited models of net CO_2 assimilation (von Caemmerer, 2000) and the in vitro data set presented by Studer et al. (2014), the predicted apparent in vivo V_{pmax} in the wild-type, heterozygous, and homozygous plants are 34, 33, and 13 μmol m^{-2} s^{-1}, respectively. These values are similar to the gas-exchange estimates of in vivo V_{pmax} presented by Studer et al. (2014), reanalyzed here using just the linear portion of the A-C_i curves as 36 ± 2, 34 ± 2, and 16 ± 1 for the wild-type, heterozygous, and homozygous plants, respectively. This demonstrates that the in vitro data of CA and PEPc activities used with the Hatch and Burnell (1990) model and the PEPc-limited models of net CO_2 assimilation (von Caemmerer, 2000) can accurately predict subtle changes in leaf gas exchange measured in plants with low CA.

Although the difference between in vivo and in vitro PEPc can generally be predicted in the example presented above, there are still unknown factors that potentially regulate in vivo PEPc activity. Of critical importance for estimating in vivo V_{pmax} are the values of g_m; however, there are no robust means for measuring g_m in C_4 plants, which also limits the accuracy of defining a CA limitation to gas-exchange measurements. Therefore, more work is needed to define the importance of CA in C_4 photosynthesis, especially in linking leaf-level gas exchange to biochemical models in order to better define carbon flux through the C_4 pathway.

CONCLUSION

Rubisco, PEPc, and CA are potentially rate-limiting steps in the photosynthetic assimilation of atmospheric CO_2 in C_4 plants. Therefore, the activity and kinetic properties of these enzymes are needed to accurately parameterize biochemical models of leaf CO_2 exchange in response to changes in CO_2 and temperature. To address this issue, the temperature responses of Rubisco carboxylation and oxygenation kinetics, PEPc carboxylation kinetics, and CA hydration reaction from the C_4 plant *S. viridis* (succession no., A-010) were analyzed using a membrane inlet mass spectrometer. These findings suggest that the C_4 Rubisco of *S. viridis* has a similar temperature response to previously measured C_3 Rubisco, the K_P of PEPc increases with temperature, and, although modeling shows that CA limits HCO_3^- availability to PEPc in *S. viridis*, it also supports previous findings that large changes in CA activity have minimal effect on net CO_2 assimilation. These results advance our understanding of the temperature response of CO_2 assimilation in C_4 plants and help to better parameterize the models of C_4 photosynthesis. However, more work is needed, including leaf gas-exchange and isotopic measurements, to determine the importance and implications of CA limitation to C_4 photosynthesis.

MATERIALS AND METHODS

Growth Conditions

Seeds of *Setaria viridis* (A-010) were planted in 7.5-L pots with LC-1 Sunshine mix (Sun Gro Horticulture). Plants were grown in environmental growth chambers (Enconair Ecological GC-16) with a temperature of 28°C/18°C day/night, photoperiod of 16/8 h day/night, and light intensity of 1,000 μmol quanta m^{-2} s^{-1} at canopy level using 50% 400-W high-pressure sodium and 50% 400-W metal halide lighting. Relative humidity was not controlled and varied from 30% to 85% during the life of the plants as measured by the growth chamber. Plants were watered as needed and fertilized twice per week using Peters 20-20-20 (J.R. Peters) and supplemented with Sprint 330 iron chelate (BASF) as needed. Plants were grown for 6 weeks after germination and sampled periodically between 3 and 6 weeks.

Sample Preparation

CA Extraction

Leaf discs were extracted on ice in a glass homogenizer in 1 mL of 50 mM HEPES (pH 7.8), 1% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM EDTA, 10 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, and 2% (v/v) protease inhibitor cocktail (P9599; Sigma-Aldrich). Crude extracts were centrifuged at 4°C for 1 min at 17,000*g*, and the supernatant was collected for immediate use in the CA assay.

PEPc Extraction

The midveins of youngest fully expanded leaves were removed, and the remaining tissue was cut into small pieces prior to grinding to a fine powder in liquid nitrogen using mortar and pestle. The frozen powder was transferred to an ice-cooled mortar containing extraction buffer and ground on ice using a pestle until fully homogenized. Approximately 2 g of leaf tissue was ground in 4 mL of 100 mM HEPES (pH 7.8), 10 mM DTT, 25 mM $MgCl_2$, 1 mM EDTA, 10 mM $NaHCO_3$, 1% (w/v) PVPP, 0.5% (v/v) Triton, 33 μL of protease inhibitor cocktail (Sigma-Aldrich) was used for every gram of leaf tissue used, and sand. The extract was spun at 17,000*g* for 10 min at 4°C. The supernatant was collected and desalted using an Econo Pac 10DG column (Bio-Rad), filtered through a Millex GP 33-mm syringe-driven filter unit (Millipore), and then centrifuged using Amicon Ultra Ultracel 30K centrifugal filters (Millipore) at 4°C for 1 h at 4,000 rpm (3,000*g*). The layer maintained above the filter unit was collected, brought to 20% (v/v) glycerol, flash frozen in liquid nitrogen, and stored at −80°C until measured. Three individual plants were used, with one extraction per plant.

Rubisco Extraction

Rubisco extraction followed that of PEPc except that most Rubisco activity was found to be in the green layer of the pellet and not the supernatant following the first centrifugation. The supernatant was discarded, and the upper portion of the pellet was collected, avoiding sand and PVPP. The pellet was resuspended in extraction buffer and spun again at 4°C, for 9 s, up to 17,000*g* to remove large particulates. The supernatant was collected and run through Econo Pac 10DG desalting columns (Bio-Rad), and the dark green fraction was collected and centrifuged at 4°C, for 10 min, at 17,000*g*. Although no pellet formed, a gradient in color, from light green at the top of the tube to dark green at the bottom, was noted. Most Rubisco activity was found to be in the bottom half of the sample. The upper light green portion of the sample was discarded to concentrate Rubisco. The sample was brought to 20% (v/v) glycerol, flash frozen in liquid nitrogen, and stored at −80°C until measured. After being thawed for use, the extract was allowed to activate in 15 mM $MgCl_2$ and 15 mM $NaHCO_3$ for 10 min at room temperature before being placed on ice for the duration of the measurement. Measurements for a single oxygen level lasted approximately 1 h, and a single aliquot was used per oxygen level. A new aliquot was thawed for each of the four oxygen levels.

Extraction for Spectrophotometry

Rubisco activity and PEPc activity at 25°C were measured on freshly collected leaf discs homogenized on ice in 1 mL of 100 mM HEPES (pH 7.8), 1% (w/v) PVPP, 1 mM EDTA, 10 mM DTT, 0.1% (v/v) Triton, and 1% (v/v) protease inhibitor cocktail using a glass homogenizer. The extract was then centrifuged at 17,000*g* for 1 min at 4°C, and the supernatant was collected. The supernatant was activated in 15 mM $MgCl_2$ and 15 mM $NaHCO_3$ for 10 min at room temperature and placed on ice.

Membrane Inlet Mass Spectrometry

CA Measurements

CA activity was measured using a membrane inlet mass spectrometer to measure the rates of $^{18}O_2$ exchange from labeled $^{13}C^{18}O_2$ to $H_2{}^{16}O$ with a total carbon concentration of 1 mM (Silverman, 1982; Badger and Price, 1989; Hatch and Burnell, 1990). The hydration rates were calculated from the enhancement in the rate of ^{18}O loss over the uncatalyzed rate with the nonenzymatic first-order rate constant for the hydration of CO_2 calculated for the assay pH 8.03 at 25°C using the equation from Jenkins et al. (1989). The measured temperature response was applied to the 25°C rate constant correcting for the change in assay pH at each temperature (Supplemental Fig. S3). The CO_2 concentration was calculated using the temperature-appropriate pKa assuming an ionic strength of 0.1 M (Harned and Bonner, 1945), and the pCO_2 was calculated using the temperature-appropriate Henry's constant (Sander, 2015). The temperature response was determined on three biological replicates separately extracted and measured three times (technical replication) from frozen leaf tissue in 5°C increments from 10°C to 40°C. Total leaf CA activity at 25°C was determined from four biological replicates measured on fresh tissue.

PEPc and Rubisco Measurements

PEPc and Rubisco assays were conducted in a 600-μL temperature-controlled cuvette linked to a mass spectrometer as described by Cousins et al. (2010). CO_2 and oxygen calibrations were made daily at the measurement temperature as described by Cousins et al. (2010), with the exception that no oxygen-zero or membrane consumption was determined, because it was assumed to be accounted for in the blank rate made immediately prior to measurement of the enzymatic rate. To determine the oxygen concentration dissolved in water during equilibrium with air, the temperature dependency of the Henry's constant for oxygen was taken into account given the measurement temperature. Because calibrations account for the concentrations of CO_2 and

total inorganic carbon, the concentration of HCO_3^- was calculated as the difference between total inorganic carbon and CO_2.

Measurements of PEPc bicarbonate kinetics were similar to the method presented by Cousins et al. (2010), except that only one oxygen level (approximately 80 μM) was used with five HCO_3^- concentrations ranging from 0 to 3,000 μM. The assay buffer contained 200 mM HEPES (pH 7.8; measured at 25°C), 20 mM $MgCl_2$, 8 μg mL^{-1} CA, 5 mM Glc-6-P, and 4 mM PEP. For all measurement points, except the lowest HCO_3^-, 30 s were sampled prior to the initiation of the reaction as the blank rate; following a 30-s mixing period after the injection of PEP to initiate the reaction, the next 30 s were sampled as the enzymatic rate (Fig. 1). The blank rate was subtracted from the enzymatic rate. For the lowest HCO_3^- concentration, the slope was found to change rapidly over the course of 30 s; therefore, the slope was calculated every 5 s during the 30-s interval. Three biological replicates were measured in 5°C increments from 10°C to 40°C.

Measurements of Rubisco kinetics were made following the methods of Cousins et al. (2010). Samples were measured at four oxygen concentrations ranging from 40 to 1,600 μM, and five CO_2 concentrations ranging from 10 to 200 μM at each oxygen level, for a total of 20 data points per measurement. Measurements were made in 5°C intervals from 10°C to 40°C, and four replicates were measured per temperature. The assay buffer contained 200 mM HEPES, 20 mM $MgCl_2$, 0.1 mM α-hydroxypyridinemethanesulfonic acid, 8 μg mL^{-1} CA, and 0.6 mM RuBP. A total of 20 to 100 μL of extract was added per measurement depending on the Rubisco concentration of the extract. One measurement initiating the reaction with RuBP was made at each oxygen concentration to test for any non-RuBP-dependent consumption of CO_2 and oxygen. Non-RuBP-dependent consumption of CO_2 and oxygen was considered negligible because it did not have a significant effect on the final calculations of Rubisco kinetic parameters.

For PEPc measurements, v_p and HCO_3^- concentration ($[HCO_3^-]$) were fit to the equation:

$$v_p = \frac{V_{pmax}[HCO_3^-]}{[HCO_3^-] + K_P} \quad (1)$$

solving for V_{pmax} and K_P. For Rubisco measurements, v_c and v_o were normalized by dividing all measured values by their average to give uniform weight during the fitting calculation. The normalized v_c and v_o, and corresponding CO_2 concentration ($[CO_2]$) and oxygen concentration ($[O_2]$), were fit simultaneously to the following equations:

$$v_c = \frac{V_{cmax}[CO_2]}{[CO_2] + K_C(1 + O_2/K_O)} \quad (2)$$

$$v_o = \frac{V_{omax}[O_2]}{[O_2] + K_O(1 + CO_2/K_C)} \quad (3)$$

solving for the parameters K_C and K_O. This was repeated using the nontransformed v_c and v_o values while holding K_C and K_O constant and solving for V_{cmax} and V_{omax}. The normalizing of data before solving for K_C and K_O improved the fits for v_o. The K_C and K_O data obtained this way match the methods of plotting the apparent K_C against oxygen concentration (von Caemmerer et al., 1994). All model fits were performed in the software package Origin 8 (OriginLab) using the nonlinear curve-fit function NLfit.

Enzyme Quantification

Rubisco Quantification

Rubisco activity was determined by enzyme-coupled spectrophotometry, monitoring the conversion rate of NAD (NADH) to NAD^+ at 340 nm (Evolution 300 UV-Vis; Thermo Fisher Scientific). The 1-mL assay contained 100 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS; pH 8 with NaOH), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 5 mM creatine phosphate, 20 mM $NaHCO_3$, 0.2 mM NADH, 12.5 units mL^{-1} creatine phosphokinase, 250 units mL^{-1} CA, 22.5 units mL^{-1} 3-phosphoglyceric phosphokinase, 20 units mL^{-1} glyceraldehyde 3-phosphate dehydrogenase, 56 units mL^{-1} triose-phosphate isomerase, 20 units mL^{-1} glycerol-3-phosphate dehydrogenase, and 10 μL of fresh leaf extract. The reaction was initiated with 0.5 mM RuBP. Rubisco content was determined from the stoichiometric binding of radiolabeled [^{14}C]carboxyarabinitolbisphosphate (^{14}CABP; Collatz et al., 1979; Walker et al., 2013). For ^{14}CABP binding assays, the extract was incubated in 1 mM ^{14}CABP for 45 min at room temperature and then passed through a chromatography column (737-4731; Bio-Rad) packed with Sephadex G-50 fine beads (GE Healthcare Biosciences). Rubisco content was determined from the Rubisco-bound fractions using a scintillation counter and the specific activity of the ^{14}CABP (Collatz et al., 1979; Walker et al., 2013).

The extract used to measure Rubisco kinetics was too viscous to move through the columns used for ^{14}CABP site quantification. To normalize the temperature response of V_{cmax} and V_{omax} from the membrane inlet mass spectrometry measurements, each extract was measured at 25°C for V_{cmax} using enzyme-coupled spectrophotometry. The 25°C V_{cmax} value was used to normalize rates obtained from 10°C to 40°C using membrane inlet mass spectrometry. There was no significant difference between the 25°C values measured on the spectrophotometer compared with the membrane inlet mass spectrometer.

PEPc Quantification

A similar enzyme-coupled spectrophotometric assay as described above for Rubisco was conducted for PEPc to determine the leaf-level V_{pmax}. The 1-mL assay contained 100 mM EPPS (pH 8 NaOH), 20 mM $MgCl_2$, 1 mM EDTA, 5 mM Glc-6-P, 1 mM $NaHCO_3$, 0.2 mM NADH, 12 units of malate dehydrogenase, and 10 μL of freshly collected leaf extract. The reaction was initiated with 4 mM PEP.

Modeling the Temperature Response

The temperature responses of the kinetic parameters k_h (for the first order rate constant for the hydration of CO_2), K_P, V_{cmax}, V_{omax}, K_C, K_O, and $S_{C/O}$ were fit to the following equation:

$$\text{parameter} = k_{25}\ e^{E_a\,(T_k - 298.15)/(298.15RT_k)} \quad (4)$$

where k_{25} is the value of the parameter at 25°C and R is the molar gas constant (Badger and Collatz, 1977). The fit was calculated by taking the natural log of the data plotted against $(T_k - 298.15)/T_k$ using the linear function in Origin 8, such that the intercept was equal to $\ln(k_{25})$ and the slope was equal to $E_a/(298.15R)$. The temperature responses of k_{CA} and V_{pmax} were modeled to include the heat of deactivation (H_d) and entropy factor (ΔS) using the following equation (Farquhar et al., 1980; Leuning, 1997):

$$\text{parameter} = k_{25}\ e^{E_a\,(T_k - 298.15)/(298.15RT_k)}\frac{1 + e^{(298.15\Delta S - H_d)/(298.15R)}}{1 + e^{(T_k\Delta S - H_d)/(T_kR)}} \quad (5)$$

This model fitting was performed using the nonlinear curve-fit function NLfit in Origin 8.

Modeling the Supply of HCO_3^- by CA

The model of Hatch and Burnell (1990) was used to calculate the CA-limited model (Fig. 7). The rates of PEP carboxylation and HCO_3^- concentration were calculated by solving for the following equations:

$$v_h = pCO_2 k_{CA} \quad (6)$$

$$v_p = \frac{[HCO_3^-]V_{pmax}}{K_P + [HCO_3^-]} \quad (7)$$

$$v_d = v_h - v_p \quad (8)$$

$$\frac{v_d}{v_h} = \frac{k_r[HCO_3^-]}{k_f[CO_2]} \quad (9)$$

$$pCO_2 = K_H[CO_2] \quad (10)$$

where v_d is the rate of HCO_3^- dehydration catalyzed by CA, k_r is the uncatalyzed rate constant for the reverse reaction, k_f is the uncatalyzed rate constant for the forward reaction, and K_H is the Henry's law constant used to determine the concentration of CO_2 for any given pCO_2 at the appropriate temperature. The pCO_2 was held constant at 12 Pa. The uncatalyzed rates of the forward and reverse reactions at 25°C and pH 7.2 were calculated using the following equations from Jenkins et al. (1989):

$$k_f = 6.22 \times 10^{-11}/[H^+] + 3.8 \times 10^{-2} \quad (11)$$

$$k_r = 2 \times 10^{-4} + 4.96 \times 10^{4}[H^+] \quad (12)$$

where the temperature response of k_f and k_r measured by Sanyal and Maren (1981) for pH 7.2 was applied.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Non-normalized temperature response of v_c and v_o.

Supplemental Figure S2. Measurement of PEPc kinetics.

Supplemental Figure S3. Measurement of CA parameters.

ACKNOWLEDGMENTS

We thank Murray Badger and Connor Bollinger-Smith for helping to establish our membrane inlet mass spectrometry protocol for Rubisco, Chuck Cody for maintaining plant growth facilities, and Susanne von Caemmerer and past and current members of Cousins laboratory for helpful and insightful discussions.

Received April 20, 2015; accepted September 15, 2015; published September 15, 2015.

LITERATURE CITED

Affek HP, Krisch MJ, Yakir D (2006) Effects of intraleaf variations in carbonic anhydrase activity and gas exchange on leaf $C^{18}OO$ isoflux in *Zea mays*. New Phytol **169:** 321–329

Badger MR, Andrews TJ (1974) Effects of CO_2, O_2 and temperature on a high-affinity form of ribulose diphosphate carboxylase-oxygenase from spinach. Biochem Biophys Res Commun **60:** 204–210

Badger MR, Collatz GJ (1977) Studies on the kinetic mechanism of ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. Carnegie Inst Wash Year Book **76:** 355–361

Badger MR, Price GD (1989) Carbonic anhydrase activity associated with the cyanobacterium *Synechococcus* PCC7942. Plant Physiol **89:** 51–60

Badger MR, Price GD (1994) The role of carbonic anhydrase in photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **45:** 369–392

Bauwe H (1986) An efficient method for the determination of K_m values for HCO_3^- of phosphoenolpyruvate carboxylase. Planta **169:** 356–360

Bernacchi C, Singsaas E, Pimentel C, Portis A Jr, Long S (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant Cell Environ **24:** 253–259

Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP (2002) Temperature response of mesophyll conductance: implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiol **130:** 1992–1998

Berry J, Farquhar G (1978) The CO_2 concentrating function of C_4 photosynthesis: a biochemical model. *In* D Hall, J Coombs, T Goodwin, eds, Proceedings of the 4th International Congress on Photosynthesis. Biochemical Society, London, pp 119–131

Björkman O, Pearcy RW (1970) Effect of growth temperature on the temperature dependence of photosynthesis *in vivo* and on CO_2 fixation by carboxydismutase *in vitro* in C_3 and C_4 species. Carnegie Inst Wash Year Book **70:** 511–520

Buchanan-Bollig IC, Kluge M, Müller D (1984) Kinetic changes with temperature of phosphoenolpyruvate carboxylase from a CAM plant. Plant Cell Environ **7:** 63–70

Burnell JN, Hatch MD (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C_4 pathway operation. Plant Physiol **86:** 1252–1256

Chen DX, Coughenour M, Knapp A, Owensby C (1994) Mathematical simulation of C_4 grass photosynthesis in ambient and elevated CO_2. Ecol Modell **73:** 63–80

Collatz G, Badger M, Smith C, Berry J (1979) A radioimmune assay for RuP_2 carboxylase protein. Carnegie Inst Wash Year Book **78:** 171–175

Cousins AB, Badger MR, von Caemmerer S (2008) C_4 photosynthetic isotope exchange in NAD-ME- and NADP-ME-type grasses. J Exp Bot **59:** 1695–1703

Cousins AB, Ghannoum O, von Caemmerer S, Badger MR (2010) Simultaneous determination of Rubisco carboxylase and oxygenase kinetic parameters in *Triticum aestivum* and *Zea mays* using membrane inlet mass spectrometry. Plant Cell Environ **33:** 444–452

Edwards G, Walker DA (1983) C_3, C_4: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis. Blackwell Scientific Publications, Oxford

Farquhar GD (1983) On the nature of carbon isotope discrimination in C_4 species. Aust J Plant Physiol **10:** 205–226

Farquhar GD, von Caemmerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta **149:** 78–90

Galmés J, Kapralov MV, Andralojc PJ, Conesa MA, Keys AJ, Parry MA, Flexas J (2014) Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends. Plant Cell Environ **37:** 1989–2001

Galmés J, Kapralov MV, Copolovici LO, Hermida-Carrera C, Niinemets Ü (2015) Temperature responses of the Rubisco maximum carboxylase activity across domains of life: phylogenetic signals, trade-offs, and importance for carbon gain. Photosynth Res **123:** 183–201

Gillon J, Yakir D (2001) Influence of carbonic anhydrase activity in terrestrial vegetation on the ^{18}O content of atmospheric CO_2. Science **291:** 2584–2587

Harned HS, Bonner FT (1945) The first ionization of carbonic acid in aqueous solutions of sodium chloride. J Am Chem Soc **67:** 1026–1031

Hatch MD, Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step of C_4 photosynthesis. Plant Physiol **93:** 825–828

Jenkins CL, Furbank RT, Hatch MD (1989) Mechanism of C_4 photosynthesis: a model describing the inorganic carbon pool in bundle sheath cells. Plant Physiol **91:** 1372–1381

Jordan DB, Ogren WL (1983) Species variation in kinetic properties of ribulose 1,5-bisphosphate carboxylase/oxygenase. Arch Biochem Biophys **227:** 425–433

Jordan DB, Ogren WL (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase: dependence on ribulosebisphosphate concentration, pH and temperature. Planta **161:** 308–313

Kubien DS, Whitney SM, Moore PV, Jesson LK (2008) The biochemistry of Rubisco in *Flaveria*. J Exp Bot **59:** 1767–1777

Leuning R (1997) Scaling to a common temperature improves the correlation between the photosynthesis parameters J_{max} and V_{cmax}. J Exp Bot **48:** 345–347

Massad RS, Tuzet A, Bethenod O (2007) The effect of temperature on C_4-type leaf photosynthesis parameters. Plant Cell Environ **30:** 1191–1204

Perdomo JA, Cavanagh AP, Kubien DS, Galmés J (2015) Temperature dependence of in vitro Rubisco kinetics in species of *Flaveria* with different photosynthetic mechanisms. Photosynth Res **124:** 67–75

Sage RF (2002) Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot **53:** 609–620

Sage RF, Seemann JR (1993) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in response to reduced light intensity in C_4 plants. Plant Physiol **102:** 21–28

Sander R (2015) Compilation of Henry's law constants (version 4.0) for water as solvent. Atmos Chem Phys **15:** 4399–4981

Sanyal G, Maren TH (1981) Thermodynamics of carbonic anhydrase catalysis: a comparison between human isoenzymes B and C. J Biol Chem **256:** 608–612

Savir Y, Noor E, Milo R, Tlusty T (2010) Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proc Natl Acad Sci USA **107:** 3475–3480

Silverman DN (1982) Carbonic anhydrase: oxygen-18 exchange catalyzed by an enzyme with rate-contributing proton-transfer steps. Methods Enzymol **87:** 732–752

Studer AJ, Gandin A, Kolbe AR, Wang L, Cousins AB, Brutnell TP (2014) A limited role for carbonic anhydrase in C_4 photosynthesis as revealed by a *ca1ca2* double mutant in maize. Plant Physiol **165:** 608–617

von Caemmerer S (2000) Biochemical Models of Leaf Photosynthesis. CSIRO Publishing, Collingwood, Australia

von Caemmerer S (2013) Steady-state models of photosynthesis. Plant Cell Environ **36:** 1617–1630

von Caemmerer S, Evans JR, Hudson GS, Andrews TJ (1994) The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. Planta **195:** 88–97

von Caemmerer S, Quick WP (2000) Rubisco: physiology in vivo. *In* RC Leegood, TD Sharkey, S von Caemmerer, eds, Photosynthesis: Physiology and Metabolism. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 85–113

von Caemmerer S, Quinn V, Hancock N, Price G, Furbank R, Ludwig M (2004) Carbonic anhydrase and C_4 photosynthesis: a transgenic analysis. Plant Cell Environ **27:** 697–703

Walker B, Ariza LS, Kaines S, Badger MR, Cousins AB (2013) Temperature response of in vivo Rubisco kinetics and mesophyll conductance in *Arabidopsis thaliana*: comparisons to *Nicotiana tabacum*. Plant Cell Environ **36:** 2108–2119

Whitney SM, Houtz RL, Alonso H (2011) Advancing our understanding and capacity to engineer nature's CO_2-sequestering enzyme, Rubisco. Plant Physiol **155:** 27–35

Wu MX, Wedding RT (1987) Temperature effects on phospho*enol*pyruvate carboxylase from a CAM and a C_4 plant: a comparative study. Plant Physiol **85:** 497–501

Yeoh HH, Badger MR, Watson L (1980) Variations in $K_m(CO_2)$ of ribulose-1,5-bisphosphate carboxylase among grasses. Plant Physiol **66:** 1110–1112