Influence of temperature on measurements of the CO\textsubscript{2} compensation point: differences between the Laisk and O\textsubscript{2}-exchange methods

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Abstract

The CO\textsubscript{2} compensation point in the absence of day respiration (\(\Gamma^*\)) is a key parameter for modelling leaf CO\textsubscript{2} exchange. \(\Gamma^*\) links the kinetics of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) with the stoichiometry of CO\textsubscript{2} released per Rubisco oxygenation (\(\alpha\)), two essential components of biochemical models of photosynthesis. There are two main gas-exchange methods for measuring \(\Gamma^*\): (i) the Laisk method, which requires estimates of mesophyll conductance to CO\textsubscript{2} (\(g_m\)) and (ii) measurements of O\textsubscript{2} isotope exchange, which assume constant values of \(\alpha\) and a fixed stoichiometry between O\textsubscript{2} uptake and Rubisco oxygenation. In this study, the temperature response of \(\Gamma^*\) measured using the Laisk and O\textsubscript{2}-exchange methods was compared under ambient (25 °C) and elevated (35 °C) temperatures to determine whether both methods yielded similar results. Previously published temperature responses of \(\Gamma^*\) estimated with the Laisk and O\textsubscript{2}-exchange methods in \textit{Nicotiana tabacum} demonstrated that the Laisk-derived model of \(\Gamma^*\) was more sensitive to temperature compared with the O\textsubscript{2}-exchange model. Measurements in \textit{Arabidopsis thaliana} indicated that the Laisk and O\textsubscript{2}-exchange methods produced similar \(\Gamma^*\) at 25 °C; however, \(\Gamma^*\) values from O\textsubscript{2} exchange were lower at 35 °C compared with the Laisk method. Compared with a photorespiratory mutant (\textit{pmdh1pmdh2hpr}) with increased \(\alpha\), wild-type (WT) plants had lower Laisk values of \(\Gamma^*\) at 25 °C but were not significantly different at 35 °C. These differences between Laisk and O\textsubscript{2} exchange values of \(\Gamma^*\) at 35 °C could be explained by temperature sensitivity of \(\alpha\) in WT and/or errors in the assumptions of O\textsubscript{2} exchange. The differences between \(\Gamma^*\) measured using the Laisk and O\textsubscript{2}-exchange method with temperature demonstrate that assumptions used to measure \(\Gamma^*\), and possibly the species-specific validity of these assumptions, need to be considered when modelling the temperature response of photosynthesis.

Key words: CO\textsubscript{2} compensation point, peroxisomes, photorespiration, photorespiratory CO\textsubscript{2} release, photosynthesis, Rubisco oxygenation, temperature.

Introduction

Models of photosynthesis are important tools for predicting the response of plants to climate change. The Farquhar, von Caemmerer, and Berry biochemical model of C\textsubscript{3} photosynthesis was first parameterized to predict photosynthetic rates at 25 °C using the kinetic parameters of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), the enzyme responsible for
initiating carbon fixation (Farquhar et al., 1980). This model has proven to be robust in predicting the effects of CO₂ availability on photosynthesis at 25 °C but has been expanded to account for the temperature response of Rubisco kinetics with mixed success (Bernacchi et al., 2001, 2002, 2003; Sage et al., 2008). For example, the temperature response of the initial slope of the photosynthetic CO₂ response (A-C) curve in Picea mariana is less than that predicted by the Nicotiana tabacum Rubisco kinetic’s temperature model (Sage et al., 2008; Way and Sage, 2008). The authors attributed this deviation to a greater deactivation of Rubisco with temperature in P mariana compared with N. tabacum or to differences in the temperature response of Rubisco kinetics between these species.

For accurate modelling, it is important to have correct Rubisco kinetics and assumptions concerning the major fluxes of CO₂ and O₂ during photosynthesis. The biochemical model of photosynthesis predicts net leaf CO₂ exchange from the balance of carbon gain through Rubisco carboxylation with carbon loss through day respiration (R_d) and photorespiration. Photorespiration releases CO₂ at a given stoichiometry of CO₂ per oxygenation (α), which is assumed to remain constant at 0.5 based on current understanding of photorespiratory biochemistry (Reumann and Weber, 2006). In C₃ plants, photorespiration releases carbon at approximately 25% the rate of gross CO₂ fixation, reducing the quantum efficiency of photosynthesis (von Caemmerer and Farquhar, 1981; Sharkey, 1988). Therefore, the CO₂ compensation point in the absence of day respiration (Γ'), which quantifies photorespiratory loss of CO₂ and the kinetic properties of Rubisco, is an essential term in models of photosynthesis (see Equations 2 and 4 below).

Γ' can be measured either biochemically through in vitro assays or in vivo using gas-exchange methods. Generally, in vivo Γ' is measured with the so-called ‘Laisk method’ as the intersection of A-C curves measured at multiple subsaturating light intensities (Γ'ₐ) (Laisk, 1977). The original method described by Laisk did not take into account mesophyll conductance of CO₂ (gₘ) (Equation 3) to adjust the intercellular CO₂ partial pressure (Cᵢ) to the CO₂ partial pressure at the site of Rubisco (Cₛ); however, several recent publications have reviewed the importance of including gₘ in estimates of Γ'ₐ and gas-exchange generally (Warren, 2008; Furbank et al., 2009).

Alternatively, mass spectrometer measurements of leaf O₂ isotope exchange can also be used as an in vivo estimate of Γ' (Γ'₀). This method does not require estimates of gₘ but does require assumptions related to leaf O₂ exchange and α (see Equations 4, 7, and 8) (Ruuska et al., 2000; Bernacchi et al., 2002). O₂ exchange is typically measured by placing a leaf disk in a sealed cuvette in an ¹⁸O₂ atmosphere attached to a mass spectrometer via a membrane inlet (Canvin et al., 1980; Beckmann et al., 2009). The exchange of O₂ in and out of the leaf is measured by following the uptake of ¹⁸O₂ and evolution of the natural abundance of ¹⁶O₂ from water splitting during photosynthesis (see Equations 7 and 8). The Γ'₀ calculations assume that α is constant at 0.5, O₂ consumption from day respiration is the same as in the dark, and rates of photoresduction of O₂ to water (the Mehler reaction) are negligible (Canvin et al., 1980; Badger, 1985). These assumptions appeared valid at 25 °C when compared with independent measurements of gas exchange and Rubisco kinetics (Ruuska et al., 2000), but their accuracy as temperature increases has not been widely characterized (Badger et al., 2000).

Unfortunately, measurements of α are inherently difficult because they require determining the rate of CO₂ release from photorespiration and the rate of Rubisco oxygenation (vₒ) while Rubisco carboxylation (v_c) and CO₂ release from R_d continue in the light. However, at 25 °C the post-illumination burst (PIB) and ¹³CO₂ release following a saturating ¹²CO₂ injection both scale with photorespiratory CO₂ release, providing an estimate of the CO₂ component of α (Doehlert et al., 1979; Delfine et al., 1999; Loreto et al., 2001; Cousins et al., 2008, 2011). Additionally, v_c can be estimated using isotopic exchange of ¹⁸O₂ and ¹⁶O₂, but these measurements are subject to the assumptions of O₂ exchange outlined previously and discussed in the theory section below (Canvin et al., 1980; Badger, 1985; Cousins et al., 2008, 2011). Recently, measurements of ¹⁸CO₂ release and ¹³O₂ and ¹⁶O₂ exchange indicated an increase in α in Arabidopsis thaliana lacking both isoforms of peroxisomal malate dehydrogenase (pmdh1pmdh2) and peroxisomal hydroxypyruvate reductase (hpr) (Cousins et al., 2008, 2011).

Despite the importance of Γ' to gas-exchange models and the value of understanding the temperature response of photosynthesis, to our knowledge there are no published comparisons of Γ'ₐ and Γ'₀ at ambient and elevated temperatures. Such a comparison would help determine whether the two methods give consistent results and identify which assumptions may need re-evaluating at elevated temperatures. Therefore, this study examined the temperature response of Γ'ₐ and Γ'₀ measured in N. tabacum (Bernacchi et al., 2001, 2002). Additionally, the temperature and O₂ response of Γ'ₐ and Γ'₀ were measured in A. thaliana wild-type (WT) and pmdh1pmdh2hpr plants. These data were used to explore the potential physiological explanations for differences between the two measurements of Γ', including increases in α and changes in O₂ exchange with temperature.

Theory

The rate of net assimilation of CO₂ (A) can be modelled by subtracting CO₂ released by photorespiration and mitochondrial respiration from Rubisco carboxylation rates:

\[ A = v_c - \alpha v_o - R_d \]  

(1)

where R_d is the rate of day respiration (Farquhar et al., 1980). Additionally, the Farquhar, von Caemmerer, and Berry biochemical model describes Rubisco-limited photosynthesis as:

\[ A = V_{max} \left( \frac{C_o - \Gamma_o}{C_o + K_c \left( 1 + O/K_o \right)} \right) - R_d \]

(2)

where \( V_{max} \), K_c, and K_o represent the maximum rate of v_c and Michaelis–Menten constants for reactions with CO₂ and O₂,
respectively (von Caemmerer, 2000). $C_c$ can be calculated from intercellular CO₂ partial pressure ($C_i$) using $g_m$ according to:

\[ C_c = C_i - \frac{A}{g_m} \]  

(3)

$\Gamma^*$, the CO₂ compensation point in the absence of day respiration is described by the Rubisco specificity for CO₂ over O₂ ($S_{(O)}$), partial pressure of O₂ ($O$) and α as:

\[ \Gamma^* = \frac{\alpha O}{S_{co}} \]  

(4)

Changes in $\Gamma^*$ affect estimates of net assimilation and, as indicated in Equation 4, are directly proportional to $O$ and $\alpha$. The CO₂ compensation point in the presence of $R_d$ ($\Gamma$) is expressed as:

\[ \Gamma = \frac{\Gamma^* + K_v (1 + O/K_o) R_d V c_{max}}{1 - R_d / V c_{max}} \]  

(5)

and is measured as the CO₂ partial pressure where $\Gamma$ is zero.

Photosynthesis at higher CO₂ partial pressures is not Rubisco limited (Equation 2) but is usually limited by the ability of the Calvin–Benson cycle to regenerate intermediates for carbon fixation due to insufficient production of NADPH. Under these conditions, photosynthesis is dependent on the maximum rate of electron transport ($J_{max}$) and energy demand of photosynthesis and photorespiration according to:

\[ A = \frac{(C_c - \Gamma^*) J_{max}}{4C_c + 8\Gamma^*} - R_d \]  

(6)

The Laisk method (Laisk, 1977) was used to measure the apparent compensation point ($\Gamma^*$) by accounting for mesophyll conductance ($g_m$) and $R_d$ according to $\Gamma^* = C^* + R_d g_m$. $g_m$ was calculated from the photosynthetic flux density of 300 μmol m⁻² s⁻¹ and 2000 μbar CO₂ (Equation 4). Both the Laisk and the O₂-exchange method rely on measurements of the net exchange of CO₂ and O₂, respectively, to determine $\Gamma^*$ assuming that CO₂ and O₂ are exchanged primarily through reactions of photosynthesis, photorespiration, and $R_d$. However, there are several other carboxylases and decarboxylations within plant cells, including phosphoenolpyruvate carboxylase and carbamoyl phosphate synthetase, that could mask the true $\Gamma^*$ with unaccounted fluxes (Raven and Farquhar, 1990). Whilst these additional fluxes are important physiologically, their rates are typically a tenth to a one-thousandth the rate of CO₂ flux through Rubisco and have a negligible impact on calculations of $\Gamma^*$.

**Materials and methods**

**Growth conditions**

WT *A. thaliana* Columbia accession and mutant pmdh1 pm dh2 hp r (Pracharoenwattana et al., 2007) were grown in a climate-controlled cabinet (Econair Ecological Chambers, Winnipeg, Canada) under a photosynthetic flux density of 300 μmol m⁻² s⁻¹ and 2000 μbar CO₂ to minimize the phenotype of pmdh1 pm dh2 hp r (Pracharoenwattana et al., 2007). Day/night cycles were 11/13 h and 23/18 °C. Seeds were cold stratified for 3 d and germinated on sterile agar plates supplemented with MS medium (Plant Media, Dublin, OH, USA) and 1% sucrose. Following cold stratification, plates were placed in the growth chamber for 1 week and the seedlings were then transferred to soil for an additional 3 weeks and fertilized weekly with Peters 20-20-20 (J.R. Peters, Allentown, PA, USA). The youngest fully expanded leaves of 31–40-d-old plants were used for gas-exchange measurements.

**Laisk CO₂ compensation points**

The Laisk method (Laisk, 1977) was used to measure the apparent compensation point ($\Gamma^*$) in WT and pmdh1 pm dh2 hp r plants under different O₂ at 25 and 35 °C. Different O₂ partial pressures (92, 184, and 368 mbar O₂) were generated using O₂ and N₂ mixed with calibrated mass flow controllers (model GFC17; Aalborg, Orangeburg, NY, USA). $A$–$C_i$ curves were measured on a leaf fully enclosed in a 2 cm² measuring head (6400–40 Leaf Chamber Fluorometer; Li-Cor Biosciences, Lincoln, NE, USA) at sub saturating light intensities using a Li-Cor 6400 XT (Li-Cor Biosciences) and the x and y coordinates of these points were used to determine $R_d$ (y coordinate) and $C^*$ (x coordinate). CO₂ diffusion through the gasket was corrected according to the manufacturer’s instructions (Li-c or 6400XT manual version 6). The CO₂ compensation point in the absence of day respiration ($\Gamma^*$′) was subsequently calculated from $C^*$ by accounting for mesophyll conductance ($g_m$) and $R_d$ according to $\Gamma^* = C^* + R_d g_m$. With $g_m$ equal to 0.2 and 0.35 mol CO₂ m⁻² bar⁻¹ at 25 and 35 °C, respectively. The value of 0.2 mol CO₂ m⁻² bar⁻¹ at 25 °C was the average of several *A. thaliana* ecotypes measured under various conditions (Tazoe et al., 2011) and this value becomes 0.35 mol CO₂ m⁻² bar⁻¹ at 35 °C according to the temperature-response model of (Bernacchi et al., 2002).

**Mass spectrometric measurements**

Rates of $\nu_v$ and $\nu_o$ were determined from measurements of $^{18}$O₂ consumption and $^{16}$O₂ evolution according to Equations 7 and 8. $^{18}$O₂ consumption in the light and dark and $^{16}$O₂ evolution in the light was measured as described previously (Canvin et al., 1980;
Parameterization and temperature-response modelling

$A$–$C_{i}$ curves were measured using a Li-Cor 6400 assuming a $g_{m}$ of 0.2 and 0.35 mol CO$_2$ m$^{-2}$ bar$^{-1}$ at 25 and 35 $^\circ$C, respectively (Bernacchi et al., 2001, 2002; Tazoe et al., 2011). Measurements were made under saturating illumination (photosynthetic flux density of 1200 mol m$^{-2}$ s$^{-1}$) and vapour pressure deficits below 15 mbar made under saturating illumination (photosynthetic flux density (Bernacchi et al., 2002; Tazoe et al., 2011). Measurements were

Statistics

A four-way analysis of variance (ANOVA; Table 1) was used to determine the influence of genotype, temperature, measurement method, and O$_2$ levels on $g_{m}$ using Statistix 9 (Analytical Software, Tallahassee, FL, USA). A three-way ANOVA (Table 2) was used to determine the influence of genotype, temperature, and O$_2$ levels on measurements of $v_{c}$, $^{13}$CO$_2$ release per $v_{c}$ and PIB per $v_{c}$ using Statistix 9. A two-way ANOVA was used to determine the significance in measured and modelled $V_{cmax}$ and $J_{max}$ values (Table 3) using R (R Foundation for Statistical Computing, Vienna Austria, http://www.R-project.org). Significance was assumed to be $P < 0.05$.

Table 1. Effects of using the Laisk or O$_2$-exchange methods to estimate $\Gamma^*$ on modelling CO$_2$ assimilation curves under elevated temperature. Maximum rate of Rubisco carboxylation ($V_{cmax}$) and electron transport ($J_{max}$) at 25 and 35 $^\circ$C calculated using standard biochemical models of leaf photosynthesis (von Caemmerer, 2000) with $\Gamma_{L}$ or $\Gamma_{O}$ from Bernacchi et al. (2001) or Bernacchi et al. (2002). The modelled $V_{cmax}$ and $J_{max}$ at 35 $^\circ$C were scaled from 25 $^\circ$C measurements using the temperature-response functions of Bernacchi et al. (2001, 2002). Results are shown as means ±SE of five leaves from separate plants. Statistical analysis was conducted using a one-way ANOVA; different superscript letters indicate significant differences between $\alpha$ assumptions and temperatures at $P < 0.05$.

| Temperature | Assumed $\Gamma^*$ | $V_{cmax}$ ($\mu$mol m$^{-2}$ s$^{-1}$) | $J_{max}$ ($\mu$mol m$^{-2}$ s$^{-1}$) |
|-------------|-------------------|---------------------------------|---------------------------------|
|             | Measured | Modelled | Measured | Modelled |
| 25$^\circ$C | $\Gamma_{L}$ | 41.3±1.5$^a$ | – | 87.6±3.1$^a$ | – |
|             | $\Gamma_{O}$ | 38.1±0.5$^b$ | – | 84.6±2.7$^b$ | – |
| 35$^\circ$C | $\Gamma_{L}$ | 85.9±5.1$^c$ | 96.5±3.6$^c$ | 107.9±3.4$^c$ | 163.2±6.2$^c$ |
|             | $\Gamma_{O}$ | 70.8±2.1$^c$ | 89.0±1.2$^c$ | 97.0±3.0$^c$ | 148.9±4.8$^c$ |

Results

Comparison of $\Gamma^*_L$ and $\Gamma^*_O$ from Bernacchi et al. (2001, 2002)

The response of $\Gamma^*_L$ to temperature was modelled as described by Bernacchi et al. (2001) using the Laisk method to estimate the temperature response assuming an infinite mesophyll conductance ($g_{m}$) (Fig. 1, dotted line). This response was then corrected for $g_{m}$ using the values reported by Bernacchi et al. (2002), the temperature response of $R_T$ from Bernacchi et al. (2001), and Equation 3 (Fig. 1, solid line). The dashed line in Fig. 1 represents the temperature response of $\Gamma^*_O$ according to Bernacchi et al. (2002), which was determined from O$_2$ exchange using a membrane inlet mass spectrometer assuming a constant stoichiometric release of CO$_2$ per oxygenation of 0.5. The 25 $^\circ$C $\Gamma^*_L$ values were greater than $\Gamma^*_O$ regardless of $g_{m}$ and were more responsive to temperature.

Effects of $\Gamma^*_L$ and $\Gamma^*_O$ on modelling of photosynthetic CO$_2$-response curves under elevated temperatures

In WT *A. thaliana*, the photosynthetic parameters $V_{cmax}$ and $J_{max}$ were calculated (Farquhar et al., 1980; von Caemmerer, 2000) from the net CO$_2$ assimilation rates as a function of CO$_2$ partial pressures at 25 and 35 $^\circ$C assuming $\Gamma^*_L$, $\Gamma^*_O$, and Rubisco kinetics from Bernacchi et al. (2001, 2002, 2003) (Fig. 2 and Table 1). $V_{cmax}$ was not significantly different when $\Gamma^*_L$ or $\Gamma^*_O$ was used at 25 $^\circ$C ($41.3±1.5$ mol m$^{-2}$ s$^{-1}$ for $\Gamma^*_L$ and $38.1±0.5$ mol m$^{-2}$ s$^{-1}$ for $\Gamma^*_O$). However, at 35 $^\circ$C, $V_{cmax}$ was significantly different depending on whether $\Gamma^*_L$ ($85.9±5.1$ mol m$^{-2}$ s$^{-1}$) or $\Gamma^*_O$ ($70.8±2.1$ mol m$^{-2}$ s$^{-1}$) was used for the calculation. Additionally, at 35 $^\circ$C, the modelled temperature response of $V_{cmax}$ was significantly different from the measured values using $\Gamma^*_O$ (Bernacchi et al., 2002) but not $\Gamma^*_L$ (Bernacchi et al., 2001). However, the calculated values of $J_{max}$ were not significantly different when using either $\Gamma^*_O$ or $\Gamma^*_L$. Additional key gas-exchange parameters, including net CO$_2$ assimilation, intercellular CO$_2$ concentration, stomatal conductance to H$_2$O, and H$_2$O transpiration rates, are presented in Supplementary Table S1 (at JXB online).
Table 2. Results of ANOVA comparing the Laik and O₂-exchange methods of measuring Γ⁺ at various oxygen partial pressures and temperatures in WT and pmdh1-pmdh2hpr A. thaliana. Method refers to the measurement technique of Γ⁺ with measurements of O₂ exchange on the mass spectrometer indicated as Mass to avoid confusion with the O effect and the mutant pmdh1pmdh2hpr referred to as 3X for convenience. O is indicated as the partial pressure in mbar (92O₂, 184O₂, and 368O₂) and temperature is in °C (25T, 35T). Asterisks indicate a significant interaction according to ANOVA (P < 0.05) and different superscript letters denote significant differences according to a Tukey’s post-hoc test (P < 0.05). Results are shown as the means ±SE of three to six leaves from separate plants.

| Parameter | Factor | F sub x, y | Interactions |
|-----------|--------|------------|--------------|
| Γ⁺        | Genotype | 72.2, a,b   | 3X25-3X35, 3X25-Mass, 3X35-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | O₂     | 397.3, a    | 3X25-3X35, 3X25-Mass, 3X35-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Temp   | 84.7, a     | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Method | 0.6, a     | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Genotype, O₂ | 6.1, a  | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Temp, Method | 4.5, a    | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Genotype, Method | 4.9, a  | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Genotype, O₂, Temp | 1.6, a  | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Genotype, O₂, Temp, Method | 15.4, a  | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | O₂, Temp, Method | 3.8, a    | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |
|           | Genotype, O₂, Temp, Method | 2.0, a  | 3X25-3X35, 3X25-Mass, 3X25-Mass, 3X25-Laisk, 3X25-Mass-Laisk, 3X25-Laisk-Mass |

CO₂ compensation point in the absence of day respiration under elevated temperature

Γ⁺ L and Γ⁺ O were measured and modelled in A. thaliana WT and pmdh1pmdh2hpr plants at 25 and 35 °C in response to various O (Fig. 3). WT values of Γ⁺ L and Γ⁺ O increased linearly with O at both 25 °C (r²=1.0000 for Γ⁺ L and r²=0.9997 for Γ⁺ O) and 35 °C (r²=0.9982 for Γ⁺ L and r²=0.9854 for Γ⁺ O) with a significantly higher Γ⁺ L compared with Γ⁺ O at 35 °C regardless of O (Fig. 3A, C and Table 2). There was also a linear response of Γ⁺ L and Γ⁺ O in the pmdh1pmdh2hpr plants to O at 25 °C (r²=0.9999 for Γ⁺ L and r²=0.9423 for Γ⁺ O) and 35 °C (r²=0.9797 for Γ⁺ L and r²=0.9773 for Γ⁺ O) (Fig. 3B, D); however, there was no significant difference between Γ⁺ L and Γ⁺ O at either temperature (Table 2). The WT Γ⁺ L and Γ⁺ O were lower than pmdh1pmdh2hpr at 25 °C regardless of O (Table 2). However, at 35 °C, there was no difference in Γ⁺ L between the two genotypes, but Γ⁺ O was significantly higher in pmdh1pmdh2hpr compared with WT at all O (Table 2).

As the model was fitted to WT values of Γ⁺ L and Γ⁺ O at 25 °C for Bernacchi et al. (2001, 2002), there was good agreement between the measured and modelled values at each O for the WT. However, at 35 °C, Γ⁺ L was slightly underestimated and Γ⁺ O was slightly overestimated by Bernacchi et al. (2001). The modelled Γ⁺ for the pmdh1pmdh2hpr plants was adjusted to a higher α of 0.8 (Equation 4). At 25 °C in the pmdh1pmdh2hpr plants, the modelled values fitted Γ⁺ L at all O and Γ⁺ O at 92 and 184 mbar O (Fig. 3B). However, at 35 °C, Γ⁺ L was underestimated by the model of Bernacchi et al. (2001) and Γ⁺ O was overestimated by the model of Bernacchi et al. (2002) (Fig. 3D).

Rates of Rubisco oxygenation and carboxylation from measurements of O₃ and of CO₂ isotope exchange

A membrane inlet mass spectrometer was used to measure rates of CO₂ and O₂ isotope exchange in WT and pmdh1-pmdh2hpr plants in response to temperature and O. From these measurements, the PIB, the release of ¹²CO₂ in a saturating ¹³CO₂ background, and the rate of Rubisco oxygenation (vₒ) were determined. At 25 and 35 °C, there was a significant response of vₒ to O for both genotypes (Fig. 4 and Table 3). However, vₒ did not respond to temperature in WT plants but was significantly different between 25 and 35°C in the pmdh1pmdh2hpr plants. In WT plants, the PIB:vₒ ratio was significantly lower at 25 °C compared with that at 35 °C, regardless of O₂ level, and was significantly lower in WT compared with pmdh1pmdh2hpr plants at 25 but not at 35°C across all O₂ levels (Fig. 5 and Table 3). In both genotypes, there was no significant response of PIB:vₒ to O₂ at 25 °C, but at 35 °C, the PIB:vₒ ratio was higher at 92 mbar compared with at 184 and 368 mbar O₂. The ¹²CO₂:vₒ ratio responded significantly to O₂, regardless of temperature and genotype, but decreased with temperature in the pmdh1pmdh2hpr plants but not in the WT plants. At 25 °C, the ¹²CO₂:vₒ ratio was greater in the pmdh1pmdh2hpr plants compared with the WT plants, but there was no difference between genotypes at 35 °C. In summary, at 25 °C the
Table 3. Results of two-way ANOVA on mass spectrometric measures of CO2 release during photorespiration in WT and pmdh1pmdh2hpr A. thaliana. ANOVA analysis between rates of Rubisco oxygenation (\(v_o\)), PIB/\(v_o\), and 12CO2 release following a saturating injection of 13CO2 (12CO2)/\(v_o\) as measured on leaf punches with a membrane inlet mass spectrometer. The mutant pmdh1pmdh2hpr is referred to as 3X for convenience, O is indicated by the partial pressure in mbar (92O2, 184O2, and 368O2) and temperature is in °C (25T, 35T). Asterisks indicate a significant interaction according to ANOVA (\(P<0.05\)) and different superscript letters denote significant differences according to a Tukey’s post-hoc test (\(P<0.05\)). Results are shown as the means ±SE of three to six leaves from separate plants.

| Parameter | Factor                        | \(F_{\text{null, adj}}\) | Interactions                                                                 |
|-----------|-------------------------------|---------------------------|-------------------------------------------------------------------------------|
| \(v_o\)  | Genotype                      | 37.7,1,72*                | WT92\(_{g_m}\)^a, WT184\(_{g_m}\)^b, WT368\(_{g_m}\)^a, 3X92\(_{g_m}\)^c, 3X184\(_{g_m}\)^b, 3X368\(_{g_m}\)^b       |
|          | Temp                          | 22.4,1,72*                |                                                                               |
|          | O2                            | 148.1,1,72*               |                                                                               |
|          | Genotype, O2                  | 14.9,1,72*                | WT25\(_{g_m}\)^a, WT35\(_{g_m}\)^b, 3X25\(_{g_m}\)^a, 3X35\(_{g_m}\)^a       |
|          | Genotype, Temp                | 37.7,1,72*                | 25\(_{g_m}\)^b, 25\(_{g_m}\), 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Temp, O2                      | 4.5,1,72*                 |                                                                               |
|          | Genotype, O2, Temp            | 2.4,1,72*                 | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
| PIB/\(v_o\) | Genotype                  | 54.3,1,72                 |                                                                               |
|          | Temp                          | 2.2,1,72                  |                                                                               |
|          | O2                            | 7.8,1,72                  |                                                                               |
|          | Genotype, O2                  | 5.9,1,72                  | WT25\(_{g_m}\)^a, WT184\(_{g_m}\)^b, WT368\(_{g_m}\)^a, 3X92\(_{g_m}\)^c, 3X184\(_{g_m}\)^b, 3X368\(_{g_m}\)^b   |
|          | Genotype, Temp                | 21.8,1,72                 | WT25\(_{g_m}\)^a, WT35\(_{g_m}\)^b, 3X25\(_{g_m}\)^a, 3X35\(_{g_m}\)^a       |
|          | Temp, O2                      | 17.2,1,72                 | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Genotype, O2, Temp            | 2.6,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Genotype                      | 4.8,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Temp                          | 0.1,1,72                  |                                                                               |
|          | O2                            | 8.9,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Genotype, O2                  | 2.4,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Genotype, Temp                | 15.9,1,72                 | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Temp, O2                      | 0.3,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |
|          | Genotype, O2, Temp            | 2.4,1,72                  | 25\(_{g_m}\)^b, 25\(_{g_m}\)^a, 25\(_{g_m}\)^a, 35\(_{g_m}\)                      |

Fig. 1. Modelled response of the CO2 compensation point in the absence of day respiration (\(\Gamma^*\)) to temperature and O2. The graph shows the temperature response of \(\Gamma^*\) from Bernacchi et al. (2001) with a correction for mesophyll conductance (\(g_{m}\)) (solid line) and with an infinite \(g_{m}\) (dotted line). The dashed line represents the response according to Bernacchi et al. (2002), which was determined assuming a stochiometric release of CO2 per oxygenation of 0.5.

Fig. 2. The CO2 response of photosynthesis at 25 °C (closed circles) and 35 °C (open circles) as measured by a Li-Cor 6400. Chloroplastic CO2 partial pressure (C\(_c\)) was determined from previously published mesophyll conductance values (with a mesophyll conductance (\(g_{m}\)) of 0.2 and 0.35 mmol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\)) at 25 °C (Tazoe et al., 2011) and 35 °C, respectively. Results are shown as means ±standard error (SE) of five leaves from separate plants.
PIB: \(v_o\) and \(^{13}\text{CO}_2 : v_o\) ratios were higher in the \(pmdh1pmdh2hpr\) plants compared with the WT plants, but at 35°C, they were not significantly different between genotypes, regardless of \(O\). Additionally, PIB: \(v_o\) was significantly higher in the WT plants at 35°C compared with 25°C but did not significantly respond to temperature in the \(pmdh1pmdh2hpr\) plants. In the WT plants, PIB: \(v_o\) was different at 92 mbar compared with 184 and 368 mbar \(O_2\) but not in the \(pmdh1pmdh2hpr\) plants.

**Discussion**

**Effects of \(R_d\) and \(g_m\) on measurements of \(\Gamma^*\) using the Laisk and \(O_2\)-exchange methods**

Bernacchi et al. (2001) measured the temperature response of \(\Gamma^*\) in \(N.\ tabacum\) using the Laisk method (\(\Gamma^*_L\)) (Laisk, 1977) to develop a temperature response model of \(\Gamma^*\). Measurements of \(\Gamma^*_L\) require no assumptions about the

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**Fig. 3.** The CO2 compensation point in the absence of day respiration (\(\Gamma^*\)) in WT (A, C) and \(pmdh1pmdh2hpr\) (B, D) \(A.\ thaliana\) plants under various \(O_2\) partial pressures at 25°C (closed symbols; A, B) and 35°C (open symbols; C, D) measured using the Laisk method for WT (circles) and \(pmdh1pmdh2hpr\) (upward triangles) plants. Measurements of \(\Gamma^*\) using \(O_2\) exchange are also shown for WT (squares) and \(pmdh1pmdh2hpr\) (downward triangles) plants. Solid lines represent predicted \(\Gamma^*\) values from Bernacchi et al. (2001) with a mesophyll conductance \((g_m)\) of 0.2 and 0.35 mmol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\) at 25 and 35°C, respectively. Dotted lines show the results from Bernacchi et al. (2002) with \(\alpha=0.8\) for the \(pmdh1pmdh2hpr\) plants. Results are shown as means ±SE of three to six leaves from separate plants. Laisk data (25°C) from WT and \(pmdh1pmdh2hpr\) plants were also presented in Cousins et al. (2011).

**Fig. 4.** Rates of Rubisco oxygenation \((\nu_o)\) at the CO2 compensation point estimated from measurements of \(^{18}\text{O}_2\) and \(^{16}\text{O}_2\) exchange as described in Materials and methods. Measurements were made at 92, 184, and 368 mbar \(O_2\) at 25 and 35°C for both WT (A) and \(pmdh1pmdh2hpr\) (B) \(A.\ thaliana\) plants. Results are shown as means ±SE of three to six leaves from separate plants.
Given that all these assumptions are correct, then \( \Gamma \) or photorespiration (Equations 4, 7, and 8) (Badger, 1985). All electrons passed to NADPH drive either photosynthesis or photorespiration, and Rubisco oxygenation, (iii) rates of O\(_2\) consumption by a direct comparison of these two methods of estimating \( \Gamma \) has not been conducted, particularly in response to temperature. As noted in several publications, it is important to account for \( g_m \) to measure \( \Gamma_L \) accurately (von Caemmerer et al., 1994; von Caemmerer, 2000; Ethier and Livingston, 2004; Furbank et al., 2009). This is because \( \Gamma_L \) is determined from the intercept of several A-C\(_i\) curves measured under subsaturating light conditions. The x value of this intercept represents \( C_i \) at \( \Gamma^* \), and the y value of \( A \) is negative and represents rates of \( R_d \) (Laisk, 1977). To estimate \( \Gamma^*_L \), the values of \( C_i \) must be corrected for \( g_m \) to obtain an accurate \( C_i \) (Equation 3) (Ethier and Livingston, 2004; Furbank et al., 2009). As \( \Gamma^*_L \) is determined when \( A \) is negative, \( \Gamma^*_L \) before accounting for \( g_m \) is lower than after correcting for \( g_m \). The \( \Gamma^*_L \) values reported by Bernacchi et al. (2001) were uncorrected for \( g_m \), meaning that they are lower than the \( g_m \)-corrected value would be. Therefore, to accurately describe \( \Gamma^* \) the model of Bernacchi et al. (2001) must be corrected for the temperature response of \( g_m \). Alternatively, at \( \Gamma \), there is no net photosynthesis and the ratio of \( A: g_m \) approaches zero regardless of \( g_m \) value (Equation 3). Therefore, because \( A \) is zero at \( \Gamma \), measurements of \( C_i \) are equal to \( C_c \). Under these conditions, stomatal conductance is similarly negligible and \( C_c \) can be determined from measured CO\(_2\) partial pressure inside the sealed cuvette without correcting for \( g_m \). Consequently, measurements of \( \Gamma^*_o \) are not sensitive to errors in \( g_m \) (Equation 4). This latter approach was used by Bernacchi et al. (2002) to measure \( \Gamma^* \) independently of assumptions of \( g_m \). To compare these two models of \( \Gamma^* \) at 25 °C, the values of Bernacchi et al. (2001) for \( \Gamma^*_L \) were corrected using \( g_m \) according to Bernacchi et al. (2002). At 25 °C, the \( g_m \)-corrected modelled values of \( \Gamma^*_L \) were higher than the modelled \( \Gamma^*_O \) (Fig. 1) and differences between \( \Gamma^*_L \) and \( \Gamma^*_O \) increased with temperature, highlighting the greater temperature sensitivity of \( \Gamma^*_L \) compared with \( \Gamma^*_O \). The difference between \( \Gamma^*_L \) and \( \Gamma^*_O \) is not explained by potential errors in assumptions of \( g_m \) because the difference is significant when \( g_m \) is assumed to be infinite (no restriction to CO\(_2\) diffusion and \( C_i=C_c \) and the discrepancy increases as \( g_m \) decreases (Fig. 1). Similarly, any assumed value of \( R_d \) (from zero to infinity) also increases the discrepancy between the values of \( \Gamma^*_L \) from Bernacchi et al. (2001) and the values of \( \Gamma^*_O \) from Bernacchi et al. (2002) (Equation 3). In summary, the temperature response and absolute values of \( \Gamma^*_L \) are higher than \( \Gamma^*_O \) even when corrected for \( g_m \) and regardless of \( R_d \). Both of these estimates of \( \Gamma^* \) are used to determine the maximum rate of Rubisco carboxylation (\( V_{\text{max}} \)) and the maximum

Fig. 5. PIB per \( V_c \) (A, B) and \( ^{12} \text{CO}_2 \) release per \( V_c \) (C, D) at various O\(_2\) partial pressures in WT (A, C) and \( \text{pmdh1pmdh2hpr} \) (B, D) \( A. \text{thal} \) at 25 and 35 °C measured from CO\(_2\) and isotopic O\(_2\) with the membrane inlet mass spectrometer. Results are shown as means ±SE of three to six leaves from separate plants.
rate of electron transport ($J_{\text{max}}$) from gas-exchange measurements of A–C curves (von Caemmerer, 2000). Additionally, the temperature response of $\Gamma$ is essential for modelling the response of these parameters and photosynthesis to changes in leaf temperatures. Therefore, it is important to determine how the difference in temperature response of $\Gamma$ between Bernacchi et al. (2001) and Bernacchi et al. (2002) influences estimates of $V_{\text{cmax}}$ and $J_{\text{max}}$ derived from A–C curves. To test this, $V_{\text{cmax}}$ and $J_{\text{max}}$ were determined from A–C curves measured at 25 and 35°C in A. thaliana with $\Gamma$ from the two Bernacchi et al. (2001, 2002) temperature-response models.

Sensitivity of $V_{\text{cmax}}$ and $J_{\text{max}}$ to $\Gamma^*$

At temperatures above 25°C, previous publications have attributed lower $V_{\text{cmax}}$ estimated from leaf gas-exchange measurements compared with modelled values as changes in the Rubisco activation state (Sage et al., 2008). However, some of this difference could also be explained by errors in $\Gamma^*$ and its modelled temperature response. For example, using the $g_m$-corrected $\Gamma^*_L$ from Bernacchi et al. (2001) to compare measured and modelled $V_{\text{cmax}}$ values from A. thaliana A–C curves, there was no significant difference at 35°C (Table 1). However, if $\Gamma^*_O$ from Bernacchi et al. (2002) was used to calculate $V_{\text{cmax}}$, then the measured values were significantly lower than the modelled $V_{\text{cmax}}$ (Table 1). This could be interpreted as deactivation of Rubisco using $\Gamma^*_O$ but not with $\Gamma^*_L$. This difference between $V_{\text{cmax}}$ calculated using $\Gamma^*_L$ versus $\Gamma^*_O$ highlights the importance of determining which method is most appropriate for modelling photosynthesis at different temperatures, as well as understanding which assumptions within the two models are valid in response to changing temperatures.

It is possible that the differences in $\Gamma^*_L$ (Bernacchi et al., 2001) versus $\Gamma^*_O$ (Bernacchi et al., 2002) are dependent on the differences in Rubisco content between genotypes used for each study. For example, Rubisco antisense plants were used by Bernacchi et al. (2001) to measure the temperature response of $\Gamma^*_L$. These plants have lowered photosynthetic rates compared with WT plants, which may have introduced errors into measuring the intercept of A–C curves at low CO₂ partial pressures (Hudson et al., 1992). Indeed, the 25°C value of $\Gamma^*_L$ of 41.9 μbar CO₂ from antisense plants measured by Bernacchi et al. (2001) is higher than other reports of $\Gamma^*_L$ measured in both WT N. tabacum and other C₃ plants. For example, $\Gamma^*_L$ values at 25°C typically range between 36.7 and 40.8 μbar CO₂ (Brooks and Farquhar, 1985; von Caemmerer et al., 1994; Laisk and Loreto, 1996).

Additionally, differences between $\Gamma^*_L$ and $\Gamma^*_O$ could be driven by errors in the assumptions used to parameterize each method. As previously discussed, $\Gamma^*_L$ must be corrected for $g_m$; however, including corrections for $g_m$ increases the difference between $\Gamma^*_L$ and $\Gamma^*_O$. Alternatively, there are several assumptions used in determining $\Gamma^*_O$ with unknown temperature responses. For example, measurements of $\Gamma^*_O$ assume that $\alpha$ is constant at 0.5 (Equation 4). Additionally, $\Gamma^*_O$ relies on measurements of $v_o$ and $v_r$, which require assumptions relating O₂ exchange to Rubisco reactions (discussed below).

Therefore, to test these assumptions at 25 and 35°C, measurements of $\Gamma^*_L$ and $\Gamma^*_O$ were made in A. thaliana WT and the photorespiratory mutant (pmdh1pmdh2hpr), previously characterized as having an increased $\alpha$, to determine which parameters contribute to the discrepancies between $\Gamma^*_L$ and $\Gamma^*_O$.

Differences in $\Gamma^*_L$ and $\Gamma^*_O$ in WT A. thaliana

Measurements of $\Gamma^*_L$ and $\Gamma^*_O$ in A. thaliana were used to confirm and characterize the differences between the two methods of measuring $\Gamma$ presented by Bernacchi et al. (2001, 2002) (Fig. 3). In A. thaliana, there was no significant difference between $\Gamma^*_L$ and $\Gamma^*_O$ at 25°C (Table 2). This is different from what was observed previously in N. tabacum where $\Gamma^*_L$ determined by Bernacchi et al. (2001) was higher than $\Gamma^*_O$ from Bernacchi et al. (2002) at 25°C (Fig. 1). The different response of $\Gamma^*_L$ and $\Gamma^*_O$ at 25°C between A. thaliana and N. tabacum could be the result of the different genotypes used in each study. As mentioned before, Bernacchi et al. (2001) used Rubisco small-subunit antisense plants, whilst Bernacchi et al. (2002) measured WT N. tabacum; however, in the current study, WT A. thaliana plants were used for both estimates of $\Gamma$.

The close agreement of $\Gamma^*_L$ and $\Gamma^*_O$ in WT A. thaliana at 25°C at a variety of $O$ values provides strong support that the independent assumptions of both methods are valid at 25°C in this species. However, at 35°C, $\Gamma^*_L$ and $\Gamma^*_O$ in A. thaliana were significantly different across all $O$ (Table 2 and Fig. 3), confirming a similar increased temperature response of $\Gamma^*_L$ over $\Gamma^*_O$ as suggested by the comparison of data from Bernacchi et al. (2001, 2002). As discussed previously, larger values of $g_m$ increase $\Gamma^*_L$ estimated from measured $C_i$ values (Equation 3); however, regardless of the $g_m$ values used (0.10 to infinity), $\Gamma^*_L$ was always larger than $\Gamma^*_O$ (data not shown). Therefore, errors in $g_m$ do not explain the differences between $\Gamma^*_L$ and $\Gamma^*_O$ at 35°C. These findings in A. thaliana confirm differences in $\Gamma^*_L$ and $\Gamma^*_O$ above 25°C, although the difference is less than reported in N. tabacum (Bernacchi et al., 2001, 2002). To determine whether the different temperature responses of $\Gamma^*_L$ and $\Gamma^*_O$ could be explained by changes in $\alpha$ the photorespiratory mutant pmdh1pmdh2hpr was compared with WT at both 25 and 35°C.

Response of $\alpha$ to temperature

Biochemical models of photosynthesis and measurements of $\Gamma^*_O$ (Equation 4) typically assume $\alpha$=0.5 under all conditions. However, there are several recent publications demonstrating changes in $\alpha$ when the traditional photorespiratory pathway is disrupted through genetic manipulation. For example, the photorespiratory mutants pmdh1pmdh2, hpr, and pmdh1pmdh2hpr had lower net photosynthetic rates under photorespiratory conditions, higher $\Gamma$ and higher $\Gamma^*_L$ than WT plants (Cousins et al., 2008, 2011). Additionally, measurements of CO₂ and O₂ isotope gas exchange in the pmdh1pmdh2 and hpr plants at 25°C confirmed that $\Gamma$ and $\Gamma^*_L$ were higher due to an increase in $\alpha$ (Cousins et al., 2008, 2011). Similar to
previously published work on hpr and pmdh1pmdh2 plants, the photorespiratory mutant pmdh1pmdh2hpr in this study had higher \( \Gamma_L \) and CO\(_2\) release per \( v_o \) compared with WT plants, indicating an increase in \( \alpha \) in the pmdh1pmdh2hpr plants at 25 °C (Fig. 5 and Table 3, discussed below). The \( \Gamma_o \) value in the pmdh1pmdh2hpr plants was modelled with \( \alpha=0.8 \) instead of \( \alpha=0.5 \), a stoichiometry that also modelled \( \Gamma \) and \( \Gamma_L \) in this and previous studies with photorespiratory mutants with increased \( \alpha \) (Equation 4 and Fig. 3) (Cousins et al., 2008, 2011). This suggests that misestimates of \( \alpha \) can lead to inaccurate calculations of \( \Gamma_o \).

Measurements of \( \Gamma_L \), which do not require assumptions of \( \alpha \), in WT plants were significantly lower than in pmdh1pmdh2hpr plants at 25 °C; however, at 35 °C, the values were not significantly different between genotypes across all \( O \) (Table 2). It is expected that the \( S_{\text{th}} \) of Rubisco is conserved between WT and pmdh1pmdh2hpr plants at a given temperature (Jordan and Ogren, 1984); therefore, differences in \( \Gamma_L \) at 25 °C could be attributed to \( \alpha \) (Equation 4). However, at 35 °C, the values of \( \Gamma_L \) were the same between WT and pmdh1pmdh2hpr plants, suggesting that \( \alpha \) may increase with temperature in WT A. thaliana to a stoichiometry similar to that in pmdh1pmdh2hpr plants. In WT plants, an increase in \( \alpha \) could also explain why \( \Gamma_L \) and \( \Gamma_o \) were the same at 25 °C but \( \Gamma_o \) was lower than \( \Gamma_L \) at 35 °C when assuming \( \alpha=0.5 \). The linear response of \( \Gamma_L \) to \( O \) at 35 °C indicated that the increase in \( \alpha \) would be constant at a given temperature, regardless of \( O \) (Equation 4 and Fig. 3).

Two putative reactions of photorespiratory intermediates within the peroxisome could explain increases in \( \alpha \). Specifically, excess glyoxylate and hydroxypyruvate could react with H\(_2\)O\(_2\) releasing CO\(_2\), formate, and glycylate with or without an enzyme catalyst (Elstner and Heupel, 1973; Halliwell, 1974). Indeed, this reaction is hypothesized to be a major source of formate in leaves (Igamberdiev et al., 1999). Formate can be further decarboxylated in the peroxisome (Halliwell and Butt, 1974) or oxidized to CO\(_2\) by formate dehydrogenase in the mitochondria (Hourtou-Cabassa et al., 1998), whilst glycylate could re-enter the photorespiratory pathway. These reactions would result in additional CO\(_2\) release per Rubisco oxygenation and divert carbon from the Calvin–Benson cycle and the regeneration of ribulose-1,5-bisphosphate.

It has also been hypothesized that, in WT plants, similar increases in \( \alpha \) occur under elevated temperatures due to an increase in glycolate oxidase activity relative to catalase within the peroxisomes (Grodzinski and Butt, 1977). Additionally, in isolated peroxisomes and mitochondria, an increase in H\(_2\)O\(_2\) can react with glyoxylate and hydroxypyruvate leading to an increase release of CO\(_2\) (Grodzinski and Butt, 1977; Grodzinski, 1978; Hanson and Peterson, 1985). Furthermore, overexpression of catalase in N. tabacum reduced the levels of H\(_2\)O\(_2\) and lowered \( \Gamma \) as temperature increased compared with WT plants (Brisson et al., 1998). These data suggest that \( \alpha \) could increase from non-catalysed decarboxylation reactions with H\(_2\)O\(_2\), decreasing the efficiency of phosphoglycolate recycling but not completely disrupting the photorespiratory pathway. Therefore, measurements of labelled CO\(_2\) and O\(_2\) isotope exchange as described by Cousins et al. (2008, 2011) were used to determine the influence of temperature on \( \alpha \) and to probe some of the assumptions of O\(_2\) exchange used to measure \( \Gamma_o \) at 35 °C.

**Rates of Rubisco oxygenation in A. thaliana WT and pmdh1pmdh2hpr plants**

Rates of \( v_o \) and \( v_o \) are determined by the relative availability of O\(_2\) and CO\(_2\). Rubisco kinetics, and the activation state of Rubisco (Salvucci and Crafts-Brandner, 2004; von Caemmerer et al., 2004; Sage et al., 2008). It has been shown that Rubisco deactivates under high temperatures, decreasing both \( v_o \) and \( v_o \) (Kobza and Edwards, 1987; Feller et al., 1998). Deactivation of Rubisco at 35 °C could explain the insensitivity of \( v_o \) to temperature across all O\(_2\) treatments in WT A. thaliana. However, in contrast to the WT, \( v_o \) increased with temperature in pmdh1pmdh2hpr plants but was constant at 184 and 368 mbar O\(_2\) at 35 °C. This is paradoxical given the apparent decrease in \( \Delta \)CO\(_2\) release per \( v_o \) in pmdh1pmdh2hpr plants under higher Rubisco oxygenation conditions when perturbations to photorespiration would be more severe (Table 3 and Fig. 5). However, this could be explained by errors in measuring \( v_o \) and/or photorespiratory CO\(_2\) release (discussed below).

Alternatively, the increase in \( v_o \) with temperature in pmdh1pmdh2hpr but not in WT plants could be attributed to changes in O\(_2\) exchange by alternative oxidations of photorespiratory intermediates traditionally not described as part of the photorespiratory pathway. For example, measurements of \( v_o \) would decrease if the 2:3 ratio of \( v_o \) to net O\(_2\) uptake used in Equation 7 decreased due to additional oxygenation of photorespiratory intermediates. This would subsequently increase the ratios of PIB and \( \Delta \)CO\(_2\) release per \( v_o \). These reactions could also explain the apparent discrepancies seen in \( v_o \) and CO\(_2\) release per \( v_o \) at 35 °C in the pmdh1pmdh2hpr plants (Fig. 5). If similar increases in \( \alpha \) occur in WT plants under elevated temperature due to non-enzymatic or enzymatic reactions, measurements of \( v_o \) and CO\(_2\) release per \( v_o \) would also be affected.

**CO\(_2\) release per Rubisco oxygenation reaction**

To measure \( \alpha \) accurately, both the flux of CO\(_2\) from photorespiration and the corresponding rates of \( v_o \) must be determined. The CO\(_2\) released from photorespiration cannot be measured directly; however, the combined flux from photorespiration and \( R_d \) can be estimated from the PIB and by the rate of \( \Delta \)CO\(_2\) evolution following a saturating injection of \( ^{13} \)CO\(_2\) on an illuminated leaf (Cousins et al., 2008, 2011). As discussed previously, the photorespiratory mutant pmdh1pmdh2hpr had a higher PIB and \( \Delta \)CO\(_2\) release per \( v_o \) at 25 °C compared with WT plants across O\(_2\), suggesting an increased \( \alpha \) in the pmdh1pmdh2hpr plants (Fig. 5 and Table 3). However, at 35 °C, the PIB per \( v_o \) was not significantly different between WT and pmdh1pmdh2hpr plants (Fig. 5 and Table 3). Curiously, PIB per \( v_o \) was significantly higher at the lowest O\(_2\) compared with the other O\(_2\) levels in both WT and pmdh1pmdh2hpr plants. This increase in CO\(_2\) release per \( v_o \) at the lowest O\(_2\) was not
expected based on the linear relationship between $\Gamma^*_{L}$ and $O$ (Fig. 3).

The discrepancy between $\Gamma^*_{L}$ and $\Gamma^*_{o}$ and the downward trend in PIB and $^{12}$CO$_2$ release per $v_o$ in response to $O$ seen in the WT plants might also be explained by errors in two major assumptions of O$_2$ exchange: (i) the O$_2$ uptake from day respiration is equal to rates of dark respiration and (ii) the rates of Mehler reaction are negligible. It is generally accepted that rates of respiration in the light ($R_o$) are less than rates of respiration in the dark (Villar et al., 1994; Lambers and Ribas-Carbo, 2005) and that $R_o$ may respond to changes in rates of photorespiration (Tcherkez et al., 2008). The Laisk measurements of $\Gamma^*_{L}$ can estimate the CO$_2$ release from $R_o$, which could be used in place of uptake of $^{18}$O$_2$ in the dark in estimates of $v_o$ assuming a stoichiometry of CO$_2$ evolution to O$_2$ uptake during respiration (Equation 7). However, when $R_o$ was used to calculate $v_o$ instead of the measured dark rates of O$_2$ consumption, there was no change in the trends of PIB and $^{12}$CO$_2$ release per $v_o$, as presented in Fig. 5, when the stoichiometry of CO$_2$ evolution to O$_2$ uptake was held constant regardless of the value (comparison not shown). Therefore, there would have to be changes in the stoichiometry of CO$_2$ evolution to O$_2$ uptake to explain the changes in Fig. 5.

In addition to differences in dark versus light respiration, higher rates of the Mehler reaction at 35 °C could introduce errors in the calculated rates of $v_o$ due to the consumption of O$_2$ independent of photosynthesis and photorespiration (Ort and Baker, 2002). This would lead to overestimations of $v_o$ (Equation 7) and underestimations of PIB and $^{12}$CO$_2$ release per $v_o$. The rates of Mehler would have to range from 10% of O$_2$ evolution at 92 mbar to 60% at 368 mbar to maintain a constant PIB and $^{12}$CO$_2$ release per $v_o$ with $O$ (calculations not shown). However, at 25 °C, the rates of Mehler in C$_3$ plants are reported to range from 0 to 30% of photosynthetic electron transport at 25 °C (Asada, 1999; Badger et al., 2000; Ruuska et al., 2000; Driever and Baker, 2011), but the temperature dependence and $O_2$ response of these reactions are not well known for A. thaliana. Measurements of O$_2$ exchange under various conditions in N. tabacum found that $v_o$ explained O$_2$ consumption under low and elevated temperatures, suggesting that the Mehler rate does not increase with temperature (Badger et al., 2000). Therefore, the temperature response of the Mehler reactions in A. thaliana would have to be significantly different compared with N. tabacum to explain the downward trend in Fig. 5.

Finally, the downward trend in PIB and $^{12}$CO$_2$ release per $v_o$ in response to $O$ seen in the WT plants at 35 °C could be explained if the CO$_2$ released from photorespiration does not scale with PIB and $^{12}$CO$_2$ release at elevated temperatures across $O$. In this situation, PIB and $^{12}$CO$_2$ release would no longer be proportional to the CO$_2$ released from photorespiration at 35 °C in response to $O$. This would lead to a decrease in the ratio PIB and $^{12}$CO$_2$ release per $v_o$ as $O$ increases that does not correspond to changes in $\alpha$. The observation that PIB saturates with increasing $O$ at 25 °C and with temperature supports this suggestion (Doehlert et al., 1979). Therefore, at 35 °C, the discrepancy between a constant $\alpha$ described by the linear response of $\Gamma^*_{L}$ and

**Conclusion**

The data presented here demonstrate differences in temperature-response models of $\Gamma^*$ from N. tabacum between the Laisk and O$_2$-exchange methods. These differences were large enough to impact both measured and modelled values of $V_{\text{max}}$ and $J_{\text{max}}$. Differences in $\Gamma^*$ determined from the Laisk and O$_2$-exchange method were also seen in A. thaliana at 35 °C. The difference in estimates of $\Gamma^*$ were probably due to errors in assumptions used in O$_2$-exchange calculations at elevated temperature. The extent of these errors and the species-specific differences in these assumptions should be considered when modelling the temperature response of photosynthesis with $\Gamma^*$ values derived from O$_2$ exchange.

**Supplementary data**

Supplementary data are available at JXB online.

**Supplementary Table S1.** Gas-exchange parameters from CO$_2$-response curves measured at 25 and 35 °C.

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