Exploiting transplastomically modified Rubisco to rapidly measure natural diversity in its carbon isotope discrimination using tuneable diode laser spectroscopy

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Abstract

Carbon isotope discrimination ($\Delta$) during C$_3$ photosynthesis is dominated by the fractionation occurring during CO$_2$-fixation by the enzyme Rubisco. While knowing the fractionation by enzymes is pivotal to fully understanding plant carbon metabolism, little is known about variation in the discrimination factor of Rubisco ($b$) as it is difficult to measure using existing in vitro methodologies. Tuneable diode laser absorption spectroscopy has improved the ability to make rapid measurements of $\Delta$ concurrently with photosynthetic gas exchange. This study used this technique to estimate $b$ in vivo in five tobacco (Nicotiana tabacum L. cv Petit Havana [N,N]) genotypes expressing alternative Rubisco isoforms. For transplastomic tobacco producing Rhodospirillum rubrum Rubisco $b$ was 23.8 ± 0.7‰, while Rubisco containing the large subunit Leu-335-Val mutation had a $b$-value of 13.9 ± 0.7‰. These values were significantly less than that for Rubisco from wild-type tobacco ($b$=29‰), a C$_3$ species. Transplastomic tobacco producing chimeric Rubisco comprising tobacco Rubisco small subunits and the catalytic large subunits from either the C$_4$ species Flaveria bidentis or the C$_3$–C$_4$ species Flaveria floridana had $b$-values of 27.8 ± 0.8 and 28.6 ± 0.6‰, respectively. These values were not significantly different from tobacco Rubisco.

Key words: C$_4$ photosynthesis, carbon isotope discrimination, Flaveria, Rubisco, tobacco, tuneable diode laser spectroscopy.

Introduction

Carbon isotope discrimination occurring during C$_3$ photosynthesis is determined by CO$_2$-diffusion processes from the atmosphere to the chloroplast and the biochemical fractionation occurring during CO$_2$ fixation by Rubisco and during respiratory and photorespiratory CO$_2$ release (Farquhar et al., 1989a). The fact that Rubisco discriminates strongly against $^{13}$C is apparent in the isotopic signature of atmospheric CO$_2$ and this has become a tool for monitoring global CO$_2$ exchange processes (Mook et al., 1983; Yakir and Sternberg, 2000). The strong $^{13}$CO$_2$ discrimination by Rubisco is the primary cause of depleted $^{13}$C levels in plant biomass. This effect has proved experimentally versatile by allowing photosynthetic carbon isotope discrimination to be used as a tool to elucidate CO$_2$-diffusion processes through stomata and from the leaf intercellular airspace to the sites of Rubisco carboxylation in the chloroplast stroma of C$_3$ plant species (Evans et al., 1986, 2009; Farquhar et al., 1989b). Interpreting $^{13}$CO$_2$ discrimination in C$_4$ plants has proved more challenging as a CO$_2$-concentrating mechanism (CCM) operates that spatially localizes Rubisco in bundle sheath compartments with reduced access to atmospheric CO$_2$. In the C$_4$ photosynthetic CCM, initial fixation of atmospheric CO$_2$ occurs via phosphoenolpyruvate carboxylase (PEPC), which discriminates less against $^{13}$C than Rubisco (Farquhar, 1983). C$_4$ acids diffuse into the bundle sheath where decarboxylation supplies CO$_2$ to Rubisco. As a result of this CCM pathway, photosynthetic carbon isotope discrimination is much less in C$_4$-plant species (Evans et al., 1986; Henderson et al., 1992).

The fractionation factor of Rubisco is difficult to measure and only a limited number of measurements exist (McNevin
et al., 2007 and references therein). Current methods rely on the purification of natural or recombinant Rubisco forms by processes that typically reduce catalytic activity (Sharwood et al., 2008). In plants, algae, and cyanobacteria, Rubisco is a 520–550-kDa L8S8 hexadecamer composed of eight ~50-kDa catalytic large (L) subunits and eight ~12–15-kDa small (S) subunits (Whitney et al., 2011a). In most applications of photosynthetic carbon isotope discrimination, the fractionation factor of plant L8S8 Rubisco is assumed to be ~29‰, a photosynthetic carbon isotope discrimination, the fractionalization (S) subunits (Whitney and O'Leary, 1984) and supported by in vivo measurements of carbon isotope discrimination in transgenic tobacco with reduced amounts of Rubisco (Evans et al., 1994). However, the evolutionary diversity in Rubisco catalysis (Yeh et al., 1981; Badger and Andrews, 1987; Tcherkez et al., 2006), even among closely related C3 species (Delgado et al., 1995; Galmes et al., 2005), brings into question the validity of this assumption. This catalytic diversity may conceivably arise from subtle variations to the reaction mechanism of Rubisco. Differences in the fractionation factor of Rubisco pose a useful means for interpreting such reaction mechanism variations (Tcherkez et al., 2006; McNevin et al., 2007; Tcherkez, 2013).

Transgenic tobaccos with altered amounts or forms of Rubisco have been used to quantify the enzyme’s kinetic properties using leaf gas exchange and photosynthesis models. This in vivo approach has been particularly successful in determining the Michaelis–Menten constants for CO2 and O2 (Kc and Ko), catalytic turnover rates (Vcmax and Vomax) and CO2/O2 specificity of tobacco Rubisco and how they vary with temperature (von Caemmerer et al., 1994; Bernacchi et al., 2002; Walker et al., 2013). The approach has also been successfully applied to catalytically altered Rubisco isoforms expressed in tobacco using chloroplast transformation technology (Whitney et al., 1999; Whitney and Andrews, 2003; Sharwood et al., 2008). More recent developments in tuneable diode laser (TDL) absorption spectroscopy have improved the ability to make rapid measurements of carbon isotope discrimination concurrently with photosynthetic gas exchange (Tazoe et al., 2011). The current study combines this technique with transplastomic tobacco lines expressing alternative Rubisco isoforms to measure the Rubisco discrimination factor in vivo. The results confirm the fractionation factors determined in vitro for Rubisco from Rhodospirillum rubrum and the mutant tobacco Leu-335-Val (L335V) Rubisco (McNevin et al., 2007) and also show that Rubisco fractionation factors for Rubisco from Flaveria bidentis (a C4 species) and Flaveria floridana (C3-C4 intermediate species) are similar to that from tobacco (a C3 species).

Materials and methods

Plant material

This study used wild-type tobacco (tob(Wt)), Nicotiana tabacum L. cv Petit Havana [N,NJ] and transplastomic mutants producing R. rubrum Rubisco (tob(Rr), Whitney and Andrews, 2001), mutant tobacco Rubisco containing the large subunit Leu-335-Val substitution (tob(L335V), Whitney et al., 1999), or hybrid Rubisco comprising tobacco small subunits and F. bidentis (tob(bid), Whitney et al., 2011b) or F. floridana (tob(flo), Whitney et al., 2011b) large subunits. As some of the transplastomic mutants could not grow in air, all plants were grown in a growth chamber supplemented with 1% (v/v) CO2. The air temperature was 25 °C with a 14-h photoperiod (400 μmol photon m–2 s–1) and 60% relative humidity.

Concurrent gas exchange and carbon isotope discrimination measurements

Gas exchange and carbon isotope discrimination measurements were made as described by Tazoe et al. (2011) using either a 6-cm2 chamber of the LI-6400 with a red-blue light-emitting diode (LED) light source (Li-Cor, Lincoln, Nebraska, USA) or a laboratory-constructed whole-leaf chamber (115 × 110 × 25 mm depth, boundary layer conductance 4 mol m–2 s–1) together with a red-green-blue LED light source (6400–18 RGB Light source, Li-Cor) and the LI-6400. The flow rate was set at 200 μmol s–1. Gas exchange was coupled to a tuneable diode laser (TDL, TGA100, Campbell Scientific, Logan, UT, USA) for concurrent measurements of carbon isotope composition. Measurements were made at 4-min intervals for 20 s and between six and eight measurements were made at each CO2 partial pressure at an irradiance of 1500 μmol quanta m –2 s–1. Other measurement conditions were O2 19 mbar, and a leaf temperature 25 °C. The LI-6400 CO2 mixing system was used to generate different CO2 concentrations. The δ13C of CO2 gas cylinders (δ13Cair) used in the LI-6400 CO2 injector system was between –13 and –3‰. Gas exchange was calculated using the equations presented by von Caemmerer and Farquhar (1981) and Δ was calculated from the equation presented by Evans et al. (1986) as:

\[
\Delta = \frac{1000\{\delta^{13}C_{\text{sam}} - \delta^{13}C_{\text{ref}}\}}{1000 + \delta^{13}C_{\text{sam}} - \xi (\delta^{13}C_{\text{sam}} - \delta^{13}C_{\text{ref}})}
\]

where δ13Csam and δ13Cref are the carbon isotope compositions of the leaf chamber and reference air of the LI-6400, respectively, ξ is Cref/Csam, where Cref and Csam are the CO2 concentrations of dry air entering and exiting the leaf chamber, respectively, measured by the TDL. The value of ξ ranged from 4.5 to 13 for tob(Wt), 15 to 25 for tob(L335V), 15 to 16 for tob(Rr), 11 to 16 for tob(bid), and 8 to 15 for tob(flo).

Biochemical measurements

Following gas exchange, replicate leaf samples (0.5 cm2) were taken from the sampling area and immediately frozen in liquid nitrogen and stored at –80 °C. Rubisco content in each sample was measured by the [14C]carboxyarabinitol–P2-binding assay procedure according to Ruuska et al. (1998). Soluble leaf protein was measured relative to BSA with a dye-binding assay (Pierce Coomassie Plus Kit). Dry mass of leaves were measured after 48 h at 80 °C. Leaf dry mass per unit area was calculated from destructive harvest data taken from 10 plants after 34 d.

Rubisco kinetic properties of Rubisco in tob(Rr) leaf protein extract was measured at 25 °C using 13C02 fixation assays as described (Whitney and Sharwood, 2007; Sharwood et al., 2008). Assays were performed in 8-ml septum capped vials containing 1 ml reaction buffer [50 mM HEPES-NaOH pH 7.8, 15 mM MgCl2, 0.25 mM ribulose biphosphate (RuBP) and varying concentrations of NaH13CO3 (9–952 μM) and O2 (0, 10, 15 and 20% (v/v), accurately mixed with nitrogen using Wosthoff gas mixing pumps). Leaf protein was extracted in activation buffer [50 mM HEPES-NaOH pH 7.8, 15 mM MgCl2, 20 mM NaH13CO3, 0.5 mM EDTA, 2 mM dithiothreitol, 1%, v/v, plant protease inhibitor cocktail (Sigma-Aldrich), and 1%, w/v, polyvinylpolypyrrolidone] and the Rubisco was activated at 25 °C for 10 min prior to using 20 μl to initiate the assays. The Michaelis constants (Kc) for CO2 (Kc) and O2 (Ko) were determined from the fitted data. The maximal carboxylation rate
extrapolated from Michaelis–Menten curve fitting was divided by
the amount of Rubisco active sites quantified by $[^14C]$carboxyarabi-
nitol-P$_2$ binding (Ruuska et al., 1998; Whitney and Andrews, 2001)
to give $k_{cat}$. 

Calculation of Rubisco fractionation and mesophyll conductance
A full description of discrimination during C$_3$ photosynthesis is
given by Evans et al. (1986). However, Farquhar and Cernusak (2012)
pointed out that while equations used to calculate gas exchange
include ternary effects of transpiration rate on the rate of
CO$_2$ assimilation through stomata (von Caemmerer and Farquhar,
1981), the equations describing carbon isotope discrimination had
been derived without the ternary effects. They introduced revised
equations, and these are used in the current calculation:

$$
\Delta = \frac{1}{1-t} a' + \frac{1}{1-t} \left( \frac{(1+t)b-a'}{C_i} \right) \frac{1+t}{1-t} \left( b-a \right) - \frac{eR_g}{(A+R_g)} \frac{A}{g_n C_e} \\
\frac{1}{1-t} \left( \frac{eR_g}{(A+R_g)C_e} (C_i - C_r) \right) + \frac{1+t}{1-t} \left( \frac{\Gamma_r}{C_e} \right)
$$

(2)

where

$$
t = \frac{(1+a')E}{2g_n^*}
$$

E denotes the transpiration rate, and $g_n^*$ denotes the total conduct-
ance to CO$_2$ diffusion including boundary layer and stomatal con-
ductance (von Caemmerer and Farquhar, 1981). $C_i$ and $C_e$ are the
ambient and intercellular CO$_2$ partial pressures and $\Gamma_r$ is the
compensation point in the absence of mitochondrial respiration. $A$ and
$R_g$ stand for CO$_2$-assimilation and mitochondrial respiration in
the light.

The mesophyll conductance to CO$_2$ diffusion from intercellular
airspace to the chloroplast, $g_n$, is given by:

$$
g_n = A/(C_i - C_r)
$$

(3)

where $C_i$ is the CO$_2$ partial pressure in the chloroplast. The sym-
bol $a$ (1.8‰) denotes the fractionation factor for hydration and
diffusion through water, and $b$ (usually ~29‰) is the fractionation
associated with Rubisco carboxylation. The symbol $a'$ denotes the
combined fractionation factor through the leaf boundary layer and
through stomata:

$$
a' = a (C_e - C_i) + a (C_i - C_r) / (C_i - C_r)
$$

(4)

where $C_i$ is the CO$_2$ partial pressure at the leaf surface, $a_0$ (2.9‰)
is the fractionation occurring through diffusion in the boundary layer
and $a$ (4.4‰) is the fractionation due to diffusion in air (Evans et al.,
1986). The current study uses the photosynthetic fractionation factor $f$
(16.2‰), determined by Evans and von Caemmerer (2013). Following
Tazoe et al. (2009), no fractionation by day respiration is assumed and $e$
is calculated as $\delta^{13}C_{\text{tank}} - \delta^{13}C_{\text{atmosphere}}$ (Wingate et al.
2007). In this study, $\delta^{13}C_{\text{tank}}$ ranged from ~13.3 to ~3‰ and
$\delta^{13}C_{\text{atmosphere}}$ was ~18‰ for plants grown in a growth cabinet with
CO$_2$ enrichment (McNeiv et al., 2007). Evans and von Caemmerer
(2013) solved equation 2 for $g_n^*$, but this study has solved it for the
Rubisco fractionation factor $b$:

$$
b = \frac{\Delta - a' \left( 1 - \frac{C_i}{C_e} \right) + \Delta_n + \Delta_f + \frac{1+t}{1-t} \left( a + \frac{eR_g}{(A+R_g)} \frac{A}{g_n C_e} \right)}{1+t \left( \frac{1}{C_e} - \frac{1}{C_i} \right) - \frac{A}{g_n C_e}}
$$

(5)

where

$$
\Delta_v = \frac{1+t}{1-t} \left( \frac{eR_g}{(A+R_g)C_e} (C_i - \Gamma_r) \right)
$$

(6)

is most of the fractionation associated with respiration and

$$
\Delta_f = \frac{1+t}{1-t} \left( \frac{\Gamma_r}{C_e} \right)
$$

(7)

is the fractionation associated with photosynthesis.

Results
Gas exchange and biochemical properties of tobacco genotypes
This study used five tobacco (N. tabacum L. cv Petit Havana
[N,N]) genotypes: wild-type [tob(Wt)] and transplastomic mutants
producing homodimeric L$_2$ R. rubrum Rubisco [tob(Rr)], Whitney
and Andrews, 2001; tobacco Rubisco containing the L-subunit
Leu-335-Val mutation [tob(L335V)], Whitney et al., 1999, or
producing chimeric Le$_S$ Rubisco comprising tobacco S-subunits and
either the F. bidentis L-subunit [tob(bid)], Whitney et al., 2011b or the
F. floridana L-subunit [tob(flo)], Whitney et al., 2011b. Table 1 summa-
izes in vitro catalytic properties of these enzymes and com-
pares them to the catalytic properties of the native enzyme.

All gas exchange measurements were made at low O$_2$
partial pressure (19 mbar, ~2% atmospheric pO$_2$) to ensure adequate
CO$_2$-assimilation rates could be measured at intercellular CO$_2$
levels between 100 and 800 µbar for all tobacco gen-
types and to minimize photorespiratory fractionation, CO$_2$
response curves of tob(Wt) show a clear transition from a
Rubisco-limited to an RuBP-regeneration-limited response,
whereas the other four genotypes remain Rubisco limited
over the measured range in intercellular pCO$_2$, with lower
CO$_2$-assimilation rates compared to wild type (Fig. 1). In
tob(bid) and tob(flo) leaves, reduced CO$_2$-assimilation rates
were associated with a 2.5-4 fold lower Rubisco content in
their leaves compared to wild type (Table 2 and Fig. 1A).
 Conversely, both tob(Rr) and tob(L335V) had slightly more
Rubisco than wild type on a leaf area basis (Table 2), but
the combination of lower $S_{c/o}$ and reduced carboxylation efficiency ($K_{ccat}/K_c$) resulted in CO$_2$-assimilation rates that
were still carboxylation limited at 800 µbar pCO$_2$ and 19
mbar pO$_2$ (Fig. 1B). Even under these low O$_2$ conditions,
both tob(L335V) and tob(Rr) have higher CO$_2$
compensation points compared with tob(Wt), consistent with their
significantly lower Rubisco CO$_2$/O$_2$ specificity ($S_{c/o}$) and
lower $K_{ccat}/K_c$ ratios (Table 1 and Fig. 1B). Although Rubisco
from tob(bid) and tob(flo) share comparable $S_{c/o}$ values with
tob(Wt) (Table 1), their lower $K_{ccat}/K_c$ ratios increase their
compensation points (Fig. 1A).

Maximum Rubisco activity, $V_{max}$, was estimated from
CO$_2$ response curves using the photosynthetic model of
Farquhar et al. (1980). In vitro Rubisco kinetic con-
stants $K_c$, $K_o$, and $S_{c/o}$ given in Table 1 were used, with

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Table 1. In vitro Rubisco kinetic constants of wild-type tobacco and Flaveria floridana, Flaveria bidentis, Rhodospirillum rubrum, and transplastomic mutants tob(flo), tob(bid), tob(Rr), and tob(L335V)

To convert values from concentrations to partial pressures, solubilities for CO2 of 0.0334 mol (l bar)^{-1} and for O2 of 0.00126 mol (l bar)^{-1} were used.

| Rubisco type         | S_{1/2} (MM^{-1}) | S_{1/6} (bar bar^{-1}) | k_{cat} (s^{-1}) | K_{c} (μM) | K_{o} (μbar) | K_{e} (μbar) | K_{ecat} (s^{-1}) | K_{ocat} (s^{-1}) | Reference       |
|----------------------|-------------------|------------------------|------------------|------------|-------------|-------------|------------------|------------------|-----------------|
| Tobacco              | 81 ± 1            | 2147 ± 27              | 3.2 ± 0.2        | 12.6 ± 0.2 | 377 ± 6     | 0.8         | 274 ± 18         | 217 ± 14         | Whitney et al. (2011) |
| F. floridana         | 82 ± 2            | 2174 ± 53              | 3.6 ± 0.1        | 14.4 ± 0.5 | 431 ± 15    | 1.1         | 374 ± 33         | 297 ± 26         |                 |
| tob(flo)             | 81 ± 2            | 2147 ± 53              | 3.7 ± 0.2        | 14.5 ± 0.3 | 434 ± 9     | 1.2         | 359 ± 22         | 285 ± 17         |                 |
| F. bidentis          | 81 ± 1            | 2147 ± 27              | 4.8 ± 0.3        | 20.4 ± 0.5 | 611 ± 15    | 1.2         | 420 ± 37         | 333 ± 29         |                 |
| tob(bid)             | 79 ± 2            | 2094 ± 53              | 4.7 ± 0.2        | 19.9 ± 0.6 | 596 ± 18    | 1.2         | 408 ± 28         | 324 ± 22         |                 |
| R. rubrum            | 9 ± 0.3           | 239 ± 8                | 12.3 ± 0.3       | 149 ± 8    | 4461 ± 240  | 1.4         | 159 ± 25         | 126 ± 19         | Mueller-Cajar et al. (2007) |
| tob(Rr)              | 12 ± 1            | 318 ± 27               | 5.4 ± 0.3        | 96 ± 5     | 2874 ± 150  | 0.34        | 72 ± 9           | 57 ± 7           |                 |
| tob(L335V)           | 20 ± 2            | 530 ± 53               | 0.8 ± 0.1        | 5.1 ± 0.8  | 153 ± 24    | 0.4         | 49 ± 11          | 38.9 ± 8.7       | Whitney et al. (1999) |

Fig. 1. (A) CO2-assimilation rate, A, as a function of intercellular CO2 partial pressure in tobacco wild type [tob(wt)] and two transplastomic mutants producing large subunits of Flaveria bidentis [tob(bid)] or F. floridana [tob(flo)]. Measurements were made on four tob(wt), three tob(bid), and three tob(flo) replicate plants and bars show standard errors. (B) CO2-assimilation rate, A, as a function of intercellular CO2 partial pressure in tob(wt) and two transplastomic mutants producing R. rubrum Rubisco [tob(Rr)] or tobacco mutant Rubisco [tob(L335V)]. Measurements were made on four tob(Wt), four tob(Rr), and seven tob(L335V) replicate plants and bars show standard errors. Gas exchange measurements were made at various CO2 partial pressures, O2 19 mbar, irradiance 1500 μmol m^{-2} s^{-1}, and leaf temperature 25 °C. Model curves have been fitted to each genotype with the following values from Tables 1 and 2 [except for tob(L335V); see text] and for K_{c} (μbar), K_{o} (μbar), Γ (μbar), R_{o} (μmol m^{-2} s^{-1}) and V_{max} (μmol m^{-2} s^{-1}). In A, for tob(Wt) 377, 217, 4.66, 1.8, 111.8, for tob(flo) 434, 285, 46.6, 1.9, 46.6, for tob(bid) 596, 324, 47.8, 1.3, 43.4, using g_{s}, 0.46 mol m^{-2} s^{-1} bar^{-1}, and J 130.8 μmol m^{-2} s^{-1}. In B, for tob(Wt) 377, 217, 4.66, 1.4, 134.3, for tob(Rr) 2874, 57, 31.44, 0.8, 112.4, for tob(L335V) [in vivo constants used, see text] 318, 55.6, 140, 1.24, 23.4, using g_{s}, 0.29 mol m^{-2} s^{-1} bar^{-1}, and J 115.6 μmol m^{-2} s^{-1}.

Carbon isotope discrimination of tobacco genotypes

This study measured the carbon isotope discrimination (Δ, ‰) concurrently with gas exchange using tuneable laser spectroscopy (Figs 2 and 3). The discrimination by both tob(bid) and tob(flo) was greater than that of tob(Wt) at all pCO2 (Fig. 2A). Under the range of pCO2 examined, carbon isotope discrimination by tob(L335V) was considerably less than tob(Wt) (Fig. 2B). In contrast, tob(Rr) had a greater discrimination at low pCO2 and became more similar to tob(Wt) at high pCO2. Discrimination is also shown against C/Ci concurrently with gas exchange using tuneable laser spectroscopy (Figs 2C and 3). The discrimination by both tob(bid) and tob(flo) was greater than that of tob(Wt) at all pCO2 conditions tested (Fig. 2).

The average values of carbon isotope discrimination at ambient pCO2 are shown in Table 3. Prior studies of carbon isotope discrimination by tobacco showed that Rubisco fractionation (b) was independent of variation in mesophyll conductance, g_m, and similar between the wild-type tob(L335V); see text] and for K_{c} (μbar), K_{o} (μbar), Γ (μbar), R_{o} (μmol m^{-2} s^{-1}) and V_{max} (μmol m^{-2} s^{-1}). In A, for tob(Wt) 377, 217, 4.66, 1.8, 111.8, for tob(flo) 434, 285, 46.6, 1.9, 46.6, for tob(bid) 596, 324, 47.8, 1.3, 43.4, using g_{s}, 0.46 mol m^{-2} s^{-1} bar^{-1}, and J 130.8 μmol m^{-2} s^{-1}. In B, for tob(Wt) 377, 217, 4.66, 1.4, 134.3, for tob(Rr) 2874, 57, 31.44, 0.8, 112.4, for tob(L335V) [in vivo constants used, see text] 318, 55.6, 140, 1.24, 23.4, using g_{s}, 0.29 mol m^{-2} s^{-1} bar^{-1}, and J 115.6 μmol m^{-2} s^{-1}.
Gas exchange and carbon isotope discrimination were measured at ambient CO₂ ~380 μbar, O₂ 19 mbar, irradiance 1500 μmol m⁻² s⁻¹, and leaf temperature 25 °C. Other measurements were made on leaf material harvested from the same leaves after gas exchange measurements. ND, not determined.

Table 2. Gas exchange and biochemical properties of wild-type tobacco and transplastomic mutants tob(Rr), tob(L335V), tob(bid), and tob(flo)

| Parameter | Set 1 | | Set 2 |
|-----------|-------|-------|
|          | tob(Wt) (n=4) | tob(Rr) (n=4) | tob(L335V) (n=7) | tob(Wt) (n=4) | tob(bid) (n=3) | tob(flo) (n=3) |
| CO₂-assimilation rate, A (μmol CO₂ m⁻² s⁻¹) | 26.0 ± 0.8 | 6.6 ± 0.2 | 7.1 ± 0.3 | 30.2 ± 0.9 | 13.4 ± 1.9 | 17.0 ± 0.4 |
| Stomatal conductance (mol m⁻² s⁻¹) | 0.57 ± 0.08 | 0.57 ± 0.04 | 0.31 ± 0.04 | 0.64 ± 0.06 | 0.52 ± 0.07 | 0.74 ± 0.07 |
| Ratio of intercellular to ambient CO₂, C/Cₐ | 0.77 ± 0.03 | 0.93 ± 0.01 | 0.86 ± 0.02 | 0.74 ± 0.03 | 0.86 ± 0.01 | 0.86 ± 0.01 |
| Dark respiration, R₂(μmol CO₂ m⁻² s⁻¹) | 1.4 ± 0.14 | ND | 1.24 ± 0.14 | 1.8 ± 0.3 | 1.3 ± 0.05 | 1.9 ± 0.3 |
| Mesophyll conductance, gₘ | 0.29 ± 0.02 | ND | ND | 0.46 ± 0.07 | ND | ND |
| Rubisco sites (mol CO₂ m⁻² s⁻¹) | 23.1 ± 1.5 | 28.2 ± 1.2 | 32.2 ± 1.8 | 24.7 ± 0.7 | 7.9 ± 0.7 | 10.9 ± 0.3 |
| Maximum Rubisco activity, Vₘₐₓ (μmol CO₂ m⁻² s⁻¹) | 134 ± 6 | 112 ± 2 | 23.8 ± 1 | 116 ± 6 | 43 ± 7 | 44 ± 2 |
| Catalytic turnover of Rubisco in vivo, k₅₅ (s⁻¹) | 5.9 ± 0.3 | 4.1 ± 0.1 | 0.75 ± 0.03 | 4.7 ± 0.2 | 5.4 ± 0.2 | 4.1 ± 0.3 |
| Soluble protein (g m⁻²) | 6.7 ± 0.4 | 6.1 ± 0.2 | 6.7 ± 0.02 | 7.4 ± 0.1 | 7.0 ± 0.2 | 6.6 ± 0.02 |
| Leaf dry mass per unit leaf area (g m⁻²) | 18.2 ± 1.5 | 19.2 ± 1.4 | 22.8 ± 2.3 | 23.1 ± 1.0 | 22.6 ± 1.2 | 25.5 ± 1.8 |

*a* Maximum Rubisco activity, Vₘₐₓ, was estimated from measurements of CO₂ response curves using kinetic parameter values given in the legend of Fig. 1.

*b* k₅₅ was calculated from the ratio of Vₘₐₓ and Rubisco site content measured on individual leaves.

in Fig. 4, which shows that estimated b-values are relatively insensitive to changes in gₘ until it is reduced below 50% of the assumed value, where b increases. If gₘ in the transplastomic lines was 25% less than in wild-type leaves, estimated b-values would increase slightly to 24, 14.3, 28.6, and 29.6‰ for tob(Rr), tob(L335V), tob(bid), and tob(flo), respectively, which is within the margin of error for the values given in Table 3.

Respiratory and photorespiratory fractionations were calculated using equations 6 and 7 (Table 3). Although CO₂-assimilation rates were lower in the four mutant tobacco genotypes (Fig. 1), the respiration rates were similar (Table 2). Consequently, the values of respiratory fractionation (Δₑ) are slightly greater for the mutants compared to tob(Wt). Photorespiratory fractionation (Δₑ) was greater in both tob(Rr) and tob(L335V) because these Rubiscos have lower S₀ values which increases flux through photosynthesis compared to tob(Wt) (Table 3). By contrast, Δₑ was similar in tob(Wt), tob(bid), and tob(flo) because of their similar Rubisco S₅₀ values (Table 3). Together, Δₑ and Δₕ are expected to account for 10–18% of the carbon isotope discrimination signal in tob(Rr) and tob(L335V) compared to 6% for tob(bid) and tob(flo).

The measured Δ values are shown with respect to C/Cₐ (Fig. 3). Theoretical lines are shown which assume infinite mesophyll conductance and ignore the influence of Δₑ and Δₕ. Taking the Δₑ and Δₕ fractionations and mesophyll conductance into account, this study found that estimates of b for tob(Rr) and tob(L335V) were significantly less than the 29‰ assumed for tob(Wt) (Table 3). In contrast, there was no significant difference in the b-values of tob(Wt), tob(bid), and tob(flo).
Tobacco is established as a model species for investigations into photosynthetic metabolism as it is readily transformable via nuclear and transplastomic techniques (Rodermel et al., 1988; Quick et al., 1991; Hudson et al., 1992; Whitney et al., 1999; Maliga, 2002). This study group have extensively characterized gas exchange and carbon isotope discrimination properties in this species (Evans et al., 1986, 1994; Yamori et al., 2010; Tazoe et al., 2011; Evans and von Caemmerer, 2013). While knowing the Rubisco discrimination factor ($b$) is pivotal for fully understanding plant carbon metabolism and the impact of photosynthesis on atmospheric carbon isotope signatures (Suits et al., 2005; Tcherkez et al., 2011), little is known about variation in $b$ as it is difficult to measure using existing in vitro methods (McNevin et al., 2006, 2007). Tuneable diode laser absorption spectroscopy allows rapid measurements of $\Delta$ to be made concurrently with photosynthetic gas exchange. The present study used this technique to estimate $b$ in vivo in a number of transplastomic tobacco genotypes. While the technique is rapid, it relies on understanding the contribution that CO2 diffusion and respiratory metabolism have on photosynthetic carbon isotope discrimination (equations 2 to 7). The impact of respiratory and photorespiratory fractionation was minimized by making measurements under high light and low $p_{O2}$ (Table 3).

Differences in $\delta^{13}C$ values of the source and measuring CO2 also influence $\Delta$, but on average did not vary with genotype. CO2 diffusion has the greatest impact on the interpretation. Lower CO2-assimilation rates in transplastomic tobacco genotypes compared to wild type were not accompanied by proportional reductions in stomatal conductance and this led to greater ratios of intercellular to ambient CO2 ($C_i/C_a$) that increased discrimination (Figs 2 and 3). Similarly, lower CO2-assimilation rates reduced the draw down in $pCO_2$ from

![Graph showing carbon isotope discrimination, $\Delta$, as a function of the ratio of intercellular to ambient CO2 partial pressure for tob(Wt), tob(bid), tob(flo), tob(Rr), and tob(L335V). Lines show theoretical relationships between $\Delta$ and $C_i/C_a$ with different Rubisco discrimination factors ($b$) which assume an infinite $g_m$ and no respiratory fracionations, but include the ternary correction with $t=0.01$ ($\Delta = +\frac{-4.2}{1.02} b^{4.2} C_i/C_a$). Transplastomic mutants are as described for Fig. 1.

![Graph showing carbon isotope discrimination, $\Delta$, as a function of the ratio of intercellular to ambient CO2 partial pressure for tob(Wt) and tob(Rr). The graph shows theoretical relationships between $\Delta$ and $C_i/C_a$ with different Rubisco discrimination factors ($b$) which assume an infinite $g_m$ and no respiratory fracionations, but include the ternary correction with $t=0.01$ ($\Delta = +\frac{-4.2}{1.02} b^{4.2} C_i/C_a$).

Table 3. Leaf carbon isotope discrimination and Rubisco discrimination ($b$) as well as carbon isotope discrimination associated with respiration ($\Delta_r$, equation 6) and photorespiration ($\Delta_f$, equation 7) in wild-type tobacco and transplastomic mutants tob(Rr), tob(L335V), tob(bid), and tob(flo)

| Parameter                                      | Set 1                  | Set 2                  |
|------------------------------------------------|------------------------|------------------------|
|                                                   | tob(Wt) ($n=4$)        | tob(Rr) ($n=4$)        | tob(L335V) ($n=7$) | tob(Wt) ($n=4$)        | tob(bid) ($n=3$)        | tob(flo) ($n=3$)        |
| $\Delta$ (‰)                                   | 16.9±1.2               | 19.4±0.6               | 10.4±0.6            | 16.9±0.6               | 21.6±0.9               | 21.8±0.3               |
| Rubisco discrimination, $b$ (‰)                | 29                     | 23.8±0.7               | 13.9±0.7            | 29                     | 27.8±0.8               | 28.6±0.6               |
| Rubisco discrimination (b) in vitro (McNevin et al. 2007) (‰) | 28.5±0.9               | 23.3±2.1               | 12.3±1.6            |                        |                        |                        |
| $\Delta_r$ (‰)                                 | 0.2±0.01               | 0.5±0.06               | 1.2±0.2             | 0.6±0.1               | 1.2±0.2               | 1.1±0.3               |
| $\Delta_f$ (‰)                                 | 0.1±0.001              | 1.4±0.02               | 0.6±0.003           | 0.2±0.001              | 0.2±0.001              | 0.2±0.001              |

* Rubisco discrimination $b$, was estimated from $\Delta$ measured at ambient CO2 of 380 μbar using equation 5 and the $g_m$ value of the wild-type control (Table 2).

*Expressed here with respect to gaseous CO2.
intercellular airspace to the chloroplasts which would reduce the
effect of mesophyll conductance on the isotope signal. Previous
measurements of transgenic tobacco with reduced amounts of Rubisco were found to have mesophyll conductances about 20–25% less than that of wild-type leaves grown under the same conditions of irradiance, temperature, and ambient CO₂ (Evans et al., 1994). When grown under elevated CO₂, as in the present case, anti- RbcS plants are indistinguishable from wild type in terms of size. Consequently, under these conditions, their mesophyll conductance would be expected to be similar. Mesophyll conductance is influenced by growth irradiance between 0.2 and 0.5 mol m⁻² s⁻¹ bar⁻¹, having been observed for tobacco at 25 °C (Table 2; Evans et al., 1994; Yamori et al., 2010; Evans and von Caemmerer, 2013). It is therefore important to measure wild-type leaves of comparable physiological age and development. Galmes et al. (2013) reported significantly lower gs values calculated from chlorophyll fluorescence for tob(bid) and tob(flo) compared to wild type. Their plants were grown without CO₂ supplementation, but under similar irradiance, photoperiod, temperature, and humidity to this study’s growth conditions. As their values for leaf dry mass per unit area, protein and Rubisco content were similar to the values measured (Table 2), the assumption that this study could use mesophyll conductance obtained from wild-type leaves needs to be kept in mind.

The lower b-values calculated for Rubisco from tob(L335V) and tob(Rr) determined in vivo from TDL measurements match those previously determined by experimentally more demanding in vitro methods for L335V and R. rubrum Rubisco (McNevin et al., 2006, 2007). For both of these enzymes, the kinetic isotopic fractionation signatures provide valuable insights into variations in the Rubisco catalytic mechanism (i.e. the carbon bond-making and -cleavage reactions; Tcherkez et al., 2006; McNevin et al., 2007). Transplastomic modification of other L-subunit amino acids that influence the carboxylation, decarboxylation, and hydrolysis/cleavage steps of Rubisco pose a useful approach for further dissection of the mechanistic features of Rubisco catalysis. It is also feasible that examining variation in ¹³C fractionation among catalytically and phylogenetically diverse Rubiscos by a transplastomic approach, such as tob(bid) and tob(flo), may also be useful in identifying mechanisms that underlie the natural variation in Rubisco catalysis. The method used here for measuring carbon isotope discrimination by leaves during photosynthesis is experimentally robust and simple. However, it requires the generation of photoautotrophic transplastomic lines suitable for leaf gas exchange analysis. This has been challenging for some tobacco L-subunit mutations and some heterologous Rubisco isoforms where limitations in the folding and assembly requirements cannot be met by tobacco chloroplasts, thereby either restricting or preventing recombinant Rubisco biogenesis (Whitney et al., 2001, 2011a; Parry et al., 2013). As shown here for all four tobacco transplastomic genotypes, even if the introduced changes to Rubisco impair its synthesis [tob(bid) and tob(flo)] or compromise catalytic activity [tob(L335V) and tob(Rr)], these can be compensated by growth at elevated pCO₂ to enable photoautotrophic growth to maturity in soil. Gas exchange conditions can be chosen to suit the modified catalytic properties to allow concurrent assessment of carbon isotope discrimination.

Prior assessment of the hybrid Rubiscos in tob(bid) and tob(flo) showed their catalytic properties matched those of the parental F. bidentis and F. floridana Rubiscos (Whitney et al., 2011b). Catalytic properties of hybrid enzymes containing tobacco S-subunits and L-subunits from either sunflower or tomato Rubisco also reflected those of the L-subunit (Sharwood et al., 2008). However, the S-subunits of Rubisco have also been shown to influence catalytic properties. Ishikawa et al. (2011) produced hybrid Rubisco with rice L-subunits and sorghum S-subunits which increased both Kc and kcat compared to wild-type rice. The b-values determined for Rubisco in tob(bid) and tob(flo) matched the wild type, suggesting that, despite the C₄-like catalysis of the hybrid Rubisco in tob(bid) (i.e. increased kcat and Kc; Table 1), there is little or no variation in the carbon isotope discrimination by these C₃, C₄-C₃, and C₄ Rubiscos in vivo. Whelan et al. (1973) measured higher average b-values for Sorghum bicolor Rubisco (33.7 ± 6.6‰), although statistically this overlaps the range of b-values calculated here for tob(bid), tob(flo), and tob(Wt). Improving the rigor of inferring Rubisco mechanistic variations from Δ measurements clearly requires reliable measurement of this parameter for Rubisco isoforms with broader catalytic spectrums (Tcherkez et al., 2006; McNevin et al., 2007). As shown here, transplastomic introduction of C₄-Rubiscos into tobacco plastids provides a feasible strategy to investigate the natural diversity in b-values for C₄ Rubiscos that are otherwise impossible to measure by in vivo approaches due to the presence of their CO₂-concentrating mechanisms. Expanding this transplastomic approach to include the catalytically distinctive Rubiscos from phylogenetically diverse sources (such as non-green algae and cyanobacteria) currently remain stymied by limitations in their folding and assembly in plant chloroplasts (Kanevski et al., 1999; Whitney et al., 2001).
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