Acclimation of Rubisco specificity factor to drought in tobacco: discrepancies between \textit{in vitro} and \textit{in vivo} estimations

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Abstract

In studies about the photosynthesis response to environmental stresses, such as drought, the Rubisco specificity factor (\(\tau\)) is assumed to be constant or derived indirectly from gas exchange measurements. However, an analysis of the acclimation of \(\tau\) to drought using \textit{in vitro} determinations is lacking. The aim of the present work was to analyse the acclimation of \(\tau\) to different drought intensities in tobacco (\textit{Nicotiana tabacum} L.). Potted tobacco plants were subjected to three different water regimes (100%, 40%, and 15% of field capacity) and new leaves were allowed to develop. When acclimated leaves were fully developed, they were sampled for gas exchange and chlorophyll fluorescence measurements, as well as for the \textit{in vitro} analysis of Rubisco kinetic properties. Relative water content and gas exchange decreased with increasing water shortage. The apparent Rubisco specificity factor as estimated \textit{in vivo} by gas exchange decreased with water stress. However, \textit{in vitro} estimates of \(\tau\) were identical among treatments, as were Rubisco specific initial activity and activation state. The reasons for the observed discrepancy between \textit{in vitro} and \textit{in vivo} estimates are profusely discussed. It is suggested that the Rubisco specificity factor does not acclimate to water stress in the short term (weeks or months) in tobacco, and the validity of the so-called Laisk gas exchange method to estimate \(\tau\) under drought is questioned.

Key words: Acclimation, drought, \textit{Nicotiana tabacum}, Rubisco, specificity factor, water stress.

Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) plays a central role in plant photosynthesis since it is involved in the uptake of CO\(_2\) by photosynthetic organisms. Besides its carboxylase activity, Rubisco also acts as an oxygenase in a reaction involving competition between O\(_2\) and CO\(_2\) for reaction with ribulose-1,5-bisphosphate (RuBP). Thus, while photosynthesis is initiated by the carboxylase activity, the oxygenase activity catalyses the first reaction in the photorespiratory pathway (Ogren and Bowes, 1971; Laing \textit{et al.}, 1974). The balance between the two competitive reactions is determined by the kinetic properties of Rubisco and the CO\(_2\) and O\(_2\) concentrations at the site of the enzyme (Laing \textit{et al.}, 1974):

\[
\frac{v_c}{v_o} = \left(\frac{V_c}{V_o}\right)\frac{[CO_2]}{[O_2]}
\]

where \(v_c\) and \(v_o\) are the velocities of carboxylation and oxygenation, respectively, \(V_c\) and \(V_o\) are the maximal velocities of the two reactions, and \(K_c\) and \(K_o\) the Michaelis constants for CO\(_2\) and O\(_2\). The substrate specificity factor, \(V_c/K_c/V_o/K_o\) (\(\tau\)), determines the relative rates of the two reactions at any given CO\(_2\) and O\(_2\) concentrations.

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Abbreviations: \(A_{lb}\) light-saturated net photosynthesis; \(A_{SAT}\) light- and CO\(_2\)-saturated photosynthesis; \(C_a\) atmospheric CO\(_2\) concentration; \(C_c\) CO\(_2\) concentration at the carboxylation site; \(C_s\) substomatal CO\(_2\) concentration; \(C_{ci}\) at the CO\(_2\) compensation point in the absence of mitochondrial respiration; ETR, electron transport rate; \(g_m\) mesophyll conductance; \(g_{st}\) stomatal conductance; LMA, leaf mass area; PGA, phosphoglyceric acid; \(R_d\) leaf respiration in the dark; \(R_l\) leaf respiration in the light; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; RWC, relative water content; \(\tau\), Rubisco specificity factor; \(\tau_c\), the apparent Rubisco specificity factor operating \textit{in vivo} on a \(C_c\) basis; \(\tau_o\), the apparent Rubisco specificity factor operating \textit{in vivo} on a \(C_o\) basis; \(V_c\), \(V_o\) maximum rates of carboxylation.

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Current models of leaf photosynthesis assume constant $\tau$ values among C$_3$ plants (Long and Bernacchi, 2003) or derive them indirectly from the CO$_2$ compensation point in the absence of mitochondrial respiration ($\Gamma^*$) after gas exchange measurements (Bernacchi et al., 2001). However, $\tau$ may not be constant under varying environmental conditions, such as light quality and quantity or water stress. Although there has been no attempt to assess this possibility. However, the molecular basis and/or structural features determining Rubisco $\tau$ are still poorly understood (Spreitzer and Salvucci, 2002; Andersson and Taylor, 2003; Spreitzer, 2003). The Rubisco holoenzyme of higher Rubisco is a hexadecamer described as four dimers of large subunits surrounded by two tetramers of small subunits. Two active sites are found at the interface of the large subunits in each dimer, i.e. eight catalytic sites per holoenzyme (Andersson and Taylor, 2003). In higher plants, the Rubisco large subunit is encoded by multiple identical copies of $rbcL$ in the chloroplast genome (Eilenberg et al., 1998), whereas an $rbcS$ gene family having 5–12 nuclear genes encodes small subunit peptides (Dean et al., 1989; Spreitzer, 2003). Thus, while the copies of the large subunit would probably be the same, the differential expression of $rbcS$ genes may depend on the environment. For instance, transcription of specific $rbcS$ genes appears to be dependent on light quality in the fern Pteris vittata (Eilenberg et al., 1990). Although large subunits have the main influence on catalytic properties, the Rubisco small subunits have also been hypothesized to affect key kinetic characteristics like $\tau$ (Roy and Andrews, 2000; Parry et al., 2003). Hence, in principle, any environmental condition capable of modulating $rbcS$ gene expression could putatively induce changes in Rubisco $\tau$.

Water stress, in particular, induces stomatal closure and a decrease in leaf internal CO$_2$ concentration, which results in increased oxygenase over carboxylase activity, thus increasing the ratio of photosynthesis to respiratory activity (Flexas and Medrano, 2002). As a consequence, Delgado et al. (1995) and Kent and Tomany (1995) hypothesized that in hot environments associated with water stress, that in hot environments associated with water stress, stomatal closure and low CO$_2$ concentrations at the site of Rubisco may impose increased selection pressure on Rubisco for improved specificity. Indeed, recent results by Galmés et al. (2005) showed that, in a survey of Mediterranean plant species, the highest values of $\tau$ were observed in Rubisco from plant species growing and adapted for growth in the driest environments. Despite these facts, when applying photosynthesis models to study the effects of water stress on photosynthetic limitations and/or on mesophyll conductance to CO$_2$ ($g_m$), Rubisco $\tau$ (or $\Gamma^*$) is usually assumed to be constant (Roupasard et al., 1995; Wilson et al., 2000; Xu and Baldocchi, 2003; Peña-Rojas et al., 2004; Warren et al., 2004). However, Bota et al. (2002) and Warren et al. (2004) have reported an apparent increase of $\Gamma^*$ under water stress. This fact merits some attention, since it may change substantially the interpretations of water stress on photosynthesis coming from studies in which $\Gamma^*$ was assumed as constant. Fluorescence estimates of $g_m$, in particular, are very sensitive to $\Gamma^*$ (Harley et al., 1992). However, estimations of $\Gamma^*$ under water stress include a number of assumptions that may not be correct and, as noted by Warren et al. (2004), it would be preferable to determine Rubisco $\tau$ (or $\Gamma^*$) independently using an alternative method. The aim of the present study is to determine whether Rubisco $\tau$ acclimates to water stress in the short term (weeks) in tobacco plants, by determining $\tau$ in vitro, and comparing it with in vivo estimates of $\Gamma^*$.

Materials and methods

**Plant material and treatments**

Thirty seeds of Nicotiana tabacum var. White Burley were germinated and grown individually in pots (20 cm height, 4.1 l volume) containing a mixture of clay–calcareous soil, horticultural substrate, and perlite (40:40:20 by vol.). The experiment was performed during spring 2004 inside a greenhouse located at the University of the Balearic Islands (Mallorca, Spain). Plants were randomly distributed for growing and spread out to avoid mutual shading and to give similar light and temperature.

All seedlings were well-watered until they had four fully expanded leaves and, presumably, an adequate root system to cope with water constraints. Irrigation treatments were started on 22 April 2004. Ten such plants were randomly selected for each of the irrigation treatments: (i) maintained at field capacity during all experiment (control treatment, C), (ii) maintained at 40% field capacity (moderate drought treatment, MD), and (iii) maintained at 15% field capacity (severe drought treatment, SD). The desired moisture levels were attained by allowing the soil to dry until close to the selected moisture level, as determined gravimetrically by weighing pots on alternate days and, from then on, compensating their daily water losses with the addition of an equal amount of Hoagland’s solution.

New leaves were allowed to develop and expand under the three irrigation treatments until 15 June 2004. Then, leaves developed during the irrigation treatments (i.e. acclimated to different water availability) were sampled to determine the leaf mass area (LMA), the relative water content (RWC), and $\tau$, total leaf soluble protein, Rubisco activity, gas exchange, and chlorophyll fluorescence.

**Rubisco purification and specificity factor measurement**

Leaves (30–50 g) of each treatment (C, MD, and SD) were collected and immediately frozen in liquid nitrogen. The leaf material was ground to a powder in a mortar, buffer was added and grinding continued from time to time as the mixture thawed. The protein extraction media used contained: 0.1 M Bicine, 10 mM Na-DIECA, 6% PEG (polyethylene glycol) 4000, 3% (w/v) PVP (polyvinylpyrrolidone) 25 000, 1 mM DTT (dithiothreitol), 1 mM benzamidine, 1 mM $\epsilon$-amino-$n$-caproic acid, and 1 mM PMSF (phenylmethylsulfonylfluoride), at pH 8.

All the purification steps were carried out at 0–4 °C. Fully thawed but still cold homogenates were filtered through butter muslin and centrifuged at 18 000 g for 20 min. The supernatant liquid was decanted through 50 µm nylon mesh and PEG 4000 was added as a 60% aqueous solution to the supernatant liquid to produce a final concentration of 20% w/v. Also, 1 M MgCl$_2$ was added to a final concentration of 20 mM followed by gentle mixing. After standing...
for 10 min the mixture was centrifuged again at 18 000 g for 20 min. The pellet was resuspended in 6 ml of column buffer (10 mM TRIS pH 8.0 with 10 mM MgCl₂, 10 mM NaHCO₃, 1 mM EDTA, and 1 mM KH₂PO₄ containing 1 mM each of DTT, PMSF, benzamidine, and ε-amino-n-caproic acid. The suspension was then centrifuged to remove insoluble material. The supernatant liquid was layered onto step gradients from 1.2 M to 0.4 M in sucrose in the column buffer. Gradients were centrifuged at 50 000 rpm for 120 min in a 70.1Ti rotor (Beckman, High Wycombe, UK). Fractions with a high protein concentration were combined and applied to two 1 ml Hitrap Q HP columns (Amersham Biosciences) connected in series previously equilibrated with column buffer and operated at 1 ml min⁻¹. The proteins were eluted using a step gradient from 0 M to 0.8 M NaCl in column buffer and fractions were collected in 1 ml intervals. Total soluble protein content in fractions was confirmed using the Bradford assay (Bradford, 1976). Rubisco-rich fractions were used to make 8–10 measurements of Rubisco specificity factor (τ) per treatment at 25 °C as follows. Rubisco solutions with a high protein concentration were desalted by centrifugation through G25 Sephadex columns (Helmerhorst and Stokes, 1980) previously equilibrated with CO₂-free 0.1 M bicine pH 8.2 containing 20 mM MgCl₂. The desalted solutions were made 10 mM with NaH¹⁴CO₃ and 0.4 mM with orthophosphate. These mixtures were incubated at 37 °C for 40 min to activate the Rubisco. Reaction mixtures were prepared in an oxygen electrode (Dual digital Model 20, Rank Brothers Ltd., Cambridge, UK) by first adding 0.93 ml of a solution of 100 mM bicine pH 8.2, 10 mM MgCl₂ containing 1.5 mg (7000 W-A units) per 100 ml of carbonic anhydrase and equilibrated with CO₂-free air at 25 °C. After adding 0.02 ml of 0.1 M NaH¹⁴CO₃ the plug was fitted to the oxygen electrode vessel. Enough activated Rubisco was then added to 40 μl for the reaction to be completed in 5 min. When the signal from the electrode was steady, the reaction was started by the addition of 10 μl of 15 mM RuBP. The final volume of the reaction mixture was 1 cm³. RuBP oxygenation was calculated from the oxygen consumption and carboxylation from the amount of¹⁴C incorporated into PGA when all the RuBP had been consumed (Parry et al., 1989). A sequence of reaction mixtures containing pure wheat Rubisco were interspersed with those containing Rubisco from the tobacco plants and the results were normalized to the average τ value obtained from wheat Rubisco (102.5 at 25 °C).

Rubisco carboxylase activity and total soluble protein
For Rubisco carboxylase activity, 3–4 samples per treatment were ground to a fine powder in a mortar, previously chilled with liquid nitrogen and homogenized in 1 ml of an ice-cold extraction medium. The extraction medium was the same as that used for Rubisco purification for τ measurements. Extracts were clarified by centrifugation (12 000 rpm at 4 °C for 2 min) and the supernatant immediately assayed at 25 °C for Rubisco activity. The initial and total activities were determined according to Parry et al. (1997). Total soluble protein was determined according to the method of Bradford (1976).

Relative water content and leaf mass area
RWC at mid-morning was determined as follows: RWC=[(Fresh weight–Dry weight)/(Turgid weight–Dry weight)]. To determine the turgid weight of the leaves, these were kept in distilled water in darkness at 4 °C to minimize respiration losses, until they reached a constant weight (full turgor, typically after 24 h). Their dry weight was obtained after 48 h at 70 °C in an oven. Six replicates per treatment were obtained.

LMA was calculated, in six replicates per treatment, as the ratio of dry mass to leaf area. Leaf area was determined in fresh leaves using an AM-100 leaf area meter (ADC, Herts, UK).

Gas exchange and chlorophyll fluorescence measurements
Leaf gas exchange parameters were measured simultaneously with measurements of chlorophyll fluorescence using an open gas exchange system (Li-6400; Li-Cor, Inc., Nebraska, USA) with an integrated fluorescence chamber head (Li-6400–40 leaf chamber fluorometer; Li-Cor, Inc.).

In light-adapted leaves, the actual photochemical efficiency of photosystem II (ϕPSII) was determined by measuring steady-state fluorescence (F₃) and maximum fluorescence during a light-saturating pulse of c. 8000 μmol m⁻² s⁻¹ (F₆₅₀) following the procedures of Genty et al. (1989):

ϕPSII = (F₆₅₀ – F₃)/F₆₅₀

The electron transport rate (ETR) was then calculated as:

ETR = ϕPSII × PFD × α

where PFD is the photosynthetically active photon flux density and α is a term which includes the product of leaf absorbance and the partitioning of absorbed quanta between photosystems I and II. α was previously determined for each treatment as the slope of the relationship between ϕPSII and ϕCO₂ obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O₂ (Valentini et al., 1995). α resulted as 0.357, with no difference between treatments.

All measurements were started at 25 °C and at 1000 μmol m⁻² s⁻¹ to ensure light saturation, with 10% blue light. Cuvette CO₂ concentration (C₄) was set at 400 μmol CO₂ mol⁻¹ air⁻¹ and the vapour pressure deficit was maintained between 1.0 and 1.5 kPa. After inducing photosynthesis under the above conditions and once steady-state was reached, photosynthesis response curves to varying substomatal CO₂ concentration (C₄) were performed. First, the C₅ was lowered stepwise from 400 to 50 μmol mol⁻¹ and then fixed again at 400 μmol mol⁻¹ until reaching a steady-state value similar to that obtained at the beginning of the curve. Then, C₅ was increased stepwise from 400 to 1500 μmol mol⁻¹. Gas exchange measurements were determined at each step after maintaining the leaf for at least 5 min at the new C₅. Measurements consisted of 12–13 measurements for each curve. Leaf respiration in the dark (R₆₅₀) was measured at the same temperature and CO₂ concentration in the same leaves after keeping them for 30 min in darkness.

The rate of non-photorespiratory CO₂ evolution in the light (R₅) and the substomatal CO₂ compensation point in the absence of mitochondrial respiration (C₅') were estimated according to the method of Laik (1977) as described by Brooks and Farquhar (1985), i.e. from the response of A₅ to C₅ at three different irradiances (750, 150, and 50 μmol m⁻² s⁻¹). Four to five replicates were made for each treatment. Finally, C₅' was converted to the chloroplast CO₂ compensation point (Γₐ) considering the effects of the internal diffusion conductance and R₅ in von Caemmerer (2000):

Γₐ = C₅' + R₅

The apparent Rubisco specificity factor operating in vivo (τₐ) was determined by the properties of Rubisco (Brooks and Farquhar, 1985) as:

τₐ = 0.5O

where O represents the oxygen molar fraction at the oxygenation site.

Cuticular conductance to H₂O was measured in four replicates per treatment as described by Boyer et al. (1997). The results showed decreasing values as drought intensified (10 mmol m⁻² s⁻¹ in C, 13 mmol m⁻² s⁻¹ in MD, and 16 mmol m⁻² s⁻¹ in SD). These values were used to correct all C₅ values and subsequent estimations.
Net photosynthesis and C_i estimations are also affected by lateral CO_2 diffusion through the IRGA’s chamber gaskets, particularly when CO_2 concentrations inside and outside the chamber are substantially different, as during measurements of A_N–C_i curves (Long and Bernacchi, 2003). To establish the magnitude of gasket leakage and correct A_N and C_i values, CO_2 response curves were made using an inert tobacco leaf thermally killed by submerging it in boiling water. The leaf was submerged in boiling water for some seconds, and then its variable chlorophyll fluorescence in the dark-adapted state was measured using a PAM-2000 (Walz, Effeltrich, Germany). This procedure was repeated until no variable chlorophyll fluorescence was detected (typically a few minutes after initiating boiling), which was taken as an evidence for total photosynthesis impairment and leaf death (Schreiber et al., 1998). Using the relationship between C_a and the ‘apparent’ photosynthesis of a chamber filled with a dead leaf, the actual A_N of live leaves was obtained at each C_a by simple subtraction of the leak flow to the obtained value. Using the corrected A_N values, C_i was recalculated using the manufacturer formulas (Long and Bernacchi, 2003).

### Estimation of C_c and g_m

From combined gas-exchange and chlorophyll fluorescence measurements, the CO_2 concentration in the chloroplasts (C_c) was calculated according to Epron et al. (1995). This model works on the assumption that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, the electron transport rate (ETR) measured by chlorophyll fluorescence can be divided into two components: ETR=ETR_A+ETR_P, where ETR_A is the fraction of yield of electron transport. Thus, the electron transport rate (ETR) assumption that all the reducing power generated by the electron transport (ETR), maximum rate of carboxylation (V_c,max), Rubisco specific initial activity, total soluble protein, and dark respiration (R_D). C_plants maintained at field capacity during all experiments (control). MD: plants maintained at 40% field capacity (moderate drought treatment). SD: plants maintained at 15% field capacity (severe drought treatment). Data are given by means and standard deviation. Different letters denote statistical differences at P <0.05 for a given parameter among treatments by Duncan Analysis.

### Results and discussion

#### Acclimation to water stress: water relations and photosynthetic parameters

Despite the acclimation of the leaves to water deficit, a decreased water supply resulted in large decreases in RWC, from 83.4% in C to 75.0 and 65.1% in MD and SD treatments, respectively (Table 1). As expected, A_N and g_s also decreased with decreasing water availability (Table 1). A_N decreased from 23.3 μmol CO_2 m^{-2} s^{-1} in C to 16.9 μmol CO_2 m^{-2} s^{-1} in MD and 7.9 μmol CO_2 m^{-2} s^{-1} in SD, while the stomatal conductance to CO_2 (g_s) decreased from 230 mmol CO_2 m^{-2} s^{-1} in C to 119 mmol CO_2 m^{-2} s^{-1} in MD and 46 mmol CO_2 m^{-2} s^{-1} in SD, ETR decreased to a much lesser extent than A_N, from 191 in C to 139 μmol m^{-2} s^{-1} in SD, possibly indicating an increased electron partitioning towards sinks other than photosynthesis, mainly photosuperoxide (Cornic and Massacci, 1996; Flexas and Medrano, 2002). Acclimation to drought resulted in an increased LMA and a somewhat increased total soluble protein content (Table 1). However, the Rubisco specific initial activity and its activation state were similar in all treatments (Table 1), as usually observed in water stress experiments, except when stress is very severe (Flexas et al., 2004). The procedure used to extract Rubisco resulted in a certain loss of the enzyme during extraction, which could explain the low in vitro Rubisco carboxylation activities compared with the maximum rate of carboxylation estimated in vivo (V_c,max), as already discussed by Bota et al. (2004) and Rogers et al. (2001). R_D remained similar (2.4±0.2 μmol CO_2 m^{-2} s^{-1}) for all treatments.

Water stress also resulted in significantly different A_N–C_i responses (Fig. 1). The operational C_i (i.e. the C_i at atmospheric C_a) declined as water stress intensified, from 261 μmol mol^{-1} in C to 170 μmol mol^{-1} in MD and 53 μmol mol^{-1} in SD, pointing to an increased stomatal limitation to photosynthesis (Cornic and Frenieu, 2002). However, light- and CO_2-saturated photosynthesis (A_SAT) also decreased apparently from 27.7 in C to 19.3 and 13.0 in MD and SD treatments, respectively, which suggested an increased non-stomatal limitation to photosynthesis in the stressed leaves (Lawlor, 2002). In addition, both in MD and SD leaves the maximum A_N value was attained at an intermediate C_s, suggesting that photosynthesis at high

### Table 1. Fluorescence, gas exchange and Rubisco assays of tobacco treatments

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>MD</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_N (μmol CO_2 m^{-2} s^{-1})</td>
<td>30.3±1.4 a</td>
<td>23.3±0.9 c</td>
<td>230±12 c</td>
</tr>
<tr>
<td>V_c,max (μmol CO_2 m^{-2} s^{-1})</td>
<td>190±17 a</td>
<td>167±4 b</td>
<td>230±12 c</td>
</tr>
<tr>
<td>g_s (mmol CO_2 m^{-2} s^{-1})</td>
<td>230±12 c</td>
<td>191±10 c</td>
<td>190±17 a</td>
</tr>
<tr>
<td>ETR (μmol e^{-} m^{-2} s^{-1})</td>
<td>167±4 b</td>
<td>191±10 c</td>
<td>190±17 a</td>
</tr>
<tr>
<td>R_D (μmol m^{-2} s^{-1})</td>
<td>2.7±0.1 a</td>
<td>2.7±0.1 a</td>
<td>2.7±0.1 a</td>
</tr>
<tr>
<td>Total soluble protein (g m^{-1} protein s^{-1})</td>
<td>7.2±0.6 a</td>
<td>8.6±0.3 b</td>
<td>7.8±0.3 ab</td>
</tr>
<tr>
<td>R__D (μmol CO_2 m^{-2} s^{-1})</td>
<td>2.5±0.4 a</td>
<td>2.1±0.2 a</td>
<td>2.4±0.2 a</td>
</tr>
</tbody>
</table>
Cᵢ was limited by triose phosphate use (Long and Bernacchi, 2003), a situation sometimes observed under water stress (Flexas et al., 2004). The maximum carboxylation capacity did not differ significantly (P > 0.05) among treatments (Table 1). All the plants maintained their operational Cᵢ at the breakpoint between CO₂-limited and RuBP- and/or TPU-limited photosynthesis, which has been interpreted as an acclimation to optimize both the photochemical and biochemical photosynthetic reactions (von Caemmerer and Farquhar, 1984).

However, the validity of A₅–Cᵢ analysis has been questioned, particularly in water-stress experiments where gₘ usually decreases in parallel with gₛ (Flexas et al., 2002, 2004; Centritto et al., 2003; Warren et al., 2004), as actually occurs in the present study (Fig. 2). These results are very similar to those often encountered using other methods for the estimation of gₘ that do not rely on previous knowledge of the in vitro τ, which supports the validity of our estimations (Loreto et al., 1992; Evans and Loreto, 2000; Flexas et al., 2004). It may be argued, however, that gₘ estimations (and A₅–Cᵢ curves) are not reliable under drought due to the incorrect estimations of Cᵢ associated with heterogeneous stomatal closure (Terashima, 1992; Buckley et al., 1997; Mott and Buckley, 1998). However, from combined gas-exchange and chlorophyll fluorescence measurements, A₅–Cᵢ curves can be obtained that are totally independent of Cᵢ estimations (Sánchez-Rodríguez et al., 1999).

Clearly, all the differences observed between treatments in the A₅–Cᵢ curves disappeared in the A₃–Cᵢ curves (Fig. 3), as already shown by Sánchez-Rodríguez et al. (1999) in water-stressed *Casuarina equisetifolia*. This is consistent with the constancy of Rubisco Vₑ,max and activation state, determined in vitro (Table 1). The only difference between treatments is the fact that MD and SD leaves attain, for the same range of Cₛ, maximum Cₑ values much lower than C leaves, due to the superimposed decreasing stomatal and mesophyll conductances to CO₂. It is interesting to note that, in A₅–Cᵢ curves, CO₂-limited and RuBP-limited regions can be differentiated only in well-watered plants, which maintain their operational Cₑ at the breakpoint between these two limitations. By contrast, MD and SD operate at the CO₂-limited region. This is due to the superimposition of two different effects, a decrease of average gₘ due to water stress and a more dynamic decrease of gₘ with increasing Cᵢ during A₅–Cᵢ curves measurements, as already demonstrated by Centritto et al. (2003).

In vivo and in vitro estimations of Rubisco specificity

The rate of respiration in the light (Rₐ) was estimated following the method described by Laisk (1977) and Brooks and Farquhar (1985). Rₐ was strongly reduced compared with Rₛ, as usually observed (Villar et al., 1994, 1995). In addition, significant differences (P < 0.05) were observed in Rₐ between plants subjected to SD (0.2 μmol m⁻² s⁻¹) and to MD and C (0.5 μmol m⁻² s⁻¹) (Table 2). It is worth noting that Rₐ estimations are strongly affected by lateral leakage through the IRGA’s chamber gaskets as well as by cuticular conductance to CO₂ and water. Thus, without correcting A₅ and Cᵢ values to account for these effects, in C plants Rₐ resulted in 1.1 μmol CO₂ m⁻² s⁻¹ but decreased to 0.5 μmol CO₂ m⁻² s⁻¹ when corrections were taken into account. Similar reductions were observed in MD and SD plants (not shown). The same method yields a value of Cᵢ at the intersection of the three A₅–Cᵢ curves (Cᵢ'), which has been used as a proxy for the CO₂ compensation point in the absence of mitochondrial respiration (Brooks and Farquhar, 1985; Villar et al., 1994, 1995). As for Rₐ, Cᵢ' estimations are also strongly
affected by lateral leakage through the IRGA’s chamber gaskets as well as by cuticular conductance to CO₂ and water. For instance, Cᵢ was in 39.5 µmol mol⁻¹ in C plants before corrections, while it was only 36.5 µmol mol⁻¹ after corrections. The magnitude of these differences was even larger in water-stressed plants. In SD, for instance, a value of 73.4 µmol mol⁻¹ was obtained prior to corrections compared with 56.7 µmol mol⁻¹ after corrections. Hence, when using the Laisk method to estimate both Rᵢ and Cᵢ, special care needs to be taken to determine leakage and cuticular conductance accurately, particularly in water-stressed plants.

Even taking into account the corrections, Cᵢ strongly differed among treatments (Table 2), from 36.5 µmol mol⁻¹ in C to 39.8 µmol mol⁻¹ in MD and up to 56.7 µmol mol⁻¹ in SD. This pattern is in accordance with that observed by Bota et al. (2002) and Warren et al. (2004) in grapevines and Douglas-fir, respectively, and may be interpreted as an indication that water stress induces changes in the Rubisco specificity factor (see estimations of in vitro apparent τᵢ from Cᵢ values in Table 2). However, it has been pointed out that a more precise estimation of the CO₂ photo-compensation point in the absence of respiration (Γ*) should take into account respiratory CO₂ flux and mesophyll conductance to CO₂ (von Caemmerer, 2000; Peisker and Apel, 2001). Warren et al. (2004) suggested that the fact that Cᵢ estimations do not take into account variable gₘ could be on the basis of the observed discrepancies between in vivo and in vitro estimations. However, when Γ* was estimated using the reported values of Cᵢ, Rᵢ, and gₘ, the discrepancies between treatments were even larger (Table 2).

In vivo apparent τ (τᵥᵢ) estimated from Γ* according to Brooks and Farquhar (1985), resulted in the following values: 103.1 in C, 92.4 in MD, and 62.1 in SD (Table 2). However, when τ was measured in vitro there were no significant differences between treatments (Table 2). The in vitro τ averaged 99.3, which did not differ significantly from that of wheat. Therefore, τᵥᵢ largely underestimated τ in SD, and resulted in being similar to it in C and MD. An improved Rubisco specificity has recently been reported for Mediterranean species inhabiting the driest and hottest areas (Galmès et al., 2005), however, the present study demonstrates that in vitro Rubisco τ is stable during acclimation to drought, which is in accordance with the use of stable values of Γ* (Roupsard et al., 1996).

Possible causes to explain the observed discrepancies between in vivo and in vitro estimations of Rubisco specificity

The large difference observed between the in vitro and the in vivo τ and the fact that in vivo estimations show a water-stress effect of Rubisco specificity factor, while in vitro estimations show a constant value, suggests that one of the two methods is not reliable, particularly under water-stress conditions. While, a priori, the misleading method could either be the in vitro or the in vivo one, the former is a well-established enzymatic method while the latter is very sensitive to small errors in the calculation of Aₛ, Cᵢ, Rᵢ, or gₘ, of which a number has been described (Long and Bernacchi, 2003). Therefore, the authors believe that the differences come from the fact that the Laisk method, although yielding quite approximate estimates in control plants, is unable to cope with the large number of different small errors that occur in the calculation of the above parameters in drought-stressed plants. The differences between Cᵢ and Γ* for a given treatment are relatively small compared with the large differences in both parameters between treatments (Table 2). Hence, misleading estimations of Rᵢ or gₘ cannot explain the observed
discrepancies between the in vivo and in vitro methods. Rather, these arise from differences in $C_i^*$, which is strongly dependent on the correct estimations of $A_N$ and, particularly, $C_i$. Among the reported limitations that could affect the estimations of $A_N$ and $C_i$ using commercial gas-exchange systems, therefore possibly inducing the observed discrepancies between the in vivo and in vitro methods, are (i) interference of cuticular conductance (Boyer et al., 1997), (ii) leaks through the IRGA chamber gaskets (Long and Bernacchi, 2003), (iii) heterogeneous (“patchy”) stomatal closure (Terashima, 1992; Buckley et al., 1997; Mott and Buckley, 1998), (iv) edge effects (Pons and Welschen, 2002), and (v) lateral flux of CO$_2$ through the internal air space (Jahnke and Krewitt, 2002; Pieruschka et al., 2005).

Cuticular conductance may have a great effect on $C_i$ at low $g_s$, but this was measured and the values corrected accordingly, as was leakage (see Materials and methods). This leaves patchy stomatal closure, edge effects, and lateral diffusion in the mesophyll as the most likely causes for the observed bias.

Patchy stomatal closure is known to impair $C_i$ calculations and to occur under certain water-stress situations (Terashima, 1992; Buckley et al., 1997; Mott and Buckley, 1998). However, it occurs mostly when stress is applied rapidly, and not under progressive water stress (Gunasekera and Berkowitz, 1992). Hence, it is unlikely that the leaves used here, which have been stress-acclimated for their entire life, suffer patchy stomatal closure. Even if some was present, its effects on $C_i$ estimations would be relatively minor, since these are important only when $g_s$ is lower than 0.03 mol H$_2$O m$^{-2}$ s$^{-1}$ (Buckley et al., 1997; Flexas et al., 2002), but perhaps important for the determination of $\Gamma^*$, where the observed range of values was small (about 20 μmol mol$^{-1}$ of difference between C and SD plants, compared with a difference in operational $C_i$ of about 200 μmol mol$^{-1}$).

Edge effects come from the fact that, due to design of leaf chambers, the gaskets cause the photosynthesizing surface to be surrounded by tissue in darkness that is respiring. This respired CO$_2$ will decrease the measured net flux, i.e. lead to underestimated $A_N$ and, consequently, erroneous $C_i$. Pons and Welschen (2002) showed that the effect was important at low light (about 25% underestimated of $A_N$) but negligible at high light. Therefore, this effect may change the slope of the $A_N$–$C_i$ curve at low light but not at high light, hence probably modifying the interception point and thus affecting $\Gamma^*$. However, an estimation using the data from Pons and Welschen (2002) reveals that $\Gamma^*$ is almost unaffected by this effect either in C, MD, or SD plants (data not shown). Therefore, it is unlikely that edge effects account for the observed differences between treatments.

Finally, lateral diffusion through air space in the mesophyll has been shown to occur, particularly in homobaric species such as tobacco (Jahnke and Krewitt, 2002; Pieruschka et al., 2005). This occurs due to the fact that illuminated mesophyll patches inside IRGA’s leaf chamber and the surrounding mesophyll patches darkened under the leaf chamber gaskets have different $C_i$, since the cells in the former patches are photosynthesizing while those in the latter are respiring. This gradient causes a lateral flux of CO$_2$ from the darkened air spaces to the illuminated air spaces. This leads to underestimations of the true photosynthesis (since a part of the CO$_2$ used by illuminated cells comes from the surrounding internal spaces of the leaves, not from the IRGA chamber) and the corresponding errors in the estimation of $C_i$. While it is difficult to estimate the magnitude of such an effect and to use its knowledge to correct the values, it is reasonable to think that this may be most important at high light (i.e. high photosynthesis leading to decreased $C_i$ in the illuminated patches, hence maximizing the lateral CO$_2$ gradient). This would lead to an almost parallel displacement of the $A_N$–$C_i$ curve at high light with a small effect on that at low light, hence displacing $\Gamma^*$ to lower values. Using similar reasoning, it is likely that this effect is much larger in water-stressed plants, since they do have a much lower $C_i$ than the controls in the illuminated mesophyll with similar $C_i$ under the gaskets, i.e. a much larger lateral CO$_2$ gradient. Therefore, lateral diffusion through the air spaces in the mesophyll is likely to cause an important part of the observed differences in $\Gamma^*$ between treatments.

Together, all these possible errors lead to misleading estimations of $\Gamma^*$, particularly under water stress. In contrast to all the uncertainties and discrepancies regarding technical problems when using the Laisk method, calculating $\Gamma^*$ from the linear relationship at low $C_e$ between gross photosynthesis (i.e. the sum of $A_N$ and $R_L$) and $C_e$ (Long and Bernacchi, 2003), resulted in a single value for all treatments (39 μmol mol$^{-1}$) which, obviously, reflected a $\tau$ value (99) very close to that determined in vitro.

Concluding remarks

In summary, the present results clearly show that the Rubisco specificity factor does not acclimate to water stress in the short term (weeks or months) in tobacco. The observations by Galmés et al. (2005) that the highest values of $\tau$ are observed in Rubisco from plants adapted to the driest environments may reflect an adaptation mechanism operating over a much longer time-scale (i.e. generations).

The comparison of in vitro and in vivo estimated $\tau$ values clearly support the conclusion by Warren et al. (2004) that, in plants with restricted photosynthesis (e.g. water stressed), it would be preferable to determine $\tau$ independently of gas-exchange measurements, using an alternative method. In vitro determinations proved to be a suitable means. An alternative would be to estimate $\Gamma^*$ in control plants using the Laisk method as corrected for $R_L$ and $g_s$. 

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(von Caemmerer, 2000), and assume that it is not affected by the applied treatments, as our data suggest.

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