Small decreases in SBPase cause a linear decline in the apparent RuBP regeneration rate, but do not affect Rubisco carboxylation capacity

Elizabeth P. Harrison1, Hulya Olcer2, Julie C. Lloyd, Stephen P. Long3 and Christine A. Raines4

Department of Biological Sciences, John Tabor Laboratories, University of Essex, Colchester CO4 3SQ, UK

Received 1 February 2001; Accepted 10 May 2001

Abstract

The response of net photosynthetic CO2 uptake (A) to increasing leaf intercellular CO2 concentration (ci) was determined in antisense Nicotiana tabacum plants, derived from six independent transformation lines, displaying a range of sedoheptulose-1,7-bisphosphatase (SBPase) activities. The maximum in vivo ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation (Vc,max) and RuBP regeneration (Jmax) rates were calculated from the steady-state measurements of the A to ci response curves. In plants with reductions in SBPase activity of between 9% and 60%, maximum RuBP regeneration capacity declined linearly (r² = 0.79) and no significant change in apparent in vivo Rubisco activity (Vc,max) was observed in these plants. No correlation between Vc,max and a decrease in capacity for RuBP regeneration was observed (r² = 0.14) in the SBPase antisense plants. These data demonstrate that small decreases in SBPase activity limit photosynthetic carbon assimilation by reducing the capacity for RuBP regeneration.

Key words: Antisense, photosynthesis, Rubisco, SBPase.

Introduction

A model of CO2 uptake (Farquhar et al., 1980) has provided a widely used method that allows the limitation of photosynthesis, under saturating light conditions, to be partitioned between Rubisco activity and the capacity for RuBP regeneration. By determining the response of net leaf CO2 uptake (A) to intercellular CO2 concentration (ci), the model may be used to partition limitation of the rate of carbon assimilation (A) between Rubisco activity (Vc,max) and maximum rate of RuBP regeneration, which is assumed proportional to the maximum rate of whole chain electron transport (Jmax). When ci is low, Rubisco activity normally controls A. However, at higher ci, control shifts to the regeneration of RuBP. This means that the formation of RuBP is limited by the maximum rate of whole chain electron transport, or co-limited by the rate of whole chain electron transport and enzymes in the regenerative phase of the Calvin cycle (Farquhar et al., 1980; von Caemmerer, 2000).

A practical value of the model of Farquhar et al. lies in the assumption that the variables Vc,max and Jmax are independent (Farquhar et al., 1980). However, a survey of data obtained from 109 species revealed a linear relationship between Jmax and Vc,max (Wullschleger, 1993). Leuning re-analysed these data by adjusting the estimates to a common temperature, using equations for the temperature dependence of these parameters (Leuning, 1997). This increased the correlation coefficient (r²) of Jmax with

Abbreviations: A, leaf CO2 uptake (μmol m⁻² s⁻¹); ci, ambient CO2 concentration (μmol mol⁻¹); ci, intercellular CO2 concentration (μmol mol⁻¹); FBPase, fructose-1,6-bisphosphatase; Jmax, maximum capacity for whole chain electron transport (μmol m⁻² s⁻¹); MS, Murashige and Skoog; PPFD, photosynthetically active photon flux density (μmol m⁻² s⁻¹); PRKase, phosphoribulokinase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (LSU large subunit, SSU small subunit); RuBP, ribulose-1,5-bisphosphate; Vc,max, apparent maximum Rubisco activity (μmol m⁻² s⁻¹); SBPase, sedoheptulose-1,7-bisphosphatase; T1, first generation of primary transformants.

© Society for Experimental Biology 2001
to 0.87. Evans showed that within a species $V_{c,\text{max}}/J_{\text{max}}$ remained more or less constant over a wide range of leaf nitrogen contents, from optimal to deficient levels (Evans, 1986). These correlations may simply be chance but may also reflect co-ordinated synthesis or activation of the photosynthetic components by parallel factors conserved across species. As such it may not be possible to have a decrease in $J_{\text{max}}$ without a decrease in $V_{c,\text{max}}$. The possibility of genetically modifying $J_{\text{max}}$ independently from $V_{c,\text{max}}$ and vice versa, has assumed added significance in considering the adaptation of C3 crops to elevated $V_{\text{c}}$ levels (Evans, 1986). Here optimization of investment within the photosynthetic apparatus for elevated $V_{\text{c}}$ would require increased investment into capacity for the regeneration of RuBP and decreased investment into Rubisco (Long and Drake, 1992). Transgenic plants with altered levels of individual Calvin cycle enzymes have provided a means to test whether $J_{\text{max}}$ may be varied independently of $V_{c,\text{max}}$.

Several studies with transgenic antisense plants have shown that an imbalance between $V_{c,\text{max}}$ and $J_{\text{max}}$, relative to wild-type plants, may be induced either by a reduction of Rubisco content or by a decrease of a component affecting RuBP regeneration. However, whilst the correlation apparent in the survey by Wullschleger is broken (Wullschleger, 1993), a decrease in $J_{\text{max}}$ has rarely been achieved without some decrease in $V_{c,\text{max}}$ and vice versa. Price et al. (1998) found that a decrease in the Calvin cycle enzyme GAPDH of more than 65% was necessary to effect any decrease in CO2 assimilation (Price et al., 1995). In their comparison of $A/c_i$ responses of wild-type and antisense plants it was shown that with a 90% decrease in GAPDH, $J_{\text{max}}$ is decreased by 57%, however, $V_{c,\text{max}}$ was also decreased by 43%. A decrease in $V_{c,\text{max}}$ was also indicated by a linear decline in the number of Rubisco binding sites per unit leaf area when GAPDH activity was reduced below 50% of wild-type activity. However, a more recent analysis of the same GAPDH transformant lines, suggested that in vivo Rubisco activity during transient RuBP saturation, was unaffected by comparison to wild-type plants (Ruuska et al., 1998). This showed clearly that potential $V_{c,\text{max}}$ was unaltered by large decreases in $J_{\text{max}}$; however, the possibility still exists for steady-state $V_{c,\text{max}}$ to be altered via feedback control. Is it possible to show the separation of $J_{\text{max}}$ from variation in $V_{c,\text{max}}$, indicated by Ruuska et al. (Ruuska et al., 1998), under steady-state conditions?

An ideal antisense approach to test whether a decrease in $J_{\text{max}}$ effects a change in steady-state $V_o$ would target an enzyme where even a small decrease will cause a significant decrease in $J_{\text{max}}$. Previous analysis of transgenic plants with small reductions in SBPase activity revealed a loss of photosynthetic capacity (Harrison et al., 1998; Raines et al., 2000). In this paper, transgenic lines have been used with a wide range of SBPase activities to test the hypothesis that steady-state RuBP regeneration ($J_{\text{max}}$) may be decreased without affecting Rubisco activity in vivo ($V_{c,\text{max}}$).

### Materials and methods

#### Plant material

The transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun) used in this study were the T1 progeny resulting from the self-fertilization of six independent antisense SBPase parents (T0) generated as described previously (Harrison et al., 1998). Seeds were germinated on sterile MS media supplemented with 3% (w/v) sucrose (Murashige and Skoog, 1962) and kanamycin (100 μg ml⁻¹) in a 14 h photoperiod at 20 °C. Four-week-old seedlings were transferred to soil (Levington F2, Fisons) and placed in a controlled-environment greenhouse under the same light dark regime at c. 26 °C during the light period and 18 °C during the dark period. The plants were illuminated with natural light supplemented with high pressure sodium lamps giving 1000–1400 μmol m⁻² s⁻¹ at leaf level (measured at midday), and a minimum of 500 μmol m⁻² s⁻¹ throughout the photoperiod. Plants were grown individually in 18 cm pots positioned at random, relocated at regular intervals, and watered daily with Hoagland’s solution (Hoagland and Arnon, 1950). Gas exchange measurements were made on each side of the mid-rib midway on the long axis of the leaf. Leaves 7 and 8, each on completion of expansion, were used for these measurements. Immediately on completion of photosynthetic measurement, discs (1.5 cm diameter) were cut from the same areas of the leaf, snap frozen into liquid N₂ and stored at −80 °C.

#### Photosynthesis measurements

Gas-exchange measurements were conducted at saturating PPFD (1000 μmol m⁻² s⁻¹) with a leaf temperature of 25 ± 1.5 °C and vapour pressure deficit of c. 1 kPa. Rates of CO₂ uptake and water vapour efflux were measured in an open gas-exchange system, incorporating infrared CO₂ and H₂O analysers and a leaf cuvette (CIRAS-1 and PLC, PP Systems, Hitchin, UK). The analyser was calibrated against a known CO₂ standard (Linde Gas, Stoke-on-Trent, UK) and water vapour concentrations provided by a water vapour generator (WG 600, ADC Ltd., Hoddesdon, Herts., UK). PPFD was measured with a quantum sensor (Skye Instruments Ltd., Wales). $A$ and $c_i$ were determined using the equations of von Caemmerer and Farquhar (von Caemmerer and Farquhar, 1981). To determine the response of $A$ to $c_i$, $A$ was first measured at the ambient CO₂ concentration ($c_a$) in which the plants had grown. To determine the initial slope of the $A/c_i$ response, $c_a$ was decreased in several steps to c. 50 μmol mol⁻¹. The $c_a$ was then returned to the growth concentration to check that the original rate could be regained and was then finally increased stepwise to 2000 μmol mol⁻¹ to complete the response curve.

The maximum Rubisco activity in vivo ($V_{c,\text{max}}$) was estimated from the initial slope of the response of $A$ to $c_i$ as described earlier (Wullschleger, 1993) and using the Rubisco kinetic parameters of von Caemmerer et al. (von Caemmerer et al., 1994). $V_{c,\text{max}}$ estimated in this way, provided a measure of the activity of Rubisco within the leaves analysed. The points above the inflection of the response curve of $A$ to $c_i$ were used to estimate $J_{\text{max}}$. 

---

1780 Harrison et al.
SBPase activity, Western blot analysis and photosynthetic pigments

Frozen leaf discs were ground to a powder at liquid N₂ temperature and then used for enzyme activity, protein and pigment assays. SBPase activity was determined by the phosphate release method as described previously (Harrison et al., 1998). SBPase and Rubisco SSU protein levels were determined by separation on 12% SDS-PAGE followed by Western blotting using a horseradish peroxidase conjugated second antibody and ECL kit (Amersham International PLC) (Harrison et al., 1998). Rubisco LSU and SSU were determined using a laser densitometric scanner (Molecular Dynamics, Sevenoaks, Kent). Chlorophylls were determined by the method of Hill et al. (Hill et al., 1985).

Results

Plant phenotypes

Fifteen T1 progeny, from six independent transgenic lines containing a tobacco antisense SBPase transgene, were found to have levels of SBPase activity decreased between 9% and 82%, relative to wild-type levels. SBPase protein levels were closely and positively correlated with SBPase activity ($r^2=0.97$). Western blot analysis of samples from seven of these SBPase antisense plants is shown in Fig. 1. These data indicated that there was no detectable change in either Rubisco SSU or LSU in the SBPase antisense plants relative to wild-type plants, except where SBPase activity was decreased by more than 60%. The antisense plants with reductions of 60% or less of wild-type SBPase activity had the same leaf appearance and chlorophyll content as the wild-type plants (examples shown in Table 1). However, plants with greater reductions in SBPase activity showed an obvious reduction in chlorophyll content.

$A/c_i$ response analysis

The response of net CO₂ uptake ($A$) to increasing intercellular CO₂ ($c_i$), the $A/c_i$ response curve, showed clear differences between antisense and wild-type plants. All of the SBPase antisense plants analysed in this study had a lower $A$ at the highest $c_i$ ($A_{max}$) (examples shown in Table 1). In contrast, the initial slopes of the response appeared identical to the wild-type plants, except in the transgenic plants where SBPase activity was decreased by more than 60%. Three typical $A/c_i$ response curves obtained from the SBPase antisense plants are shown in Fig. 2. From these data it can be seen that reductions in SBPase activity to 38% and 57% of wild-type plants decreased RuBP-limited photosynthesis ($J_{max}$), but not Rubisco-limited ($V_{c,max}$) photosynthesis. In contrast, in plants with more severe reductions in SBPase activity, both $J_{max}$ and $V_{c,max}$ were reduced (Fig. 2). This is in keeping with the Western blot analysis of Rubisco LSU and SSU that showed that the amount of Rubisco protein was reduced in plants with SBPase activities decreased by 60% or more relative to wild-type plants (Fig. 1). In four of the antisense plants SBPase activity was reduce by more than 60% which clearly caused pleiotropic effects on Rubisco and photosynthetic pigments; for this reason these plants were eliminated from the analysis of dependence of $J_{max}$ on $V_{c,max}$.

![SBPase, SSU, LSU](image)

**Fig. 1.** SBPase and Rubisco protein levels in SBPase antisense tobacco plants. Soluble leaf proteins, loaded on an equal leaf area basis, were separated using SDS-PAGE. SBPase, Rubisco SSU and LSU were detected using ECL after Western blotting and sequential probing with polyclonal antiserum against SBPase and Rubisco holoenzyme. These data are examples taken from the analysis of five different wild-type plants and, for the transgenic plants, samples were taken from each plant from two separate leaves.

Table 1. SBPase activities, light-saturated photosynthetic rates ($A_{max}$), chlorophyll and carotenoid content in the same two fully expanded leaves (7, 8) of seven antisense SBPase plants with reductions in activity of between 9–82% of wild type

Data are means ±SE. Samples were for five different plants for the wild type. For the individual transgenes samples were taken from two separate leaves.

<table>
<thead>
<tr>
<th>Plant</th>
<th>SBPase activity (µmol m⁻² s⁻¹)</th>
<th>% Decrease in SBPase activity</th>
<th>Chlorophyll (µg g⁻¹ FW)</th>
<th>Carotenoids (µg g⁻¹ FW)</th>
<th>$A_{max}$ (µmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>12.0 ± 0.5</td>
<td>0</td>
<td>598 ± 39</td>
<td>294 ± 19</td>
<td>54.9 ± 1.2</td>
</tr>
<tr>
<td>1.11-22</td>
<td>10.9 ± 0.3</td>
<td>9</td>
<td>584 ± 31</td>
<td>274 ± 16</td>
<td>48.3 ± 1.2</td>
</tr>
<tr>
<td>1.14-26</td>
<td>9.1 ± 0.5</td>
<td>24</td>
<td>611 ± 35</td>
<td>302 ± 21</td>
<td>46.2 ± 0.9</td>
</tr>
<tr>
<td>1.13-30</td>
<td>7.9 ± 0.5</td>
<td>34</td>
<td>608 ± 41</td>
<td>311 ± 26</td>
<td>43.8 ± 1.5</td>
</tr>
<tr>
<td>1.14-29</td>
<td>7.5 ± 0.7</td>
<td>38</td>
<td>575 ± 21</td>
<td>314 ± 18</td>
<td>42.6 ± 0.5</td>
</tr>
<tr>
<td>1.13-29</td>
<td>5.1 ± 0.1</td>
<td>57</td>
<td>578 ± 19</td>
<td>276 ± 12</td>
<td>33.1 ± 0.8</td>
</tr>
<tr>
<td>1.13-27</td>
<td>3.8 ± 0.4</td>
<td>69</td>
<td>430 ± 19</td>
<td>265 ± 13</td>
<td>17.3 ± 0.7</td>
</tr>
<tr>
<td>1.15-25</td>
<td>2.2 ± 0.2</td>
<td>82</td>
<td>351 ± 14</td>
<td>190 ± 10</td>
<td>13.6 ± 0.6</td>
</tr>
</tbody>
</table>
each plant.

the transgenes two separate leaves were measured at full expansion on

the mean of measurements on five individual wild-type plants and for

transformants and on five wild-type plants. Each datum point represents

SBPase antisense tobacco plants. Measurements were made on single

transport calculated from gas exchange (as reflected in the maximum rate of whole chain electron

in vivo

steady-state

Discussion

Using the model of Farquhar et al., values for in vivo

Rubisco activity ($V_{c,\text{max}}$) and RubP regeneration ($J_{\text{max}}$)

were calculated from the A/$c_i$ response curves of 11 antisense plants with reductions in SBPase activity of between 9% to 60% (Farquhar et al., 1980). In these plants $V_{c,\text{max}}$ was unchanged, while $J_{\text{max}}$ declined linearly with SBPase activity (Fig. 3). A strong linear and significant relationship between $J_{\text{max}}$ and SBPase activity was found with a slope of 0.69 ($r^2 = 0.79, p < 0.01$; Fig. 3). In contrast, there was no significant correlation of SBPase activity with $V_{c,\text{max}}$ ($r^2 = 0.25, \text{ns}$) in these plants (Fig. 3). Additionally, in the transgenic plants with reductions in SBPase activity between 9% and 60%, there was no correlation of $V_{c,\text{max}}$ with $J_{\text{max}}$ ($r^2 = 0.14, \text{ns}$).

Analysis of dependence of $J_{\text{max}}$ on $V_{c,\text{max}}$

all involved large reductions in the target protein, typically $>60\%$. Here a complete independence of steady-state $V_{c,\text{max}}$ from $J_{\text{max}}$ has been shown determined from steady-state $A/c_i$ responses (Fig. 3). Even the small decreases in $V_{c,\text{max}}$ indicated (Fig. 3) are likely to be over-estimates of the true decrease in in vivo Rubisco activity, because diffusive conductance of CO$_2$ within the mesophyll has been shown to decline with photosynthetic capacity in tobacco with decreased photosynthetic capacity (Evans et al., 1994). The present results show that steady-state $J_{\text{max}}$ can be decreased by up to 50% without having a significant effect on, or correlation with apparent steady-state $V_{c,\text{max}}$.

Many gas exchange studies have found that there is a remarkably strong correlation between $J_{\text{max}}$ and $V_{c,\text{max}}$ (reviewed by Wullschleger, 1993; Leuning, 1997). Manipulation of photosynthetic capacity using either shade conditions or nitrogen deficiency appears to result in the co-ordinate reduction of $V_{c,\text{max}}$ and $J_{\text{max}}$ such that control remains shared equally between Rubisco activity and RuBP regeneration (Evans, 1986; Evans and Farquhar, 1991). This could imply that either there are feedback control mechanisms or that synthesis of Rubisco and components of the photosynthetic apparatus determining RuBP regeneration are so tightly linked, that one cannot be varied without affecting the other. This present study, together with that of Ruuska et al. (Ruuska et al., 1998), has demonstrated that RuBP regeneration can be varied substantially using genetic engineering without affecting Rubisco activity. This potential flexibility in the

Fig. 2. Response of CO$_2$ assimilation rates to intercellular CO$_2$

concentration ($c_i$) at saturating PPFD (1000–1400 µmol m$^{-2}$ s$^{-1}$) in

SBPase antisense tobacco plants. Measurements were made on single

transformants and on five wild-type plants. Each datum point represents

the mean of measurements on five individual wild-type plants and for

the transgenes two separate leaves were measured at full expansion on

each plant.

Fig. 3. The relationship of percentage reduction in $V_{c,\text{max}}$ and in $J_{\text{max}}$
to percentage reduction in SBPase activity. $V_{c,\text{max}}$ and $J_{\text{max}}$ were determined from the $A/c_i$ relationships of 11 antisense plants with SBPase activities reduced to between 9–60% (examples plotted in Fig. 2). Each point represents the mean data for an individual transformant (two separate leaves were measured at full expansion for each transformant); the value for 0% reduction in SBPase activity represents the mean of five wild-type plants. The broken lines indicate the 95% confidence limits. The control co-efficient for SBPase activity over $J_{\text{max}}$ was derived from the gradient of line obtained by plotting the % decrease in $J_{\text{max}}$ against the % decrease in SBPase activity.
relationship between Rubisco activity and the rate of RuBP regeneration could be of benefit under conditions of rising atmospheric CO₂ concentration where the limitation on carbon assimilation shifts from Rubisco to RuBP regeneration (Stitt, 1991; Drake et al., 1997). Plants may be able to optimize investment in the photosynthetic apparatus such that Rubisco levels would decrease and/or investment in the enzymes involved in regeneration would increase.

Interestingly, the data here also showed that $J_{\text{max}}$ is very sensitive to small decreases in SBPase activity, with an apparent linear dependence, indicating a control coefficient for SBPase activity over $J_{\text{max}}$ of 0.69. It has been assumed that RuBP regeneration at light saturation is controlled by the potential whole chain electron transport rate, rather than by the enzymes of the Calvin cycle downstream of Rubisco (Farquhar et al., 1980; von Caemmerer, 2000). This assumption has been supported by the results obtained from the analysis of plants with antisense decreases in other individual enzymes of the Calvin cycle. Large decreases (> 60%) in GAPDH (Price et al., 1995), FBPase (Kossman et al., 1994), PRKase (Paul et al., 1995, 2000) were necessary to decrease photosynthesis and regeneration of RuBP. In contrast, only a small decrease in SBPase (9%) was needed to effect a decrease in the apparent in vivo maximum rate of RuBP regeneration ($\alpha J_{\text{max}}$). The assumption that the limitation to $J_{\text{max}}$ is within the electron transport pathway could only be consistent with these findings if SBPase activity is just slightly in excess of the activity needed to support the observed wild-type $J_{\text{max}}$.

The cytochrome b/f complex is commonly regarded as a limiting step in whole chain electron transport, but analysis of antisense plants with reduced levels of this complex have not resolved this issue. A decrease in electron transport rate was not apparent in the data given for these plants by Price et al. (Price et al., 1998) until cytochrome f content was decreased by more than 50%. The results of the present study suggest that wild-type SBPase activities may be close to controlling $J_{\text{max}}$ and that any de-activation of this thioredoxin-regulated enzyme would decrease, or at least co-limit, $J_{\text{max}}$ calculated from the $A/c_i$ response.

In conclusion, this study has shown from the steady-state $A/c_i$ response, that $J_{\text{max}}$ may be varied substantially without affecting $V_{c,\text{max}}$. The data presented here also indicated that SBPase may be unusual among the Calvin cycle enzymes downstream of Rubisco, including the closely related FBPase, in being just sufficient in activity to support observed rates of RuBP regeneration in vivo.

Acknowledgements

This work was supported by grant number P01723 from the Biotechnology and Biological Sciences Research Council (EPH).

We are grateful to Dr. Martin Parry for the kind gift of Rubisco antibodies and to Dr. Susanne von Caemmerer for discussion of this work.

References


Leuning R. 1997. Scaling to a common temperature improves the correlation between the photosynthesis parameters $J_{\text{max}}$ and $V_{c,\text{max}}$. Journal of Experimental Botany 48, 345–347.


