Increased Sedoheptulose-1,7-Bisphosphatase Activity in Transgenic Tobacco Plants Stimulates Photosynthesis and Growth from an Early Stage in Development[1]

Stephane Lefebvre, Tracy Lawson, Mike Fryer, Oksana V. Zakhleniuk, Julie C. Lloyd, and Christine A. Raines*

Department of Biological Sciences, University of Essex, Colchester CO4 3SQ, United Kingdom

Activity of the Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase (SBPase) was increased by overexpression of an Arabidopsis (*Arabidopsis thaliana*) cDNA in tobacco (*Nicotiana tabacum*) plants. In plants with increased SBPase activity, photosynthetic rates were increased, higher levels of Suc and starch accumulated during the photoperiod, and an increase in leaf area and biomass of up to 30% was also evident. Light saturated photosynthesis increased with increasing SBPase activity and analysis of CO_2 response curves revealed that this increase in photosynthesis could be attributed to an increase in ribulose 1,5-bisphosphate regenerative capacity. Seedlings with increased SBPase activity had an increased leaf area at the 4 to 5 leaf stage when compared to wild-type plants, and chlorophyll fluorescence imaging of these young plants revealed a higher photosynthetic capacity at the whole plant level. Measurements of photosynthesis, made under growth conditions integrated over the day, showed that mature plants with increased SBPase activity fixed 6% to 12% more carbon than equivalent wild-type leaves, with the young leaves having the highest rates. In this paper, we have shown that photosynthetic capacity per unit area and plant yield can be increased by overexpressing a single native plant enzyme, SBPase, and that this gives an advantage to the growth of these plants from an early phase of vegetative growth. This work has also shown that it is not necessary to bypass the normal regulatory control of SBPase, exerted by conditions in the stroma, to achieve improvements in carbon fixation.

The photosynthetic carbon reduction (Calvin) cycle is the primary pathway for fixation of atmospheric CO_2. This cycle plays a central role in plant metabolism, providing intermediates not only for starch and Suc biosynthesis, but also for isoprenoid metabolism and shikimic acid biosynthesis (Geiger and Servaites, 1994). Recently, a major focus has been to identify the individual steps that control carbon flux through the Calvin cycle. To address this question, antisense plants have been produced in which the levels of individual enzymes in the cycle have been reduced and the response of photosynthesis in these plants has been measured. This has allowed the contribution that individual enzymes exert over the control of flux through the Calvin cycle to be quantified (for review, see Stitt and Schulze, 1994; Raines, 2003). A number of interesting and surprising results have emerged from these analyses. Firstly, it was shown that for plants grown in moderate light and temperature conditions, more than 50% of wild-type levels of Rubisco could be removed before any significant effect on ambient photosynthesis was observed. However, under conditions of high light, reductions in photosynthesis were proportionate to decreases in Rubisco activity (Quick et al., 1991; Stitt et al., 1991; Hudson et al., 1992; Krapp et al., 1994; Stitt and Schulze, 1994). Reductions of greater than 50% in the levels of glyceraldehyde-3-P dehydrogenase, Fru-1,6-bisphosphatase (FBPase), phosphoribulokinase (PRKase), and plastid aldolase were also needed before photosynthetic capacity was affected (Kossmann et al., 1994; Stitt and Schulze, 1994; Paul et al., 1995; Price et al., 1995; Haake et al., 1998). Although the level of control exerted by any single enzyme in the cycle varied with environmental conditions and developmental status, the data from the transgenic plants strongly indicated that a number of enzymes in the Calvin cycle are present in excess. The implication from this is that the levels of Rubisco, glyceraldehyde-3-P dehydrogenase, FBPase, and PRKase are not close to limiting carbon fixation through this cycle and therefore would not be useful targets for overexpression to increase photosynthetic capacity.

In contrast, photosynthesis has been shown to be sensitive to small reductions in the levels of the enzymes transketolase and sedoheptulose-1,7-bisphosphatase (SBPase; Harrison et al., 1998; Raines et al., 2000; Henkes et al., 2001; Olcer et al., 2001). Transketolase catalyzes three reactions in the regenerative phase of the Calvin cycle. A small decrease (20%–30%) in transketolase resulted in a reduction of photosynthetic carbon fixation, and flux to phenylpropanoid metabolism was also decreased, making this enzyme a potential target for overexpression. The enzyme SBPase functions at a branch point in the Calvin cycle between regeneration of the acceptor molecule ribulose 1,5-bisphosphate (RuBP) and export to starch biosynthesis, where it catalyzes the dephos-

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* Corresponding author; e-mail rainc@essex.ac.uk; fax 44–(1206)–872592.

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phosphorylation of SBPase. RuBP regenerative capacity (Jmax) responded linearly to reductions in SBPase activity, and total biomass was also reduced in antisense SBPase plants (Harrison et al., 2001; B. Bryant and C.A. Raines, unpublished data) These findings suggest that SBPase levels in wild-type tobacco (*Nicotiana tabacum*) plants are only just sufficient to maintain carbon flux through the Calvin cycle and that it may be possible to increase photosynthetic capacity by increasing SBPase activity. Further evidence to support this hypothesis has come from the finding that transgenic tobacco plants expressing a bifunctional cyanobacterial FBPase/SBPase had increased photosynthetic carbon fixation and biomass in plants grown in hydroponic culture (Miyagawa et al., 2001). This work has demonstrated that it is possible to increase photosynthetic capacity in response to direct manipulation of the Calvin cycle. Although these data are very interesting, the question remains as to whether this effect is due to differences in regulatory properties between the cyanobacterial enzyme and the native plant FBPase and SBPase enzymes. Alternatively, simultaneous expression of the two bisphosphatase activities may be having a synergistic effect. In this paper, we present work that aims to test the hypothesis that increasing the levels of higher plant SBPase activity alone has the potential to increase photosynthetic capacity in tobacco plants.

RESULTS

Production and Selection of Tobacco Transformants

A full-length Arabidopsis (*Arabidopsis thaliana*) SBPase cDNA (Willingham et al., 1994) was used to prepare a sense gene construct in the binary vector pMOG22 containing the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1A). Following leaf disc transformation of tobacco, 60 primary transformants (T_0 generation) were rooted on hygromycin-containing medium, subsequently transferred to soil, and grown until maturity. Expression of the transgene in the T_0 plants was confirmed by reverse transcription-PCR (data not shown). Following this screening step, proteins were extracted from the newest fully expanded leaf of the T_0 plants expressing the Arabidopsis SBPase RNA, and western-blot analysis revealed that a number of plants with increased levels of SBPase protein had been produced (Fig. 1B). Western-blot analysis of T_1 progeny from 4 selected SBPase overexpressing lines (60, 11, 30, and 6) confirmed that no significant changes in the levels of the Calvin cycle enzymes, FBPase, and PRKase had occurred (Fig. 1C). Analysis of total extractable SBPase activity in newly fully expanded leaves of T_1 and T_4 progeny of the SBPase sense plants revealed that plants with a range of SBPase activity had been produced and that this was maintained in subsequent generations (Fig. 1D). SBPase expression segregated in all the lines of both the T_1 and T_4 progeny with some plants having SBPase

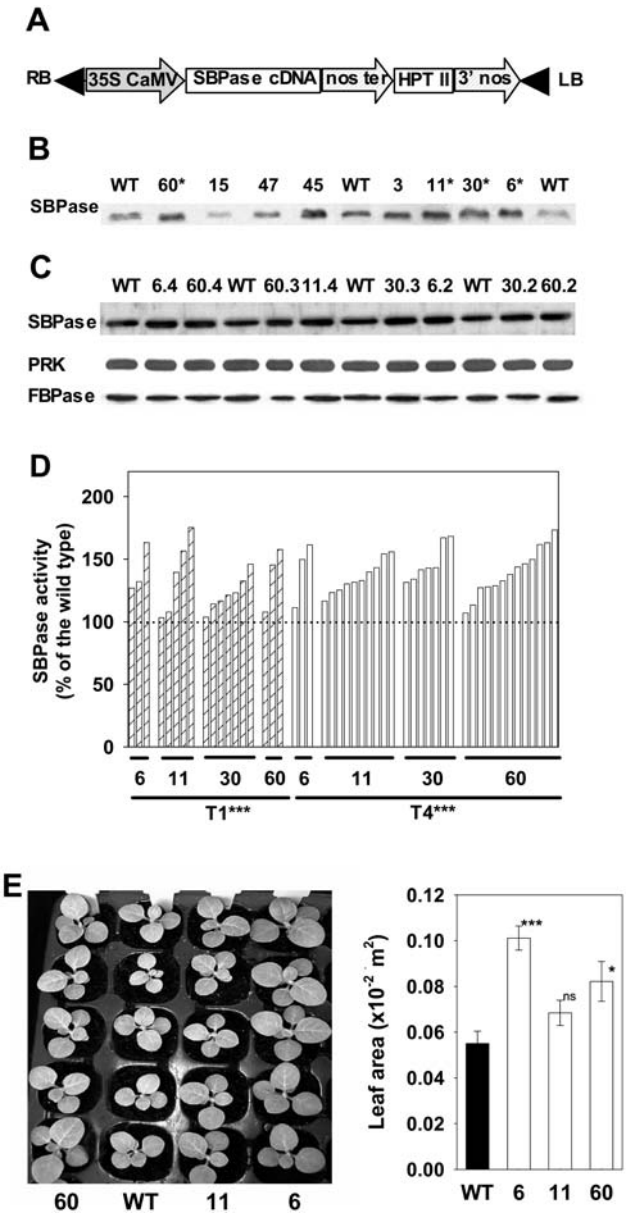

Figure 1. Production and selection of SBPase sense transgenic tobacco plants. A, The sense construct contained a full-length Arabidopsis SBPase cDNA (1,350 bp) driven by the CaMV 35S promoter and the nopaline synthase termination sequence. B, Western-blot analysis of primary transformants (T_0); 25 μg of leaf protein samples from the newest fully mature leaves were separated by SDS-PAGE and polyclonal antibodies used to detect SBPase protein. The plants marked with an asterisk were selected for further analysis. C, Western-blot analysis of wild-type and individual T_1 progeny of SBPase sense plants. The same blots were stripped and then reprobed using polyclonal antibodies raised against PRKase and chloroplastic FBPase. Each lane represents a sample taken from one individual plant, the first number denotes the line and the second number indicates an individual segregant from that line. D, Increase in total SBPase activity in individual transformants of first (T_1) and fourth (T_4) generation transgenic plants as compared to wild type (100%). E, Leaf area of wild-type and transgenic seedlings after 3 weeks of growth in a controlled environment chamber. The results are the mean $\pm$ SE for wild-type ($n = 5$) and SBPase sense ($n > 4$) plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

actvities close to wild type, while in others an increase of as much as 150% was evident. The average SBPase activity of the T_1 and T_4 population of transgenic plants was significantly different from the population of wild-type plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns >0.05) after ANOVA, post hoc Tukey test analysis. In the T_1 progeny of SBPase sense lines 6 and 60, a significant increase in the total leaf area of 3-week-old seedlings was evident (Fig. 1E).

The Impact of Increased SBPase Activity on Photosynthesis, Carbohydrate Levels, and Growth in Plants Grown in Controlled Environment Conditions

Fluorescence Imaging Analysis of Photosynthetic Capacity in Young Seedlings

A cohort of T_1 transgenic progeny (from three independent transgenic lines) and wild-type tobacco plants were grown in a controlled environment chamber until they had four to five true leaves, and chlorophyll fluorescence imaging was used to assess the photosynthetic performance of whole seedlings. The operating efficiency of PSII (F'_q/F'_m) was higher for the plants with increased SBPase activity (Table I); this indicated a higher quantum efficiency of linear electron transport through PSII in the transgenic plants. Nonphotochemical quenching (NPQ) in the SBPase sense plants was not significantly different from wild-type plants, providing evidence that the nonradiative decay (heat loss) from PSII and its antennae was equivalent between transgenic and wild-type plants. Together with the significant increase in F'_q/F'_v (which is mathematically equivalent to the coefficient of photochemical quenching, *qP*) for the transgenic plants compared with the wild-type plants, this suggested that the light energy absorbed by PSII antennae may be being used to support higher rates of photosynthetic carbon assimilation.

CO_2 Assimilation Rates in Leaves at Different Stages of Development

Gas exchange measurements were used to investigate whether the increased photosynthetic capacity observed in young seedlings was maintained in mature tobacco plants. CO_2 assimilation rates were determined, under the light level and CO_2 concentration in which the plants were grown, for young expanding leaves (12 and 13), a leaf approaching full expansion (11), and new fully expanded leaves (9 and 10) in wild-type and SBPase sense plants when 25 to 27 leaves had been produced (Fig. 2A). In wild-type plants, photosynthetic activity was lowest in the youngest leaves and increased to a maximum in the newest fully expanded leaves. A similar pattern of development of photosynthetic capacity was observed in the leaves of SBPase sense plants. However, in the SBPase overexpressing plants, the photosynthetic rate in the youngest leaves (12 and 13) was higher than wild type, but in the fully expanded leaves (9 and 10) no significant increase in photosynthesis was evident (Fig. 2A). In leaf 11 (close to full expansion), photosynthesis was only increased in the plants with the highest levels of SBPase activity. This may reflect the fact that leaf 11 is making the transition to full expansion where no significant increase in photosynthesis is evident, even in the group of plants with the highest increase in SBPase activity.

Diurnal Carbohydrate Levels

In parallel with photosynthetic analysis, diurnal patterns of carbohydrate accumulation and turnover were determined (Fig. 2B). In both the wild-type and SBPase sense plants, Suc and starch accumulated during the light period in both young expanding and newly fully expanded leaves. A correlation between increased carbohydrate accumulation and increased SBPase activity was evident, and lines with the highest

Table I. *Chlorophyll fluorescence image analysis of PSII efficiency in 3-week-old wild-type and SBPase sense plants*

Plants were grown in a controlled environment chamber under a light level of 600 μmol m^{-2} s^{-1}. Fluorescence measurements were taken at an irradiance of 500 μmol m^{-2} s^{-1} after a series of fluorescence pulses as described in "Materials and Methods." SBPase activity was determined in the newest fully expanded leaf. The values represent mean ± SE for wild-type plants ($n = 3$) and for SBPase sense line 11 ($n = 4$), line 60 ($n = 4$), and line 30 ($n = 4$). Asterisks indicate that mean values are significantly different between wild-type and SBPase sense plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ns >0.05). F_q'/F_m', the quantum yield of PSII in the light; F_q'/F_v', PSII efficiency factor and NPQ, which is determined from (F_m/F_m') −1. n.d., Not determined.

Plant Line	PSII Efficiency	F_q'/F_v'	NPQ	SBPase Activity
	F_q'/F_m'			$\mu mol\ m^{-2}\ s^{-1}$
11	0.504*** ± 0.002	0.708*** ± 0.006	0.71^{ns} ± 0.03	9.905 ± 0.998
30	0.495*** ± 0.004	0.684** ± 0.004	0.69^{ns} ± 0.02	9.260 ± 0.468
60	0.482** ± 0.004	0.674* ± 0.007	0.74^{ns} ± 0.02	n.d.
Wild type	0.460 ± 0.003	0.642 ± 0.005	0.68 ± 0.04	7.594 ± 1.000

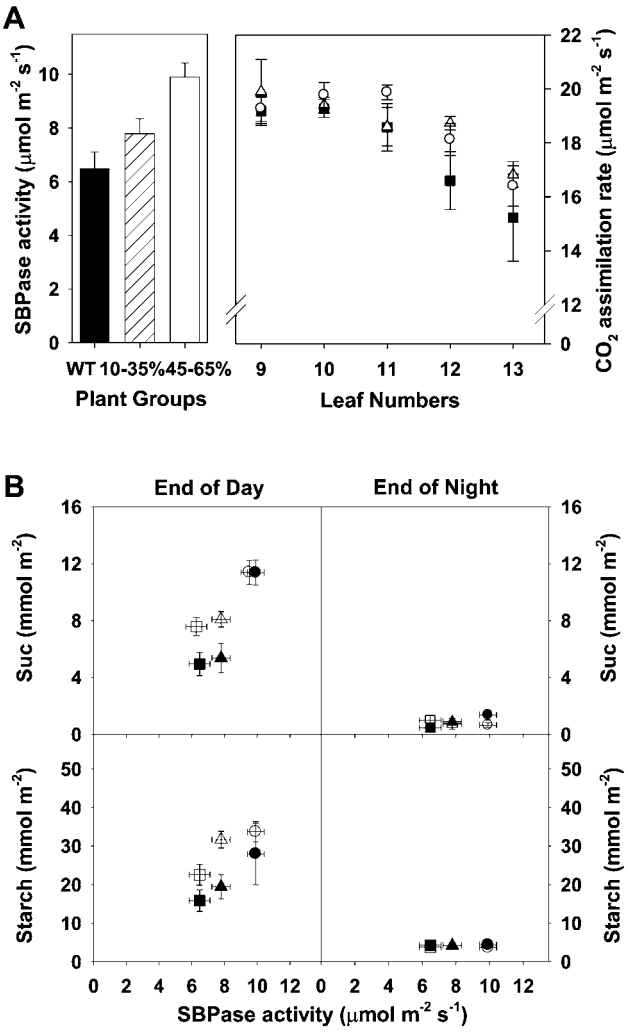

Figure 2. Ambient photosynthesis and diurnal carbohydrate accumulation in wild-type and T_1 SBPase sense tobacco plants. Plants were grown in a controlled environment chamber with a light level of 600 μmol m^{-2} s^{-1} and ambient CO_2 concentration until 25 leaves were produced. A, Total SBPase activity was determined in newly fully expanded leaves (9 and 10) on each plant and for subsequent analysis plants were divided into 2 groups with increases in SBPase activity of 10% to 35% and 45% to 65%. Values represent activities ± SE (n = 4 separate plants). Photosynthesis was determined under growth conditions in 5 different leaves (numbers 9, 10, 11, 12, and 13) of wild-type (black square) and SBPase sense plants (white circle, activity increased 10%–35%; white triangle, activity increased 45%–65%). Data points represent the mean ± SE of four different plants. B, Suc and starch levels were determined in 2 newly fully expanded leaves (9 and 10, black symbols) and 2 young expanding leaves (12 and 13, white symbols) 10 h after the onset of the light period (end of day) and at the end of the night. Symbols are as used in A. The data points represent the mean ± SE (n = 4 separate plants).

SBPase activity accumulated up to 50% more Suc and starch than equivalent wild-type plants. Suc and starch levels at the end of the night were low in both the wild-type and SBPase overexpressing plants, indicating that the carbohydrate accumulated in the light was being used and/or remobilized at night (Fig. 2B). Although photosynthetic capacity of the mature leaves (9 and 10) was not increased, the levels of both Suc and starch were higher in the transgenic plants with the highest SBPase activities.

Effect of Increased SBPase Activity on Growth

In the SBPase sense plants grown in controlled environment conditions, significant increases in growth rate and biomass production were evident. The transgenic SBPase sense plants were harvested when 27 leaves had been produced. The shoot biomass was increased by 40%, while the root biomass was essentially unchanged (Fig. 3, A and B). The change in shoot biomass in the SBPase sense plants was due to both an increase in stem and leaf dry weights (Fig. 3, C and F). Although the length of the stem was increased, this did not account for the entire increase in the stem weight, indicating that the stem diameter was also greater (Fig. 3D). Total leaf area and leaf biomass increased in parallel in the SBPase sense plants with both these parameters being up to 30% greater than in wild-type plants (Fig. 3, E and F). This was supported by the absence of any significant change in the specific leaf area (Fig. 3G).

Determination of Photosynthetic Capacity and Growth of Plants Grown in Greenhouse Conditions

Previous work on transgenic plants with altered Calvin cycle enzyme activity has shown that the environmental conditions under which the plants are grown influences the impact of any changes in enzyme activity on photosynthesis and growth. To assess the changes in photosynthetic parameters of the SBPase sense plants in a more natural environment, two separate cohorts of T_4 SBPase sense plants were grown in a controlled environment greenhouse where they were subject to normal daily changes in light and temperature.

Response of Photosynthesis to Changes in CO_2

The response of carbon fixation to increasing intercellular CO_2, the A/Ci response curve, was determined in the newest fully expanded leaf of plants that were approximately 7 weeks old and had a maximum of 14 leaves (Fig. 4A). In the transgenic plants with increased SBPase activity, the photosynthetic response to increasing CO_2 was different from the wild-type plants. The SBPase overexpressing lines had higher rates of photosynthetic carbon fixation than wild-type plants at Ci levels in the range between 150 μmol mol^{-1} to 1,200 μmol mol^{-1}. The relationship between increases in SBPase activity and photosynthetic carbon fixation, measured at both prevailing and saturating CO_2 concentrations, is shown in Figure 4B. These data showed that there was a linear relationship between SBPase activity and assimilation rates at both the CO_2 concentrations under which the plants were grown (400 μmol mol^{-1}) and saturating (1,200 μmol mol^{-1}) CO_2 concentrations.

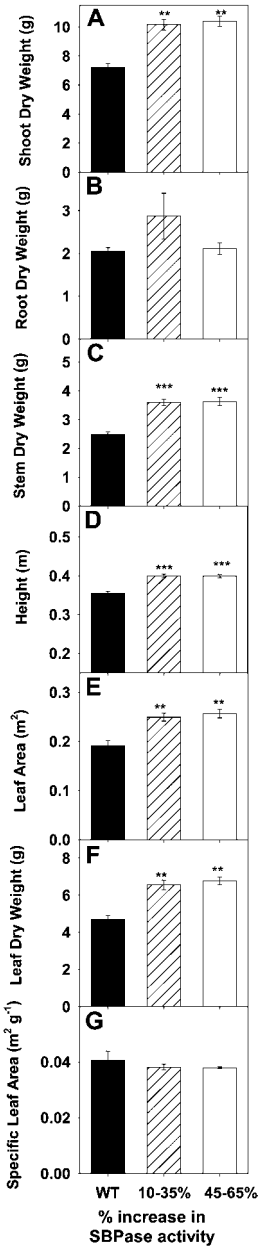

Figure 3. Growth analysis of controlled-environment-grown wild-type and SBPase sense plants. Plants grown in the same conditions as for Figure 2 were harvested after 7 weeks of growth. Results are means ± SE ($n = 4$); plants with increased SBPase activity were grouped as for Figure 2. Asterisks indicate that mean values are significantly different between wild-type and SBPase sense plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

The data from the A/Ci curve were used to calculate the apparent in vivo maximum Rubisco activity (Vc, max) and Jmax for all of the SBPase sense and wild-type plants using the equations developed by von Caemmerer and Farquhar (1981; von Caemmerer, 2000). In the plants with increased SBPase activity, the Jmax was increased by as much as 30% in response to increasing SBPase activity (Fig. 5A). Interestingly, Vc, max was 10% above that for wild-type plants (Fig. 5B).

Daily Progression of Photosynthesis

The data obtained from the A/Ci response analysis revealed that under saturating light a stimulation of photosynthesis was evident in the newest fully expanded leaves. To investigate this further, the in situ response of photosynthesis to increased SBPase activity was determined by measuring photosynthesis under the prevailing light and CO_2 conditions in a young

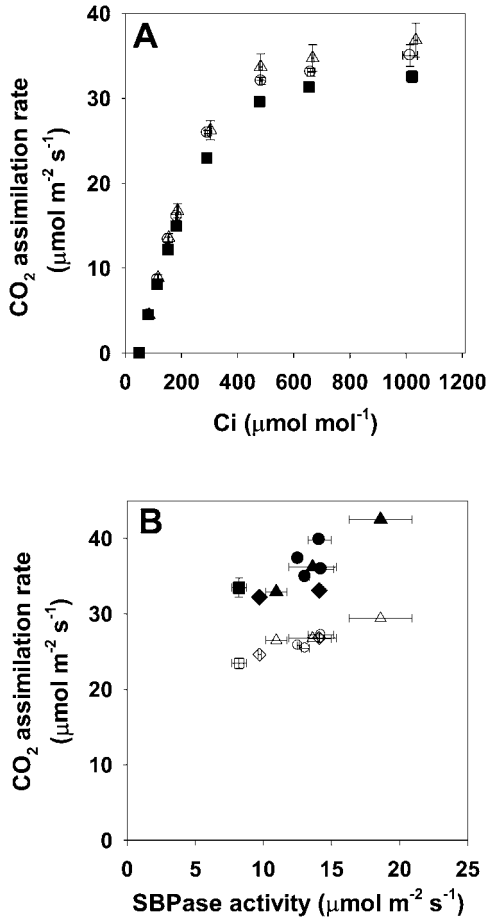

Figure 4. Photosynthetic capacity of greenhouse-grown wild-type and SBPase sense plants. Plants were grown in a controlled-environment greenhouse with light levels of 600 to 1,600 μmol m^{-2} s^{-1} and measurements made after 7 weeks of growth. A, Photosynthetic carbon fixation rates were determined in the newest fully expanded leaf, as a function of increasing CO_2 concentration at saturating-light levels (1,200 μmol m^{-2} s^{-1}; A/Ci response curve). Wild-type plants (■) and sense plants with increased SBPase activity of 25% to 50% (○) and 60% to 100% (△). Values represent the mean of at least three plants ± SE. B, The relationship between SBPase activity (determined in the same leaf area used for photosynthesis) and light-saturated photosynthetic carbon assimilation rates at 400 μmol mol^{-1} (white symbols) and 1,200 μmol mol^{-1} (black symbols) external CO_2 derived from the A/Ci curves. Individual measurements of photosynthesis are shown for each plant, and SBPase activity is the mean ± SE of triplicate assays on 2 extracts from each plant, line 6 (◇), line 11 (△), line 60 (○), and wild type (□).

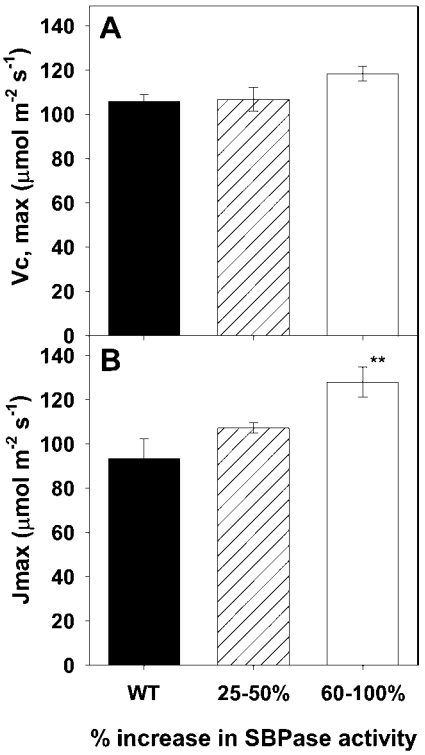

Figure 5. Rubisco activity and Jmax derived from the A/Ci response curves shown in Figure 4 using the equations of von Caemmerer and Farquhar (1981). A, Maximum velocity of carboxylation (Vc, max). B, Maximum in vivo rates of Jmax. Values are the mean ± SE in wild-type plants ($n = 7$) and SBPase sense plants (increase in activity 25%–50%, $n = 6$; increase in activity 50%–100%, $n = 7$). Asterisks indicate the mean values that are significantly different between wild-type SBPase sense plants (**, $P < 0.01$).

expanding and newly fully expanded leaf on wild-type and transgenic plants throughout the photoperiod. The SBPase sense lines had slightly higher rates of photosynthesis over the course of the day than equivalent wild-type plants (Fig. 6). This stimulation of photosynthesis was greatest in the young leaves, in agreement with the analysis of the plants grown in the controlled environment cabinet (Fig. 2; Table I). Integrating ambient photosynthesis measurements over the day revealed that in the SBPase sense plants the daily carbon fixed in young leaves was increased by 12% ($P < 0.05$) and, even in the mature leaves, a 6% increase was evident. No significant differences in SBPase activity were evident between the young and old leaves of the wild-type, or of SBPase sense, plants.

Growth Response to Increased SBPase Activity

In the SBPase sense plants grown in greenhouse conditions, only small differences in shoot biomass were found when plants were harvested at the floral bud stage of development (Fig. 7A). However, although stem height was increased significantly (Fig. 7C), only small changes were seen in stem and leaf dry weights and these were not statistically significant (Fig. 7, B and D). Increases in total leaf area and leaf biomass increased in parallel in the SBPase sense plants by 10% (Fig. 7, D and E), resulting in no change in specific leaf area (Fig. 7F).

In addition to analysis of total leaf area and biomass, the area and weight of all of the individual leaves were determined for the wild-type and SBPase sense plants. The average area and weight of leaves on the SBPase sense plants produced during early- to mid-vegetative phase (I, II) was about 50% greater than those of wild-type plants. During phase III, small increases in both leaf area and leaf weight were evident but the level of significance was just above $P = 0.05$. In contrast, the average area and weight of leaves produced later in the vegetative phase and just prior to flowering (IV) were similar to those of the wild-type plants (Fig. 8, A and B).

DISCUSSION

Transgenic plants expressing an Arabidopsis SBPase sense construct were produced and a number of lines identified with increased SBPase activity. Photosynthesis and growth rates were increased in the SBPase overexpressing plants grown in both controlled envi-

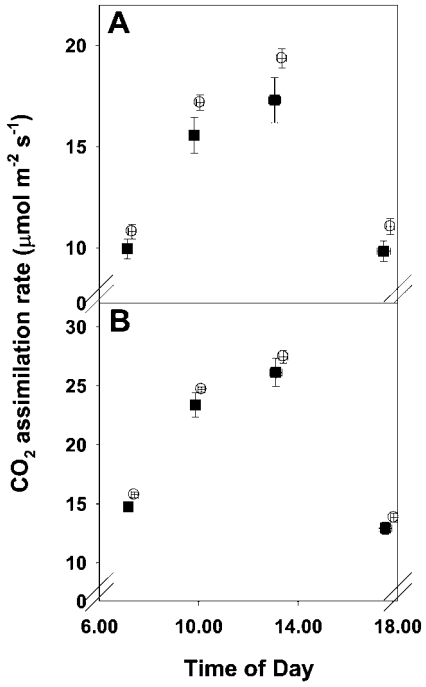

Figure 6. Diurnal progression of leaf photosynthesis in greenhouse-grown SBPase sense plants. Plants were grown in a controlled-environment greenhouse with light levels of 600 to 1,600 μmol m^{-2} s^{-1} photosynthetic carbon fixation rates determined under ambient conditions in 2 different leaves. A, Young expanding leaf. B, Newly fully expanded leaf of wild-type and SBPase sense plants. For the wild type (■), each symbol represents the mean ± SE of eight plants. For the transgenic plants (○), each symbol represents the mean ± SE of 16 plants. The SBPase activity in the young leaves of the wild-type plants was 12.57 ± 0.67 and 16.6 ± 0.64 for the transgenic plants and in mature leaves of wild-type plants was 13.76 ± 1.18 and 18 ± 0.42 for the transgenic plants.

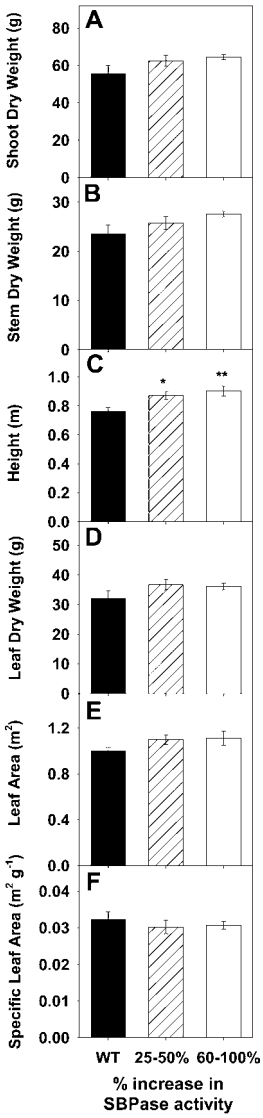

Figure 7. Growth analysis of greenhouse-grown wild-type and SBPase sense plants. Plants grown in a controlled-environment greenhouse (and analyzed in Figs. 4 and 5) were harvested when the first flower buds appeared. Values are the mean ± SE in wild-type plants ($n = 8$) and SBPase sense plants (increase in activity 25%–50%, $n = 6$; increase in activity 60%–100%, $n = 7$). Asterisks indicate that mean values are significantly different between wild-type and SBPase sense plants (*, $P < 0.05$; **, $P < 0.01$).

ronment chambers and the greenhouse. Light-saturated rates of photosynthesis, measured under prevailing and saturating CO_2 concentrations, increased linearly in response to increased SBPase activity. However, this increase in photosynthesis was not directly proportionate to the change in total extractable SBPase activity, indicating that the relationship between SBPase activity and photosynthesis is not simple. This could reflect regulatory properties of the system, resulting in photosynthetic carbon fixation being limited at another point in the Calvin cycle (Stitt and Schulze, 1994; Raines, 2003). This may be due, in part, to the fact that SBPase activity is subject to regulatory feedback by its product and also by redox via thioredoxin (Raines et al., 1999). Analysis of A/Ci response curves in the SBPase sense plants, using the equations developed by von Caemmerer and Farquhar (1981), showed that the increase in photosynthetic capacity could be attributed to an increase in the rate of Jmax in the SBPase sense lines as compared to wild-type plants. These results fit well with the location of SBPase in the regenerative part of the Calvin cycle and also with our previous results showing that Jmax declined linearly with reductions in SBPase activity (Harrison et al., 2001). Interestingly, the values for Vc, max were also increased by about 10%. Rubisco activation state and RuBP levels were also shown to be higher in transgenic plants where photosynthetic capacity has been increased by the overexpression of a bifunctional cyanobacterial FBPase/SBPase (Miyagawa et al., 2001).

Our data have shown clearly that, under light-saturating conditions, photosynthetic rates on a leaf area basis were increased in response to increased SBPase activity. In addition, using fluorescence imaging of whole plants, we have also shown that young seedlings (4–5 leaf stage) had significantly higher photosynthetic capacity than wild-type plants. These data show that whole-plant photosynthesis can be increased during the early stages of vegetative growth by increasing SBPase activity. Analysis of the daily progression of photosynthesis, measured under the same light and CO_2 concentrations that the plants were

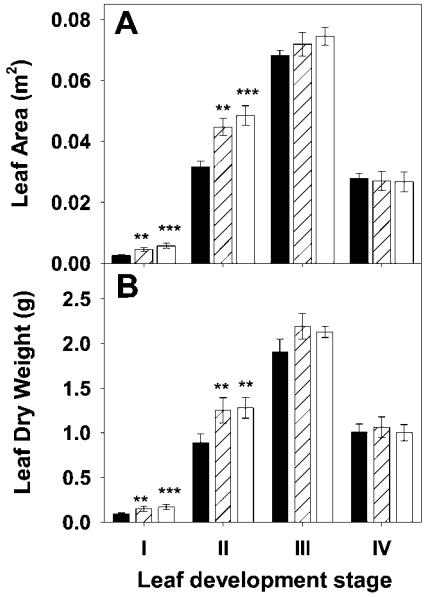

Figure 8. Leaf area profiles for wild-type and SBPase sense plants grown in a controlled-environment greenhouse. A, Average leaf area and (B) average leaf dry weight were determined for I, leaves 3 to 6; II, leaves 7 to 10; III, leaves 11 to 16; and IV, leaves 17 to 32 in the same plants used for the analyses in Figures 4 to 6. Values are the mean ± SE in wild-type plants (black bar, $n = 7$) and SBPase sense plants (hatched bar, increase in activity 25%–50%, $n = 6$; white bar, increase in activity 60%–100%, $n = 7$). Asterisks indicate that mean values are significantly different between wild-type and SBPase sense plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

grown in, revealed an increase in total carbon fixed during the photoperiod in the SBPase sense lines. Interestingly, this increase in photosynthesis was greater in young expanding leaves (12%) than in fully expanded leaves (6%) in the SBPase sense plants compared to the equivalent leaves on wild-type plants. This difference in the response of photosynthesis to increased SBPase activity (between young expanded and newly fully expanded leaves) was also observed for the plants grown in controlled environment chambers. This result was somewhat unexpected and cannot be accounted for by differential increases in SBPase activity in the young leaves. It is possible that, in the mature leaves of SBPase overexpressing lines, additional limiting factors, such as the availability of water, control the flux of carbon through the Calvin cycle. These differences might be expected from previous studies of Calvin cycle antisense plants that have also shown that the relative importance of any individual enzyme over carbon fixation is not fixed and will vary depending on growth condition and development (for review, see Stitt and Schulze, 1994; Raines, 2003).

Analysis of diurnal changes in carbohydrate accumulation revealed that the levels of both Suc and starch increased at the end of the day in SBPase sense plants grown in controlled environment conditions. In expanding leaves (12 and 13; Fig. 2A), a clear correlation was evident between SBPase activity, increased photosynthesis, and increased accumulation of Suc and starch. In the fully expanded leaves (9 and 10) of the transgenic plants with the greatest increase in SBPase activity, increased levels of both Suc and starch were also found; however, no significant increase in photosynthesis was observed in these leaves (Fig. 2, A and B). This apparent discrepancy may be due to the fact that these photosynthetic measurements were made at a single time point in the day, but the measurements of Suc and starch were at steady state, giving the total daily accumulation. In support of this suggestion, the daily time course measurements of photosynthesis in the fully expanded leaves of the SBPase sense plants revealed a small increase (6%) in the daily carbon fixed in the mature leaves (Fig. 6).

In the young expanding and newly fully expanded leaves of both the wild-type and SBPase sense plants, only low levels of sugar and starch remained in the predawn samples. These results showed that the additional carbohydrate produced in the SBPase sense plants was being used in the dark period, possibly for growth. Indeed, the total shoot biomass and total leaf area of the SBPase sense plants grown in the controlled environment chamber were increased by up to 25%, compared to wild-type plants. A similar trend toward increased growth was evident in the greenhouse-grown plants; however, in this case, the impact was less substantial and an increase of between 7% and 12% in leaf area and leaf biomass was evident. This increase could be attributed to an increase in leaf area and biomass that occurred in the leaves produced during the early- to mid-vegetative phase of the life cycle, when the average leaf area and mass was increased by 25% compared to equivalent leaves on the wild-type plants. In contrast, the leaves produced later in the vegetative phase and just prior to flowering had a similar biomass and leaf area to that of wild-type plants. These analyses suggested that increases in SBPase activity impact photosynthesis and growth from an early stage of seedling development. This finding is in contrast with data from plants expressing a bifunctional FBPase/SBPase enzyme where increases in growth were most evident in older plants. It is possible that these differences could be due to the fact that these plants were grown in hydroponic culture, under low light and/or that the tobacco cultivar used was *xanthii* (Miyagawa et al., 2001). When the SBPase sense plants described in this paper were grown in greenhouse conditions in winter with shorter day length and lower light levels, no increase in photosynthesis or plant yield was evident (S. Lefebvre, J.C. Lloyd, and C.A. Raines, unpublished data). It is likely that, under these low-light conditions, photosynthesis was limited by components of the electron transport chain such as the cytochrome *b/f* complex or ferredoxin NADPH reductase (Price et al., 1998; Hajirezaei et al., 2002).

The relationship between photosynthetic capacity and yield is not clear, and in fact, photosynthesis per unit leaf area has remained constant even in high-yielding crop varieties (Evans and Fischer, 1999; Loomis and Amthor, 1999; Richards, 2000). In this paper, we have shown that photosynthetic capacity per unit area and plant yield can be increased by overexpressing a single native plant enzyme, SBPase. This work has also shown that it is not necessary to bypass the normal regulatory control of SBPase, exerted by conditions in the stroma, to achieve improvements in carbon fixation. However, our study has also indicated that this manipulation of SBPase activity will not provide an advantage to plants under all growth conditions. Nevertheless, our results, together with that of Miyagawa et al. (2001), provide evidence suggesting that it may be possible to improve crop yield by manipulating photosynthetic carbon fixation capacity. Although improvement in crop yields have been achieved through changes in biomass allocation and light interception, it is unlikely that any future improvements can be made to these parameters (Long et al., 2005). Given this situation, it is clear that alternative strategies will be needed in the future if crop yields are to be increased. It will therefore be of value to test if overexpression of SBPase activity can also lead to increases in photosynthesis and yield in crop species grown under field conditions.

MATERIALS AND METHODS

Generation of the Transgenic Plants

An Arabidopsis (*Arabidopsis thaliana*) full-length SBPase cDNA (Willingham et al., 1994) was cloned into the pMog 22 vector containing the CaMV 35S

promotor and *nos* terminator sequences (Fig. 1). The recombinant plasmid was introduced into tobacco (*Nicotiana tabacum*) L. cv Samsun using *Agrobacterium tumefaciens* LBA4404 via leaf-disc transformation (Horsch et al., 1985). Shoots were regenerated on selective medium containing hygromycin (300 mg L^{-1}) and primary transformants (T_0) were allowed to self-fertilize.

Plant Growth Conditions

Wild-type tobacco plants and T_1 progeny resulting from self-fertilization of transgenic plants were germinated in sterile agar medium containing Murashige and Skoog salts supplemented with 1% (w/v) Suc (plus hygromycin 300 mg L^{-1} for the transformants). Seeds were germinated in controlled environment chambers at an irradiance of 40 μmol photons m^{-2} s^{-1}, 22°C, relative humidity of 60%, in a 16-h photoperiod. After 2 weeks, plants were transferred to soil (Levington F_2, Fisons, Ipswich, UK) and grown in either a controlled environment chamber with a 16-h photoperiod, 500 to 600 μmol m^{-2} s^{-1}, at 25°C day/20°C night, or a controlled environment greenhouse (16-h photoperiod, 25°C–30°C day/20°C night, and natural light supplemented with high-pressure sodium light bulbs, giving between 600–1,600 μmol m^{-2} s^{-1} from the pot level to the top of the plant, respectively). Positions of the plants were changed daily and watered with a nutrient medium (Hoagland and Arnon, 1950). Leaf discs (0.8-cm diameter), for analysis of SBPase activities, carbohydrate, and protein, were taken from the same areas of the leaf used for photosynthetic analysis, immediately plunged into liquid N_2, and stored at −80°C.

Determination of Biomass and Growth Parameters

Leaf areas were determined immediately after harvest using a Scan Jet 5370C (Hewlett-Packard, Palo Alto, CA). Leaves, stems, and roots were dried at 60°C until a constant weight was obtained (4 d) and final dry weights determined.

Gas Exchange Measurements

A/Ci response curves were made using a portable gas exchange system (LI-COR 6400; LI-COR, Lincoln, NE). The gas exchange system was zeroed daily using anhydrous calcium carbonate (Drierite, W.A. Hammond Drierite, Xenia, OH) to remove water and using soda lime (sofnolime granules, Morgan Medical, Kent, UK) to remove CO_2 from the air entering the cuvette. Leaf temperatures were set at 25°C for all measurements, though actual temperature ranged from 25°C to 30°C. Leaves were illuminated using a red-blue light source attached to the gas-exchange system, and light levels were maintained at 1,200 μmol m^{-2} s^{-1} for the duration of the A versus C_i response curve. Leaf vapor pressure deficits were maintained between 0.5 to approximately 1.6 kPa. Measurements of photosynthetic carbon assimilation (A) were made starting at 400 μmol mol^{-1} CO_2 surrounding the leaf, decreased stepwise to 50 μmol mol^{-1}, returned to 400 μmol mol^{-1}, and increased stepwise to 1,600 μmol mol^{-1} CO_2. Each complete curve consisted of at least nine separate measurements. Values for A and C_i were calculated using the equations of von Caemmerer and Farquhar (1981) and were used to solve for Vc, max and Jmax.

Diurnal Photosynthesis

The diurnal response of leaf photosynthesis was measured beginning at 6:30 AM and finishing at 6:30 PM; photosynthesis of a newly fully expanded leaf and a young expanding leaf were measured for all plants at approximately 3-h intervals. Measurements were made using a portable gas exchange system (LI-COR 6400). Light levels at each time point were set to reflect the ambient light over the day: 6:30 AM to 8 AM, 400 μmol m^{-2} s^{-1}; 9:30 AM to 11 AM, 1,000 μmol m^{-2} s^{-1}; 12.30 PM to 2 PM, 1,500 μmol m^{-2} s^{-1}; 4:30 PM to 6:30 PM, 500 μmol m^{-2} s^{-1}. Photosynthesis measurements were recorded at steady state.

Chlorophyll Fluoresence Imaging

Chlorophyll fluorescence measurements were performed on 3-week-old tobacco seedlings that had been grown in a controlled environment chamber providing 500 to 600 μmol m^{-2} s^{-1} light and a CO_2 concentration of 360 μmol mol^{-1}. Chlorophyll fluorescence parameters were obtained using a CF Imager chlorophyll fluorescence imaging system (Technologica, Colchester, UK; Barbagallo et al., 2003; Baker and Rosenqvist, 2004). Seedlings were dark adapted 15 min and the minimal fluorescence from a dark-adapted leaf (F_o) was determined using three weak measuring pulses of light and following a saturating pulse of 4,800 μmol m^{-2} s^{-1} for 800 ms, the maximal fluorescence from a dark-adapted leaf (F_m) obtained. The plants were then maintained for 15 min under actinic light (300 μmol m^{-2} s^{-1}) and F_m' monitored by applying saturating light pulses (4,800 μmol m^{-2} s^{-1} for 800 ms) 3 times at 5-min intervals. The actinic light was then increased to 500 μmol m^{-2} s^{-1} and F_m' measurements repeated; plants were then returned to the dark for 15 min and F_m and F_o determined again. F_q' (photochemical quenching of fluorescence due to open PSII centers) was calculated from the difference in fluorescence between F_m' and F', where F' is the fluorescence emission from a leaf adapted to actinic light. F_v' (variable fluorescence level of leaves in light) was determined from the difference between F_m' and F_o', where F_o' is the minimal fluorescence from a light-adapted leaf. For each determination of fluorescence parameters, three plants were measured simultaneously (two transgenic and one wild type). The data represented in Table I correspond to the third measurement under 500 μmol m^{-2} s^{-1} actinic light. NPQ was determined from $(F_m/F_m') - 1$.

Protein Extraction and Western Blotting

Leaf discs sampled as described above were ground in liquid nitrogen and protein quantification determined (Harrison et al., 1998). Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed using antibodies raised against SBPase, FBPase, and PRKase. Proteins were detected using horseradish peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham, Buckinghamshire, UK).

Determination of SBPase Activity by Phosphate Release

SBPase activity was determined by phosphate release (Harrison et al., 1998). Immediately after photosynthesis measurement, leaf discs were isolated from the same leaves and frozen in liquid nitrogen. For analysis, leaf discs were ground to a fine powder in liquid nitrogen in extraction buffer (50 mM HEPES, pH8.2; 5 mM MgCl; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.1% Triton X-100; 2 mM benzamidine; 2 mM aminocapronic acid; 0.5 mM phenylmethylsulfonylfluoride; 10 mM dithiothreitol) and the resulting solution centrifuged 1 min at 14,000g, 4°C. The resulting supernatant (1 mL) was desalted through an NAP-10 column (Amersham) and the eluate aliquoted and stored in liquid nitrogen. For the assay, the reaction was started by adding 20 μL of extract to 66 μL of assay buffer (50 mM Tris, pH 8.2; 15 mM $MgCl_2$; 1.5 mM EDTA; 10 mM dithiothreitol; 2 mM SBP) and incubated at 25°C for 5 min. The reaction was stopped by addition of 50 μL of 1 M perchloric acid and centrifuged for 10 min at 14,000g, 4°C. Samples (30 μL) and standards (30 μL, PO_4^{3-} 0.125−4 nmol) were incubated 25 min at room temperature with 300 μL of Biomol Green (Affiniti Research Products, Exeter, UK) and the A_{620} was measured using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA).

Determination of Suc and Starch

Carbohydrates and starch were extracted from leaf discs sampled 2 times, 10 h into the light period and 7 h into the dark period, on leaves 9, 10, 12, and 13 of 7-week-old plants. The leaf discs were incubated in 80% (v/v) ethanol for 30 min at 80°C and then washed 4 times with ethanol 80% (v/v). Suc was measured from the extracts in ethanol using an enzyme-based protocol (Stitt et al., 1989), and the starch contents were estimated from the ethanol-insoluble pellet according to Stitt et al. (1978), with the exception that the samples were boiled for 1 h and not autoclaved.

Statistical Analysis

All statistical analyses were done by comparing ANOVA, using Sys-stat, University of Essex, UK. The differences between means were tested using the Post hoc Tukey test (SPSS, Chicago).

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CORRECTION

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Lefebvre S., Lawson T., Zakhleniuk O.V., Lloyd J.C., and Raines C.A. Increased Sedoheptulose-1,7-Bisphosphatase Activity in Transgenic Tobacco Plants Stimulates Photosynthesis and Growth from an Early Stage in Development.

Plant Physiology regrets that, due to an error in the revision process for this article, Mike Fryer was mistakenly excluded from the list of authors. Additionally, two references were excluded from the Literature Cited:

Ballou CE (1963) Preparation and properties of D-erythrose 4-phosphate. Methods Enzymol **6:** 482–484

Tsolas O (1975) Sedoheptulose 1,7-bisphosphate. Methods Enzymol **41:** 77–79