Plant Biotechnology Journal (2004) **2**, pp. 71–82 Doi: 10.1046/j.1467-7652.2003.00053.x

Genetic modification of photosynthesis with *E. coli* genes for trehalose synthesis

Till K. Pellny[1], Oula Ghannoum[2], Jann P. Conroy[3], Henriette Schluepmann[4], Sjef Smeekens[4], John Andralojc[1], Klaus Peter Krause[5], Oscar Goddijn[5] and Matthew J. Paul[1,*]

[1]*Crop Performance and Improvement, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK;* [2]*Molecular Plant Physiology Group. Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, 2601 ACT, Australia;* [3]*Centre for Horticulture and Plant Sciences, Hawkesbury Campus, University of Western Sydney, South Penrith DC NSW 1797, Australia;* [4]*Molecular Plant Physiology, Utrecht University, Padualaan 8, 3584CH Utrecht, The Netherlands;* [5]*Syngenta Mogen, Einsteinweg 97, 2333 CB Leiden, PO Box 628, 2300 AP Leiden, The Netherlands*

Received 29 August 2003; revised 10 October 2003; accepted 13 October 2003.
**Correspondence* (fax +44 (0)1582 763010; e-mail matthew.paul@bbsrc.ac.uk)

Summary

Improvement in photosynthesis per unit leaf area has been difficult to alter by breeding or genetic modification. We report large changes in photosynthesis in *Nicotiana tabacum* transformed with *E. coli* genes for the trehalose pathway. Significantly, photosynthetic capacity (CO_2 assimilation at varying light and CO_2, and quantum yield of PSII electron transport) per unit leaf area and per leaf dry weight were increased in lines of *N. tabacum* transformed with the *E. coli* gene *otsA*, which encodes trehalose phosphate synthase. In contrast, transformation with *otsB*, which encodes trehalose phosphate phosphatase or *Trec*, encoding trehalose phosphate hydrolase, produced the opposite effect. Changes in CO_2 assimilation per unit leaf area were closely related to the amount and activity of Rubisco, but not to the maximum activities of other Calvin cycle enzymes. Alterations in photosynthesis were associated with trehalose 6-phosphate content rather than trehalose. When growth parameters were determined, a greater photosynthetic capacity did not translate into greater relative growth rate or biomass. This was because photosynthetic capacity was negatively related to leaf area and leaf area ratio. In contrast, relative growth rate and biomass were positively related to leaf area. These results demonstrate a novel means of modifying Rubisco content and photosynthesis, and the complexities of regulation of photosynthesis at the whole plant level, with potential benefits to biomass production through improved leaf area.

Keywords: photosynthesis, trehalose 6-phosphate, genetic modification, metabolism, Rubisco, growth.

Introduction

The improvement of crop yields was one of the major technological achievements of the 20th century. This was achieved by two methods: (i) by breeding, which increased the proportion of biomass in the harvested product, i.e. by increasing the harvest index; (ii) by improving growth through agronomic practice, in particular with nitrogenous fertilisers. This enabled yield potential to be achieved by creating conditions favouring high photosynthetic rates and increased leaf area. Improvement of yield has been associated with an environmental improvement in photosynthesis to achieve existing genetic potential rather than genetic improvement of this potential. Despite intense selection pressure for yield through the breeding of new crop cultivars, photosynthesis per unit leaf area has remained the same or fallen for the majority of crops (Evans, 1993; Richards, 2000).

With improvements in yields by conventional means reaching a limit, increasing photosynthetic capacity per unit leaf area has been a target to further increase yield potential. Strategies employed in C_3 plants have been to target key Calvin cycle enzymes, particularly Rubisco (Whitney and Andrews, 2001) and to incorporate components of the C_4 pathway into C_3 species (Ku *et al.*, 1999). None of these has proved straightforward because of the number of genes that need to be transformed simultaneously, problems of Rubisco assembly (Madgwick *et al.*, 2002) and the complexity of the regulation of photosynthesis at the whole plant level.

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Transformation with a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphoshatase gene has produced the only report of genetic enhancement of photosynthetic capacity through direct targeting of the Calvin cycle (Miyagawa, Tamoi and Shigeoka, 2001). Recently, Lieman-Hurwitz *et al*. (2003) reported improved photosynthesis through the expression of *ictB* involved in HCO_3^- accumulation.

These former targets constitute 'push' approaches to the problem of improving photosynthesis, i.e. direct targeting of the photosynthetic apparatus. 'Pull' approaches, i.e. attempts to engineer photosynthesis indirectly through feedback processes that regulate photosynthesis have received less attention, largely because these mechanisms are complex and poorly understood. The redox signals that determine the ceiling for maximum photosynthesis (Horton, 2000) are one example. Others are the signals and feedback processes that cause end-product limitation of photosynthesis. Rubisco content and photosynthesis respond strongly to sugars (Krapp and Stitt, 1995; Paul and Driscoll, 1997; Van Oosten and Besford, 1996), indicating that alteration of this feedback mechanism could be a step towards modifying Rubisco content and thus photosynthesis. The mechanism has been linked most closely with hexokinase activity and the flux of sugars through invertase and hexokinase (Jang *et al*., 1997; Moore *et al*., 1998, 1999, 2003).

A recent discovery has given valuable insight into the regulation of carbohydrate metabolism in plants. The trehalose pathway, which was once thought to be of physiological relevance in only a few specialized resurrection plants (Muller *et al.,* 1995; Zentella *et al*., 1999) is now known to be ubiquitous (Leyman, van Dijk and Thevelein, 2001) and in *Arabidopsis*, indispensable for carbohydrate utilization (Eastmond *et al*., 2002). There is very strong evidence that this indispensable role is played by trehalose 6-phosphate (T6P), which has emerged as a metabolic signalling molecule in plants (Schluepmann *et al*., 2003). Trehalose itself has been shown to improve stress tolerance in transgenic tobacco (Pilon-Smits *et al*., 1998) and rice (Garg *et al*., 2002) engineered with *Escherichia coli* genes for the pathway.

In this work we demonstrate a dramatic effect on photosynthesis as a result of altering T6P in transgenic tobacco. Increasing T6P content increased photosynthetic capacity per unit leaf area, something that has been extremely hard to achieve through conventional breeding or other molecular approaches. This was related to higher maximum Rubisco activity relative to other Calvin cycle enzymes. Higher photosynthetic capacity did not improve biomass, however, because of an inverse relationship between photosynthetic capacity and leaf area. The work has important implications for engineering photosynthesis and productivity.

Results

Genetic modification of the trehalose pathway

Genes encoding different steps in the trehalose synthesis pathway have been transformed into tobacco (Figure 1A). Expression of the *E. coli* gene *otsA* encoding trehalose

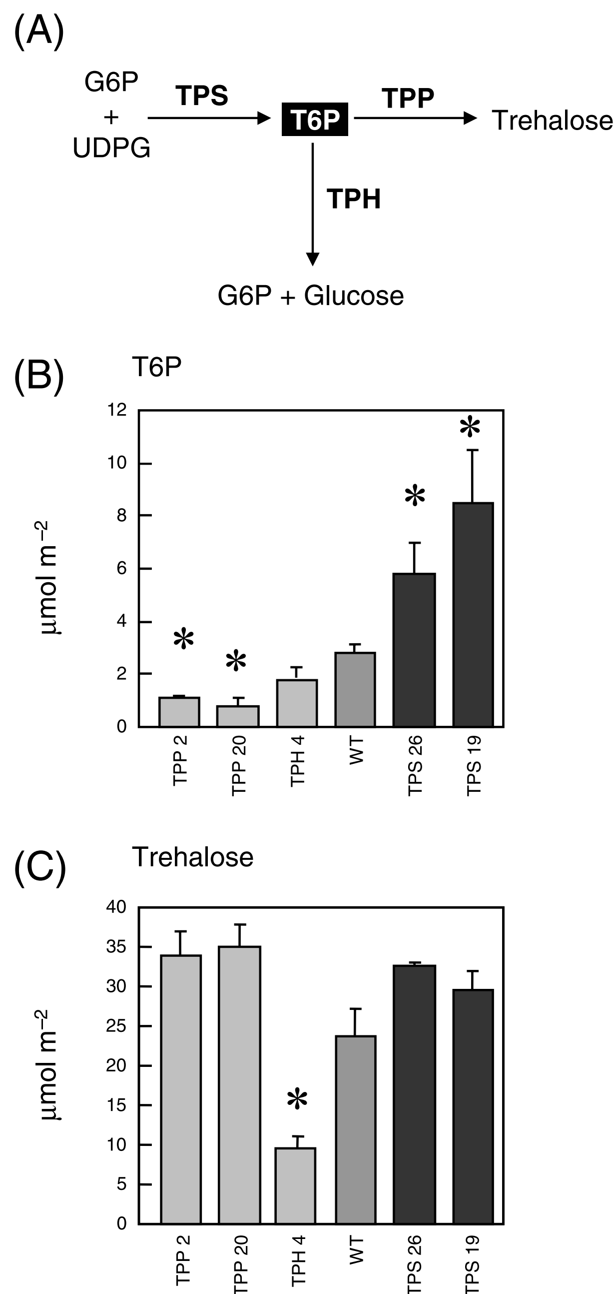

Figure 1 (A) The trehalose pathway. The over-expressed genes are TPS (trehalose phosphate synthase), TPP (trehalose phosphate phosphatase) and trehalose phosphate hydrolase (TPH). (B) Trehalose 6-phosphate (T6P) and (C) trehalose content in transgenic TPP, TPH, TPS and wild-type lines. *Statistically significant differences compared to wild-type at $P < 0.05$.

phosphate synthase (TPS) increased the amounts of T6P in leaves compared to wild-type up to threefold, but had little effect on trehalose content (Figure 1B,C). The expression of *E. coli* genes *otsB* and *Trec*, encoding trehalose phosphate phosphatase (TPP) and trehalose phosphate hydrolase (TPH), respectively (Figure 1A) decreased the amounts of T6P down to half of the wild-type (Figure 1B), with little effect on trehalose content, with the exception of TPH where trehalose content was decreased twofold compared to wild-type (Figure 1C). T6P content rather than trehalose content related to altered photosynthesis.

Photosynthetic capacity per unit leaf area and dry weight is altered

Measurements of rates of photosynthesis were carried out on numerous occasions on sets of transgenic plants between the 5 and 15 leaf stage. Each time very consistent data were obtained, representative values for which are presented (Table 1, Figure 2). Rates of CO_2 assimilation per unit leaf area under saturating light were up to 47% higher than the wild-type in TPS lines and a similar magnitude lower in TPP and TPH lines (Table 1). Smaller but statistically significant differences were also observed under growth irradiance (330 μmol/m^2/s, Table 1). Light response curves from the lines presented in Table 1 have been pooled in Figure 2A. Light-saturated photosynthesis per unit dry weight was up to 27% higher than in wild-type in TPS lines 10 and TPS 26, and a similar magnitude lower in TPP and TPH lines (Table 1). Differences were also apparent at low and high CO_2 (Figure 2B). Differences in CO_2 assimilation were accompanied by altered relative quantum yields of PSII electron transport rates (Table 1).

Rubisco and other enzyme activities

Rubisco activities paralleled the changes in photosynthesis. Maximum activities of Rubisco were up to 40% higher per unit leaf area than wild-type in lines TPS 26, TPS 19 and a similar magnitude lower in lines TPP 2, TPP 20 and TPH 4. Changes in Rubisco biochemistry were then examined in detail in lines TPS 26, TPP 2 and TPH 4 compared to wild-type (Figure 3). Amount of Rubisco per unit leaf area was up to 40% higher in TPS 26 and up to 40% lower in TPP and TPH lines grown under low light conditions (Figure 3A). Under high light growth conditions the magnitude of increase in the amount of Rubisco in TPS or decrease in TPP and TPH relative to wild-type was 20% (Figure 3A). There were no changes in *rbcS* transcript abundance (not presented). There were parallel changes in the amount of carboxyarabinitol 1-phosphate (CA1P) (Figure 3B). Initial and maximum activities of Rubisco were measured under growth irradiance (330 μmol/m^2/s) and under saturating irradiance (1500 μmol/m^2/s) (Figure 3C,D). There were clear differences in these activities, particularly under high light conditions, where close to 100% Rubisco activation was observed in all lines. Under the growing conditions, 65% to 80% activation was observed. The increase in Rubisco activity in TPS lines was not accompanied by increases in maximum activities of other Calvin cycle or

Table 1 CO_2 assimilation under growth irradiance (330 μmol/m^2/s) and saturating irradiance (1500 μmol/m^2/s) measured on fully expanded leaves of independent transformed lines

Line	CO_2 assimilation			Relative quantum yield of PSII electron transport rate ΦPSII at saturating light
	Growth light (μmol/m^2/s)	Saturating light (μmol/m^2/s)	Saturating light (μmol/g DW/s)	
TPS 10 pc	12.2 ± 1.1*	25.7 ± 1.3*	1.12*	
TPS 19 pc	10.8 ± 0.3*	21.9 ± 1.2*	0.86	0.24 ± 0.1*
TPS 26 35S	11.8 ± 0.1*	23.2 ± 0.3*	1.01*	
TPS 40 35S	10.9 ± 0.1*	18.1 ± 0.9	0.79	
TPP 2 35S	7.6 ± 0.8	12.7 ± 1.4*	0.74*	
TPP 20 pc	7.7 ± 0.1*	13.3 ± 1.5*	0.77*	
TPP 24 35S	8.7 ± 0.7	13.9 ± 2.0*	0.81	
TPH 4 pc	6.8 ± 0.2*	12.7 ± 1.0*	0.74*	0.09 ± 0.1*
TPH 7 pc	6.2 ± 0.3*	9.9 ± 2.8*	0.57*	0.08 ± 0.1*
TPH 17 pc	6.9 ± 0.1*	11.9 ± 1.1*	0.70*	
WT	9.0 ± 0.1	17.5 ± 0.2	0.88	0.16 ± 0.1

TPS encoded by *otsA*, TPP encoded by *otsB*, TPH encoded by *Trec* under the control of plastocyanin (pc) or 35S promoters. Data are expressed per unit leaf area (μmol/m^2/s) and per unit dry leaf dry weight (μmol/g DW/s). Means are of at least four measurements from four plants of each line. *Significant compared to wild-type at $P < 0.05$. Data are summarized in Figure 2.

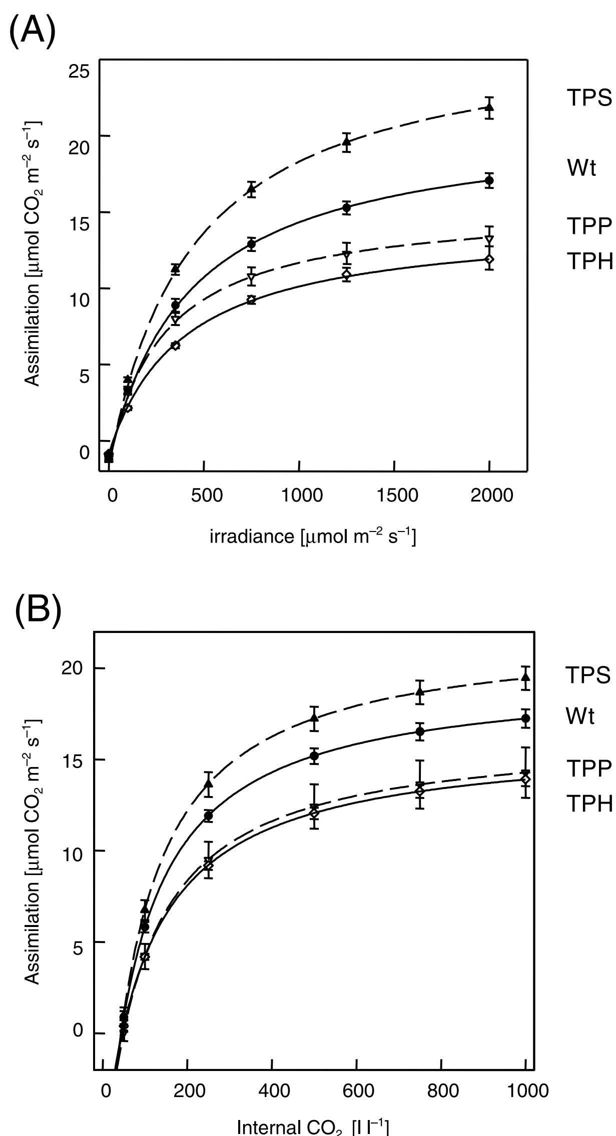

Figure 2 Photosynthetic capacity. Assimilation of CO_2 as a function of light (A) and CO_2 (B) in TPS lines 10, 19, 26, 40, wild-type (Wt), TPP lines 2, 20 and 24 and TPH lines 4, 7 and 17. Means are presented of four replicates of most recently fully expanded leaves from four plants of each line, with standard errors of the mean. For analysis, a regression curve of the form $y = y_o + (ax)/(b + x)$ was calculated for each data set using the Marquardt–Levenberg algorithm (Marquardt, 1963). From this, values for specific data points were calculated to enable statistical analysis. Factorial analysis of variance was conducted using the GenStat statistics program and the results were tested for significant differences using a *t*-test. TPS, TPP and TPH lines are significantly different from wild-type at $P < 0.05$ and above the growth irradiance of 350 µmol/m²/s (A) and at and above 100 µL/L internal CO_2 (B). Light response was performed under ambient CO_2; CO_2 response at the growth irradiance (330 µmol/m²/s).

cytosolic enzymes: phosphoribulokinase, phosphoglycerate kinase, transketolase, stromal fructose 1,6-bisphosphatase, glyeraldehyde phosphate dehydrogenase, hexokinase and cytosolic fructose 1,6-bisphosphatase (Figure 4). The apparent change in aldolase activity in TPS and TPP lines was not proven statistically. There were changes in the amounts of protein and chlorophyll in all lines tested (10 in all) compared to wild-type (representative data presented in Figure 3E,F).

Table 2 Amounts of soluble carbohydrates (mmol hexose equivalents/m²), ribulose-1,5-bisphosphate (RuBP) and 3-phosphoglyceric acid (3-PGA) (µmol/m²) measured in the middle of the photoperiod under growth conditions (330 µmol/m²/s)

	Glucose	Fructose	Sucrose	RuBP	3-PGA
TPP 20	0.60 ± 0.16	0.25 ± 0.05	1.17 ± 0.16*	66.1 ± 3.7	35.3 ± 3.0
TPH 4	0.45 ± 0.10	0.21 ± 0.04	1.01 ± 0.11*	ND†	ND†
Wild-type	0.45 ± 0.08	0.25 ± 0.04	0.63 ± 0.08	67.0 ± 3.0	38.4 ± 5.7
TPS 26	0.16 ± 0.03*	0.11 ± 0.02*	0.51 ± 0.06	57.1 ± 6.8	42.5 ± 1.2

Means are replicates of four samples from four plants of each line.
*Significant difference compared to wild-type at $P < 0.05$.
†Not determined.

Amounts of soluble carbohydrate and phosphorylated intermediates measured in the dark are increased in plants with low photosynthetic capacity

There was a trend to higher soluble carbohydrate in TPP and TPH lines than in the wild-type, particularly for sucrose, and to lower soluble carbohydrate content than wild-type in TPS lines, particularly glucose and fructose (Table 2). Amounts of glucose 6-phosphate were higher than the wild-type in the light in TPS 19. Under elevated light, the amounts of glucose 6-phosphate were higher than the wild-type in TPP 20 and TPS 19 and fructose 6-phosphate higher than wild-type in TPS 19 and TPS 26. Trends in abundance were not strong across the lines at ambient or elevated light (Figure 5). There were no differences in ribulose-1,5-bisphosphate or 3-phosphoglyceric acid (Table 2). In the dark, however, consistent differences emerged. Amounts of glucose 6-phosphate in particular were higher than the wild-type in TPP and TPH lines, but lower in TPS lines. Similar but smaller trends were observed for fructose 6-phosphate, glucose 1-phosphate and UDP-glucose.

Growth positively related to leaf area not photosynthetic capacity

Plants expressing TPP had larger leaves than the wild-type, particularly of leaves 5–15 (Figure 6A,B). Consequently the whole plant leaf area was larger (Figure 6C). Leaves and whole plant leaf area were correspondingly smaller in TPS plants (Figure 6A–C). Leaf mass per area was lower than wild-type in TPP plants and highest in TPS plants in the first two harvests (Figure 6D). Leaf area ratio (plant leaf area/plant

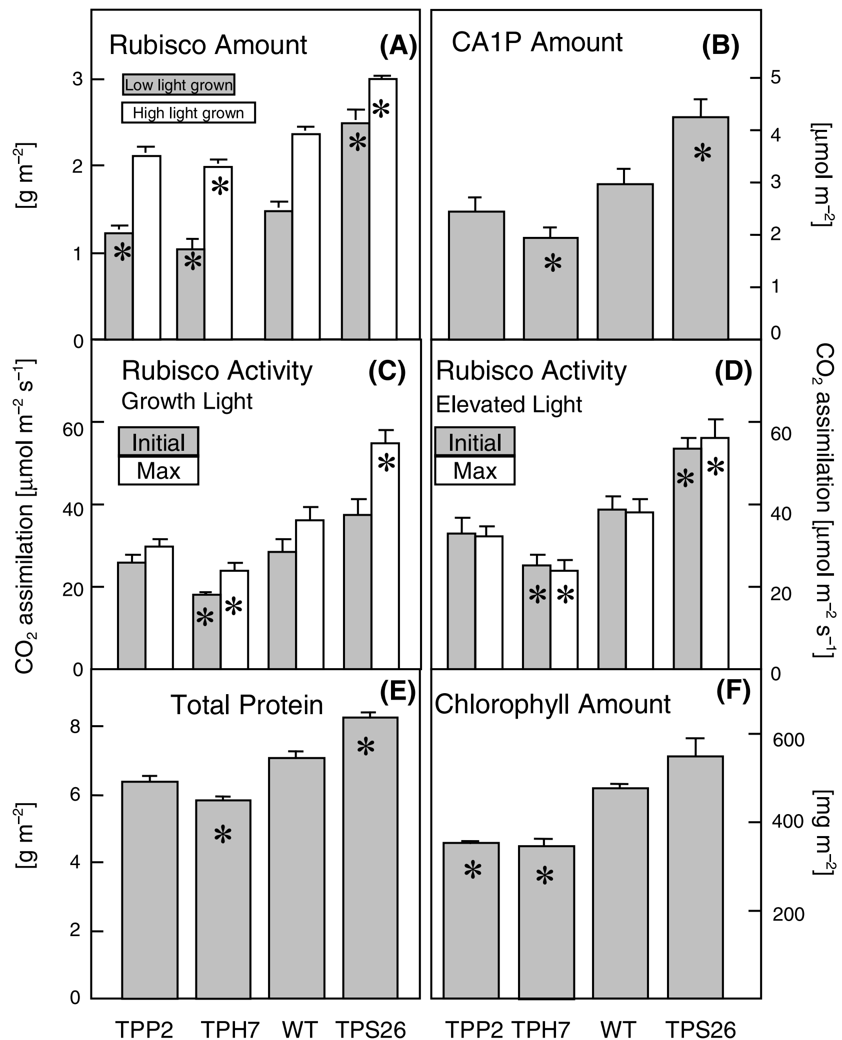

Figure 3 Rubisco biochemistry, protein and chlorophyll in low and high light grown plants (A) and in low light grown plants (B–F). (A) Amounts of Rubisco (g/m^2). (B) 2-carboxyarabinitol 1-phosphate (CA1P, μmol/m^2). (C, D) initial and maximum Rubisco activities (μmol/m^2/s) measured at growth light (330 μmol/m^2/s, C) and saturating light (1500 μmol/m^2/s, D), protein (E) (g/m^2) and

dry mass) was higher than wild-type in plants expressing TPP throughout the growth period (Figure 6E). Relative growth rate in TPP plants was higher than wild-type over the last two harvest periods (Figure 6F), which was reflected in a greater biomass at final harvest (Figure 6G).

Discussion

As a direct consequence of earlier attempts to engineer trehalose metabolism in plants (Goddijn *et al*., 1997) this once obscure pathway is now known to have an indispensable role in plants (Eastmond *et al*., 2002; Schluepmann *et al*., 2003). We now report that a further consequence of modifying T6P content is altered photosynthesis.

Improvements in photosynthetic capacity have been difficult to achieve through conventional breeding or through molecular approaches. Here we show a large increase in photosynthetic capacity per unit leaf area and dry weight in TPS transgenics with elevated T6P and Rubisco activity (Table 1 Figures 1–3). In spite of a small increase in leaf mass per unit leaf area in TPS transgenics (Figure 6D), the increase in photosynthetic capacity is still apparent when it is expressed per unit leaf dry mass and cannot be explained through increased leaf thickness. Furthermore, there were no changes in maximum activities of other Calvin cycle enzymes (Figure 4), showing that there was a specific increase in Rubisco abundance rather than a general increase in catalytic machinery. The large effect of Rubisco on photosynthetic capacity was consistent with the strong correlation between light-saturated photosynthesis and maximum Rubisco activity seen across many species (Bjorkman, 1981), and confirmed in studies of metabolic control analysis (Hudson *et al*., 1992; Stitt and Schulze, 1994). The increases in amount of Rubisco were observed in both low and high light grown plants (Figure 3A). Our work represents the first report of an increase in photosynthetic capacity resulting from elevated Rubisco content as

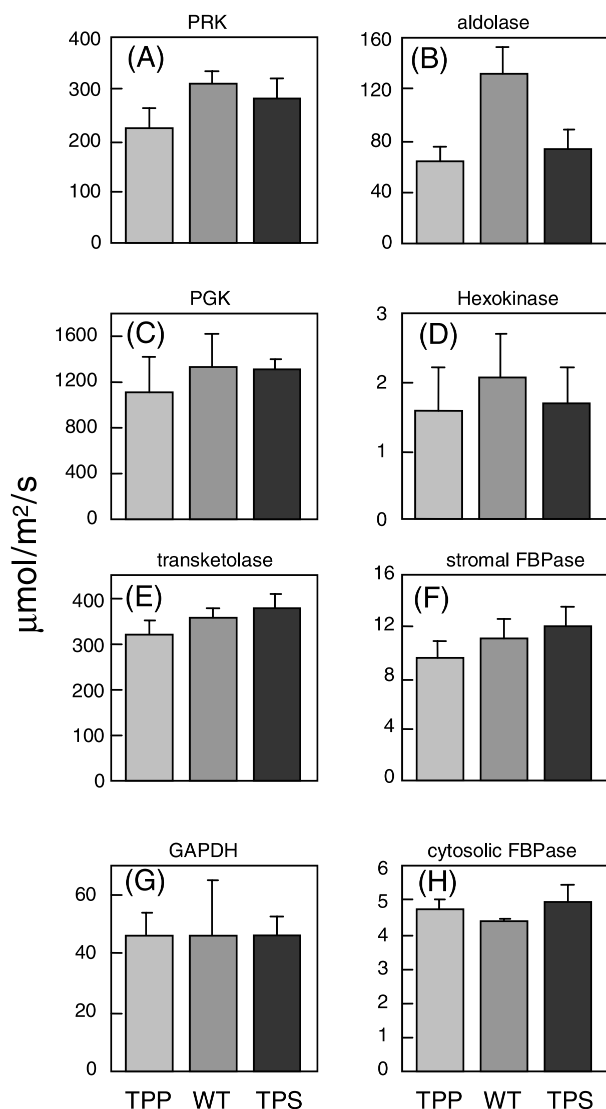

Figure 4 Maximum activities (μmol/m^2/s) of Calvin cycle and cytosolic enzymes. (A) phosphoribulokinase, PRK; (B) aldolase; (C) phosphoglycerate kinase, PGK; (D) hexokinase; (E) transketolase; (F) stromal fructose-1,6-bisphosphatase, FBPase; (G) glyceraldehyde phosphate dehydrogenase, GAPDH; (H) cytosolic fructose-1, 6-bisphosphatase, FBPase.

a result of genetic modification of metabolism. It demonstrates that it is possible to increase the amounts of this already abundant protein to improve photosynthetic capacity above Rubisco contents and photosynthetic rates normally found in wild-type plants (Banks *et al*., 1999; Paul *et al*., 2000; Ruuska *et al*., 2000). It also shows that photosynthesis can be increased as a result of increasing the abundance of just one enzyme in the Calvin cycle rather than the whole cycle (Paul, Pellny and Goddijn, 2001). Rubisco is the enzyme in this case, but this has also been demonstrated for sedoheptulose-1,7-bisphosphatase (Raines, 2003) and for sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase combined (Miyagawa, Tamoi and Shigeoka, 2001). It is likely that the enzyme activation states of enzymes such as fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase may have increased and contributed to enhanced ribulose-1,5-bisphosphate regeneration for Rubisco. Accompanying increased CO_2 fixation there was an increase in the relative quantum yield of photosystem II electron transport (Table 1). There was no evidence of large changes in light capture as chlorophyll a : b ratio was unaltered (not presented) and there was no large change in the initial slope of the light response curve (Figure 2A).

Introduction of the transgenes altered the trace abundance of T6P much more than of trehalose (Figure 1), which was closely related to effects on photosynthesis. Goddijn *et al*. (1997) found that it was possible to enhance trehalose content by inhibiting trehalase. This suggests that trehalose content is dependent more on trehalase activity than on activities of enzymes in the synthetic segment of the pathway. The more drastic impact of the transgenes on T6P levels, however, suggests that activities of enzymes in the synthetic part of the pathway exert more control on the abundance of this metabolite. Moreover, it also makes it more likely that T6P rather than trehalose is responsible for the phenotype. This becomes particularly compelling when plants expressing different transgenes, TPP and TPH, display the same phenotype and similar amounts of T6P, but dissimilar amounts of trehalose (Figure 1). Effects on T6P were also paralleled by changes in phosphorylated intermediate content in the dark (Figure 5). This confirms previous work that demonstrated a large impact of T6P on carbohydrate utilization (Schluepmann *et al*., 2003). A further unexpected spin-off, in addition to the impact on carbohydrate utilization, is the effect on photosynthesis in the present study.

Carbohydrate utilization and feedback control of photosynthesis have long been linked and this provides a starting point for understanding the mechanistic basis for altered photosynthesis in this study. Beyond direct cycling of Pi back to the chloroplast, which exerts short-term feedback control (Paul and Pellny, 2003), longer-term feedback control has been closely linked with the entry of carbon through hexokinase (Jang *et al*., 1997; Moore *et al*., 1998, 1999). This has been linked to sugar content and the cycling of sucrose through invertase and hexokinase. The work of Jang *et al*. (1997) suggests a direct role for hexokinase as a sugar sensor. In yeast, T6P directly inhibits hexokinase. No evidence for such a direct interaction has been found in plants (Eastmond *et al*., 2002). However, dark levels of metabolites (Schluepmann *et al*., 2003; Figure 5) suggest an effect of T6P downstream of hexokinase. It therefore appears that the known association

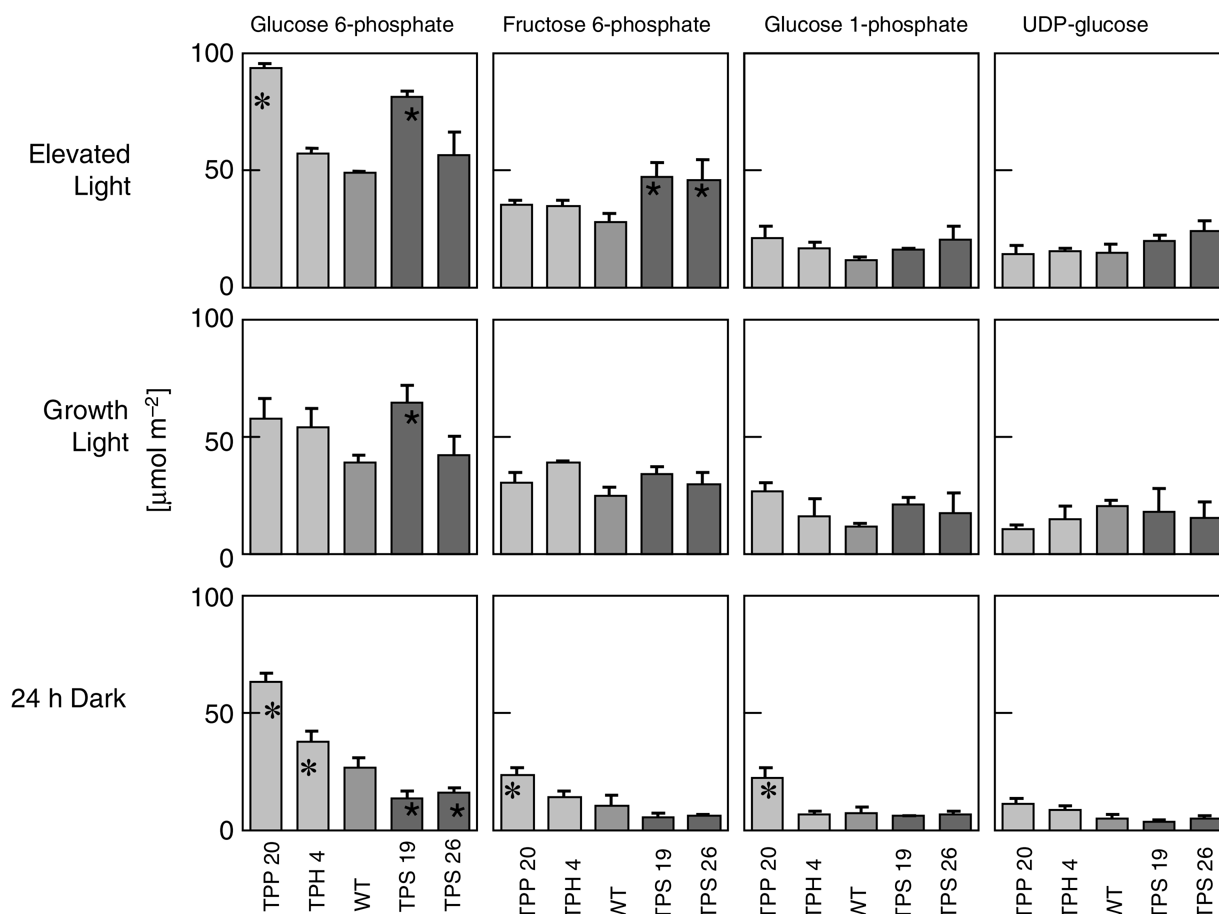

Figure 5 Phosphorylated intermediates (μmol/m^2) in most recently fully expanded leaves measured under growth light (330 μmol/m^2/s), after a 4 h period of elevated light (800 μmol/m^2/s) and in the dark.

between carbohydrate utilization and photosynthetic regulation (Jang *et al*., 1997; Moore *et al*., 1998, 1999, 2003) can be causally linked through the effects of T6P on carbohydrate utilization (Schluepmann *et al*., 2003) as a mechanism that regulates photosynthesis. The sugar contents of leaves (Table 2) were increased in TPP and TPH transgenics with a lower photosynthetic capacity consistent with a sugar repression model. It is possible that soluble sugar content may be a consequence of metabolic factors linked to this mechanism of photosynthetic regulation, rather than necessarily directly causally involved in photosynthetic regulation (Tsai *et al*., 1997). Definitive evidence of the precise cause and effect awaits further elucidation and is not the object of this paper. The large changes in Rubisco abundance protein per unit leaf area (Figure 3A) were not, however, paralleled by changes in Rubisco transcript abundance. It is possible that Rubisco abundance may be regulated downstream of transcription at the level of translation or turnover of Rubisco protein. The tight-binding inhibitor of Rubisco, CA1P, has been found to protect Rubisco against proteolytic breakdown and hence to determine the Rubisco content of leaves (Khan *et al*., 1999). The amounts of CA1P related closely to Rubisco content in this study (Figure 3B), and may play a role in regulating Rubisco abundance.

Accompanying the striking increase in photosynthetic capacity in TPS lines was a corresponding decrease in leaf area (Figure 6A–C). Surveys of variations in photosynthesis per unit leaf area of many crops demonstrates a strong negative relationship with leaf area (Bhagsari and Brown, 1986) and is therefore not surprising. It was referred to as the dilution hypothesis by Hesketh *et al*. (1981) and balancing leaf area with the photosynthetic capacity of leaves represents a central strategy of photosynthesis regulation at the whole plant level. Many factors, particularly light, determine this trade off. The impact of carbohydrate metabolism on the balance between photosynthetic capacity and leaf area production has been little studied. Our data suggest that it is likely that the effect of T6P on carbohydrate utilization (Schluepmann *et al*., 2003) is another factor influencing this process.

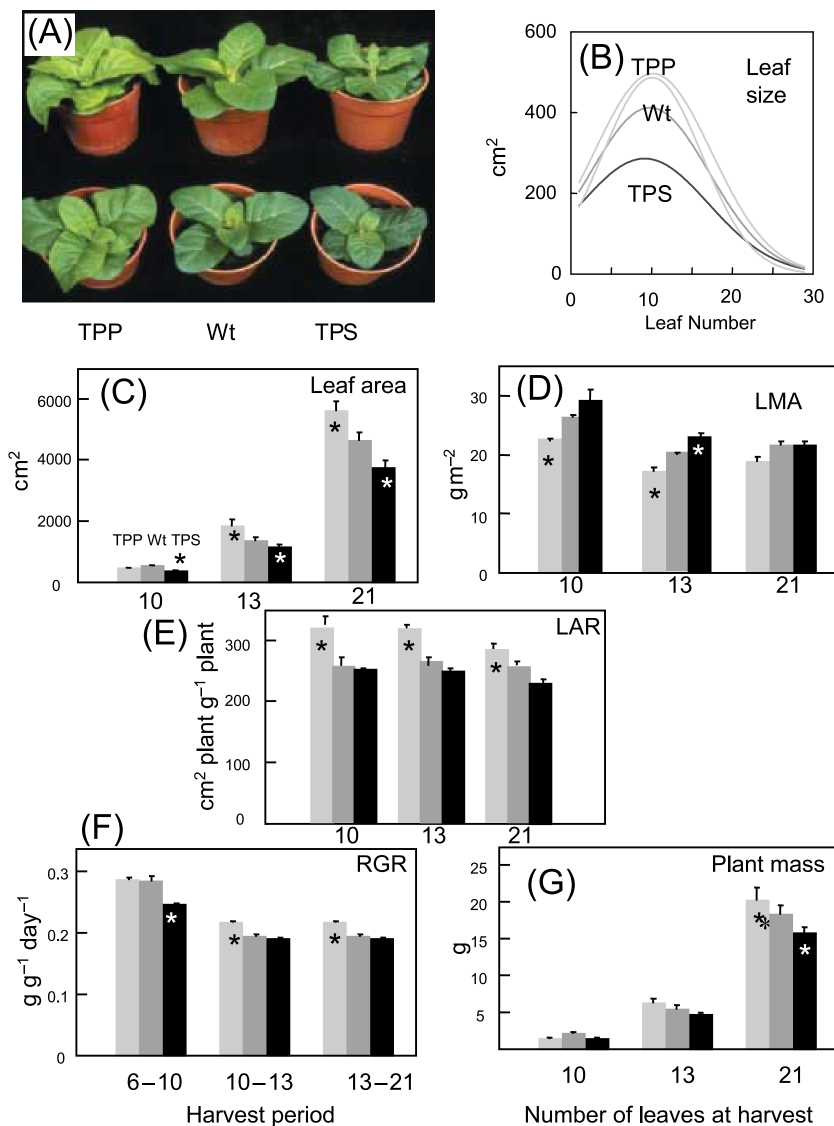

Figure 6 Phenotype and growth parameters of TPP 20, wild-type and TPS 26 lines. Plants were harvested when they had 6, 10, 13 and 21 leaves (28, 38, 44 and 53 days after sowing). (A) Visual appearance of 5-week-old plants. (B) Leaf area. (C) Total leaf area. (D) Leaf mass per leaf area (LMA). (E) Leaf area ratio (LAR). (F) Relative growth rate (Rgr; g plant/g/day), calculated over the growth intervals 6–10 leaves, 10–13 leaves and 13–21 leaves. (G) Plant dry mass (g). *Statistically significant differences ($P < 0.05$) compared to wild-type. Data are means of at least 12 replicate plants per time point which are representative data for analyses that were performed three times. ■, TPP; ■, wild-type; ■, TPS.

We found that plant leaf area favoured relative growth rate and biomass (Figure 6). These observations confirm the many studies showing that leaf area and low leaf mass per unit area are more closely related to relative growth rate and productivity than the photosynthetic capacity of individual leaves (Poorter and de Jong, 1999; Poorter and Pothmann, 1992). This also tends to confirm studies on the relationship between photosynthesis and yield, which overwhelmingly emphasized that there was little positive relationship between photosynthetic rate per unit leaf area and yield (Evans, 1993). For many crops, the highest photosynthetic rates per unit leaf area were recorded for low-yielding wild progenitors. Traditional crop breeding has increased leaf area at the expense of photosynthetic capacity as a means of improving yield. Our data confirmed that maximizing leaf area was a good strategy for maximizing whole plant photosynthesis and growth, at least under the growing conditions used in this study (high N and full irrigation). The data raise the possibility that targeting leaf photosynthesis on its own may not be the best approach to improving production, unless it can be uncoupled from the negative effects on leaf area. Miyagawa *et al.* (2001) showed that this is possible, although mechanisms that allow the separation of the two are not known. Increased photosynthetic capacity combined with greater leaf area could achieve large benefits to production if sustained by large nitrogen inputs. It should also be remembered that a different trade off between photosynthetic capacity and leaf area may have advantages under different environments. For example, under conditions of low water availability less leaf area with higher photosynthetic capacity may favour water conservation. The demonstrated effect of T6P on carbohydrate utilization and signalling (Schluepmann *et al.*, 2003)

offers a new opportunity for targeting photosynthetic capacity and leaf area production to improve biomass production.

Experimental procedures

Plant material

Nicotiana tabacum Samsun NN was transformed using Agrobacterium-mediated leaf disc transformation with plasmids as described in patents WO95/01446 and WO97/42326, and in cited methods (Goddijn *et al*., 1997). Plasmids harboured genes *otsA*, *otB* and *treC* from *E. coli* encoding trehalose phosphate synthase (TPS, EC 2.4.1.15), trehalose phosphate phosphatase (TPP, EC 3.1.3.12) and trehalose phosphate hydrolase (TPH, EC 3.2.1.93), respectively (Giaever *et al*., 1998; Kaasen *et al*., 1992; Rimmle and Boos, 1994). Genes were driven by double-enhanced CaMV35S promoter (Guilley *et al*., 1982) (TPS lines 3, 26 and 40; TPP lines 2 and 24) or plastocyanin promoter (TPS lines 10 and 19, TPP line 20 and TPH lines 4, 7 and 17) with nos terminator. Plasmid pVDH275 harbouring TPS driven by the plastocyanin promoter is a derivative of pMOG23 (Sijmons *et al*., 1990). The plastocyanin promoter was transferred using PCR amplification and primers with suitable cloning sites. To construct plasmid pVDH321 harbouring the TPP gene and plastocyanin promoter, the *Bam*HI site of plasmid pTCV124 was removed by *Bam*HI, filling in with subsequent re-ligation. Digestion with *Hin*dIII and *Eco*RI yielded a DNA fragment comprising the TPP coding region and PotPiII terminator. *Bam*HI linkers were added, and the resulting fragment in the binary expression vector pVDH275 digested with *Bam*HI yielding pVDH 321. Neomycin phosphate transferase II (nptII) conferring resistance to kanamycin was the selectable marker. Several lines of independent primary transformants were obtained. Seeds from the T2 generation of homozygous lines with one copy of the expressing transgene were used in experiments. The lines with the lowest numbers had the highest expression of transgene. Seeds were sown on moist filter paper at 25 °C and after 1 week seedlings were transferred to peat-based compost and a controlled environment providing 330 μmol photons/m^2/s, 14-h photoperiod, constant 25 °C and 70% r.h. For growth analysis the plants were grown in a glasshouse during the summer (28 °C day, 22 °C night, irradiance up to 1500 μmol photons/m^2/s on sunny days). All plants were grown with full nutrition (Banks *et al*., 1999).

Leaf gas exchange

Rates of CO_2 fixation were measured in the laboratory using a six-chamber open-circuit gas exchange system with automatic data handling. The partial pressure of CO_2 was controlled by a gas blender (Signal Instruments Co., Croydon, UK) and measured with an infra-red gas analyser Mark 3 (ADC, Hoddesdon, UK). The humidity of the air before and after passage over the leaf was determined with capacitance sensors (Vaisala). All measurements were made on 10 cm^2 areas of leaves attached to plants with a leaf temperature of 25 °C. The gas flow rate was 9 mL/s. Stomatal conductance to water vapour, transpiration and CO_2 concentration within the leaf (Ci) was calculated following the methods of Von Caemmerer and Farquhar (1981). Leaves were allowed to equilibrate for 30 min prior to measurement.

Protein and chlorophyll

Soluble proteins were extracted in phosphate buffer (50 mM sodium phosphate, pH 7.0) containing 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) triton X-100. Protein determination followed the method of Bradford (1976). Aliquots were transferred to 80% acetone for chlorophyll determination (Arnon, 1949).

Metabolite measurements

Leaf samples were snap-frozen in the light or dark and ground to powder in pestle and mortar. Powder was extracted in 5 volumes 5% perchloric acid on ice for 30 min, then neutralized with 5 M KOH in 1 M triethanolamine and metabolites measured using enzyme-linked assays. Glucose-6-P, fructose-6-P, glucose-1-P and UDP-glucose were assayed together in 50 mM Hepes, pH 7, with 5 mM $MgCl_2$, 0.25 mM NADP and 0.12 mM Na pyrophosphate. Metabolites were measured by the sequential addition of 0.1 U each of glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, phosphoglucomutase and UDPG pyrophosphorylase. All measurements were performed on a dual-wavelength spectrophotometer 340 nm/410 nm, on 50–100 μL tissue extract in 1 mL of the above reagents.

T6P was extracted from frozen pulverized leaf tissue. The powder was extracted for 20 min with 5 volumes of 80% boiling ethanol, centrifuged for 10 min at 12 000 ***g*** and the supernatant obtained was vacuum desiccated. The dry sample was extracted with 1.2 mL boiling 0.1 M NaOH for 1 h, centrifuged for 10 min at 12 000 ***g***, and the supernatant obtained was neutralized with 10 volumes of 200 mM triethanolamine, pH 7.6, and then split into two equal portions. One unit of alkaline phosphatase, 10 mM $MgCl_2$ and 1 mM $ZnCl_2$ were added to one portion to efficiently remove T6P at 25 °C for 20 min followed by 90 °C for 45 min and

centrifugation to remove the phosphatase activity. T6P was assayed based on the inhibition of *Y. lipolytica* hexokinase (Blazquez *et al.*, 1993); assays were done in quadruplicate on microtitre plates and included an internal standard curve of 0–800 pmol T6P for each sample. *S. cerevisiae* expressing this hexokinase, but lacking its own, was used (Petit and Gancedo, 1999). Yeast cake was extracted in 20 mM imidazole buffer by vortexing with glass beads, centrifuged for 10 min at 700 ***g***, and the supernatant used for hexokinase inhibition assay with 0.2 U phosphoglucose isomerase, 0.2 U glucose-6-phosphate dehyrogenase, 1 mM ATP, 0.5 mM NADP and 0.1 mM fructose final concentration. Assays were linear for 30 min and could resolve T6P levels of less than 50 pmol T6P. Recoveries of T6P were in excess of 80%. Amounts of T6P quantified this way were confirmed by HPLC using a method modified from Paul *et al*. (2000). Trehalose was determined as in the assay for soluble sugars (see below) with the addition of maltase after the invertase and then trehalase to determine trehalose.

To measure carbohydrate, discs were cut with a cork borer and extracted in 100 mM imidazole-HCl buffer, pH 6.9, and an aliquot was immediately added to 80% ethanol at 70 °C for 20 min. Samples were then kept at 20 °C until analysis. Glucose, fructose and sucrose were measured through the reduction of NADP by glucose-6-P dehydrogenase after the sequential addition of hexokinase, phosphoglucose isomerase, and invertase (Jones *et al.*, 1977). The method was adapted for use on an ELISA plate, using reduced volumes and a microplate reader to measure absorbance changes.

Rubisco activity amounts

Leaf samples were snap frozen in the light as was done by Paul *et al*. (2000). For the determination of Rubisco activity, samples were rapidly extracted (3 cm^2/mL following buffer) 50 mM Bicine-NaOH, pH 8.2, 20 mM $MgCl_2$, 1 mM EDTA, 10 mM $NaHCO_3$ (omitted for determination of initial assays), 2 mM benzamidine, 5 mM ε-aminocaproic acid (6-amino-n-hexanoic acid carboxypeptidase and plasmin inhibitor, 50 mM 2-mercaptoethanol, 10 mM DTT, 1 μM pepstatin, 10 μM E64 (cys protease inhibitor), 2 mM PMSF and 2% (w/v) insoluble PVP. Aliquots were taken for the immediate assay of initial and maximum activities. The assay buffer was 100 mM Bicine-NaOH, pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH[^{14}C]CO_3$ (0.5 μCi/μmol). Assays were started immediately with 33 mM RuBP to give a final concentration of 0.33 mM (initials), or after pre-incubation with extract for 2 min (maximums) at 25 °C for 60 s and then quenched with 10 M formic acid.

For the assay, 150 μL of Rubisco supernatant from the original extract was added to 150 μL $[^{14}C]$CABP binding solution incubated for 15–30 min at 0 °C, and then 214 μL 60% (w/v) PEG 4000 was added. After 30 min at 0 °C, the PEG precipitate was centrifuged at 10 000 ***g***, 4 °C for 10 min and the supernatant removed by aspiration. This step was then repeated and the pellet re-dissolved in 500 μL 1% (v/v) TX-100 and the amount of bound $[^{14}C]$CABP determined by scintillation counting.

CA1P

Amounts of CA1P were determined as in Khan *et al*. (1999).

Other enzyme assays

Phosphoribulokinase, aldolase, transketolase, stromal and cytosolic FBPase, PGK and GAPDH were assayed according to Paul *et al*. (2000) and Haake *et al*. (1999). Hexokinase was assayed as in Renz *et al.* (1993).

Determination of growth parameters

Growth analysis was performed on three different occasions and the data are presented from the last most detailed set of characterizations, which confirmed the trends observed in the earlier measurements. Plants were glasshouse grown (see above) and at least 12 plants per time point were sampled at intervals when plants had 6, 10, 13 and 21 leaves (28, 38, 44 and 53 days after sowing, respectively). Relative growth rate (g plant/g/day) was calculated over the growth intervals 6–10 leaves, 10–13 leaves and 13–21 leaves. Leaf area was measured using an automated planimeter (Delta-T Devices, Burwell, Cambridge, UK). Dry weight was measured after the plant material had been dried in an oven at 70 °C. Leaf mass per area was calculated as the amount of leaf area generated per unit dry weight invested in the leaf, and the leaf area ratio was calculated as the amount of plant leaf area per plant total dry weight.

Acknowledgements

Rothamsted Research receives grant-aided support from the Biotechnological and Biological Sciences Research Council of the UK. Till Pellny acknowledges a BBSRC case studentship awarded to Rothamsted and Syngenta Mogen. We thank Deborah Keith at Syngenta for continuing enthusiastic discussions. Our gratitude extends to Carlos Gancedo (Madrid, Spain) for providing us with *Yarrowia lipolytica* hexokinase

and to Silvia Rudmann and Paul Holford for help with the growth analysis.

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