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Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: effect on CO_2 assimilation and growth in low irradiance

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Summary

To quantify the importance of the Calvin cycle enzyme phosphoribulokinase (PRK) in photosynthesis and to perturb photosynthesis without large direct reductions in leaf protein content, tobacco plants (*Nicotiana tabacum* L.) were transformed with an inverted cDNA encoding tobacco PRK. A population of plants expressing antisense RNA and a range of PRK activities from wild-type to less than 5% of wild-type were obtained. CO_2 assimilation under the growing conditions (330 μmol photons m^{-2} sec^{-1}, 350 μbar CO_2, 25°C) was not inhibited until more than 85% of PRK activity had been removed. With reduction in PRK activity of between 85 and 95%, assimilation rates and amounts of chlorophyll compared with wild-type were reduced by up to half. Decreased absorption of light by leaves with less chlorophyll accounted for only a small part of the reduction in assimilation rate. When PRK activity was below 15% of wild-type, amounts of ribulose-5-phosphate, ribose-5-phosphate, ATP and fructose-6-phosphate were 1.5- to fivefold higher and levels of ribulose-1,5-bisphosphate, 3-phosphoglyceric acid and ADP 1.5- to fourfold lower than in wild-type. It is estimated that these changes maintained flux through PRK to realise the assimilation rates observed. A possible shift of control within the Calvin cycle towards fructose-1,6-bisphosphatase in plants with low PRK is discussed. Amounts of hexoses and starch in particular were reduced in plants expressing the lowest PRK activities; amounts of sucrose were little affected. Lower CO_2 assimilation in plants with low PRK activity correlated with reduced relative growth rate of shoots and delayed flowering, but there was no effect on specific leaf area. It is concluded that (i) in wild-type plants grown in constant low light, PRK has a flux-control coefficient for CO_2 assimilation of zero, and that even when amounts of PRK are reduced 20-fold relative to wild-type, altered amounts of metabolites compensate for much of the reduction in PRK protein; (ii) in plants where there is a 95% reduction in amounts of PRK, photosynthesis was reduced twofold without large changes in leaf protein content or leaf geometry.

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Introduction

The Calvin cycle is a complex series of 13 reactions catalysed by 11 enzymes. Reactions shown to be substantially displaced from equilibrium and hence potential sites of metabolic regulation within the Calvin cycle are those catalysed by ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and ribulose-5-phosphate kinase (phosphoribulokinase, PRK). This evidence comes from measurements of metabolites and enzyme activities and their compartmentation, and characterization of enzyme properties (Bassham and Krause, 1969; Leegood *et al.*, 1985; Stitt *et al.*, 1989; Woodrow, 1986; Woodrow and Berry 1988; Woodrow and Mott, 1993).

A recent approach to analysing control of flux through metabolic pathways has been to use transgenic plants with altered amounts of particular enzymes as a result of the expression of antisense gene constructs. Providing there are no pleiotropic effects, this is the most direct way of determining the importance of a particular enzyme in controlling flux. Furthermore, it enables effects at the metabolic level to be linked with those at the whole plant level. This has been done for Rubisco (Hudson *et al.*, 1992; Quick *et al.*, 1991a; Stitt *et al.*, 1991) enabling flux-control coefficients to be assigned in different short- and long-term conditions. However, because Rubisco constitutes such a large proportion of the protein in a leaf (up to 40%, Woodrow and Berry, 1988) and a reduction in amounts of Rubisco substantially reduces leaf protein content and disrupts the plant nitrogen balance (Quick *et al.* 1991a, 1991b), it is more difficult to establish direct links between photosynthesis and growth and allocation in these plants than if there were a more specific alteration in the rate of photosynthesis.

To examine the effect of reduced photosynthesis on growth and allocation without large direct alterations in leaf protein content and to quantify the importance of PRK in photosynthesis, tobacco was transformed with antisense

gene constructs to reduce the activity of PRK, an enzyme which constitutes less than 1% of leaf protein. PRK synthesizes ribulose-1,5-bisphosphate (RuBP) from ribulose 5-phosphate (Ru5P) and ATP and is rapidly activated in the light as a result of reduction of thiol groups via thioredoxin and ferredoxin-thioredoxin reductase, the kinetics of which is influenced by stromal concentrations of H^+, Mg^{2+} and metabolites (Laing *et al.*, 1981). PRK is a potential site of metabolic regulation and light-activated PRK is competitively inhibited with respect to Ru5P by 6-phosphogluconate, RuBP, 3-phosphoglyceric acid (3-PGA) and phosphate; fructose-1,6-bisphosphate (FBP) is also inhibitory (Gardemann *et al.*, 1983).

This paper documents the effect of reduced PRK activity on photosynthesis and shoot growth in transgenic tobacco under constant low-light (330 μmol m^{-2} sec^{-1}) growing conditions.

Results

Transgenic plants

Tobacco plants containing decreased PRK activity were obtained by transformation with an antisense PRK construct under the control of the tobacco *rbc*S promoter. Transgenic plants were examined for the presence of PRK RNA by Northern blot analysis and for PRK activity. There was a strong correlation between the amount of PRK RNA and PRK activity (data not shown). Transgenic plants covering a 20-fold range of PRK activities were used to examine the effects on CO_2 assimilation and growth measured in low-light conditions.

Photosynthesis

The rate of CO_2 uptake on a leaf area basis measured under the plant growth conditions was not affected until the activity of PRK was less than 25 μmol m^{-2} sec^{-1} (less than 20% of the wild-type PRK activity) (Figure 1a). Between 20 and 5 μmol m^{-2} sec^{-1} PRK activity there was a strong reduction in the rate of CO_2 fixation corresponding to a flux-control coefficient of about 0.6. Amounts of chlorophyll per unit leaf area also decreased when PRK activity was below 20 μmol m^{-2} sec^{-1} (Figure 1b). The amount of light absorbed by leaves was 86.4% ± 0.5 in wild-type compared with 84.5% ± 0.6 in plants with 5% of wild-type PRK (n= 12). Reduction in assimilation rate per unit leaf area was also accompanied by a higher Ci (Figure 1c). Transpiration rates and stomatal conductance remained unaltered (data not shown) and hence instantaneous water use efficiency was reduced (Figure 1d), because less CO_2 was fixed per unit of water transpired. Care was taken to space out plants as they grew, because mutual shading reduced differences in measured CO_2 assimilation between wild-type and plants

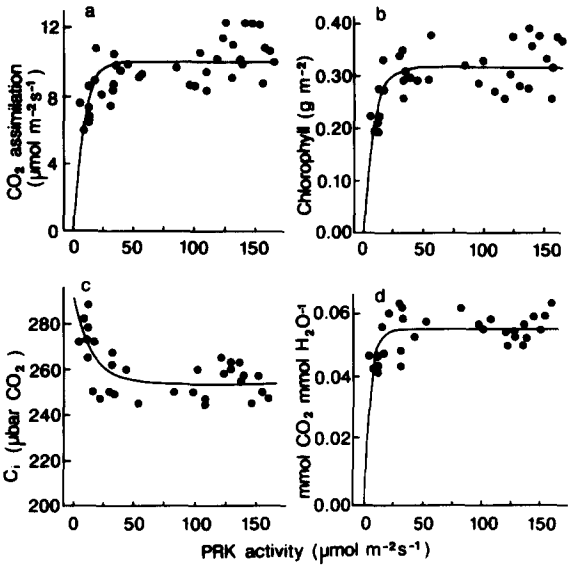

Figure 1. Response of gas exchange and chlorophyll measured under the growing conditions to altered PRK activity.
(a) CO_2 assimilation; (b) chlorophyll; (c) leaf internal CO_2 concentration (Ci); (d) instantaneous water-use efficiency (mmol CO_2 assimilated per mmol H_2O transpired).

with the lowest activity of PRK (data not presented). Also, differences in assimilation rates and chlorophyll content between wild-type and plants with the lowest activities of PRK diminished as plants got older (data not presented).

Carbon metabolism

Amounts of RuBP and 3-PGA were reduced more than twofold in plants with very low PRK activity; the ratio of these two metabolites was not affected by a large reduction in PRK activity (Figure 2). Triose phosphate (TP) levels were maintained over the range of PRK activity despite the low amount of RuBP and 3-PGA at the lowest PRK activity. Amounts of ATP and ADP were up to twofold higher and lower, respectively, relative to wild-type in plants with the lowest activities of PRK. Amounts of Ru5P and R5P were up to fivefold higher in plants with the lowest PRK activity compared with wild-type; their amounts relative to each other were unchanged. There was no consistent difference in amounts of FBP over the range of PRK activity. G6P and F6P levels were higher than in wild-type at the lowest activity of PRK. Amounts of F6P rose much more than G6P, and the ratio of G6P:F6P fell from about 2 in wild-type to 0.7 in plants with the lowest PRK activity. Using these data in the equation of Woodrow and Mott (1993) (see Experimental procedures), it is estimated that flux through PRK in wild-type was 17.1 compared to measured assimilation of about 10 (Figure 1a), and 11.3 and 5.4 compared to measured assimilation of 10 and 6.4 in plants with 50 and 10 μmol m^{-2} sec^{-1} PRK activity, respectively.

Amounts of starch and glucose in plants with 7% of wild-

type PRK activity were about a third of the amounts in wild-type plants (Table 1). Amounts of sucrose in the transformants were only slightly reduced (80% of wild-type) and amounts of fructose were about half the amounts in the wild-type. Hence, the ratio of sucrose to starch and of glucose to fructose in plants with 7% of wild-type PRK activity was twofold higher than in wild-type plants. During this first 6 h of the photoperiod, accumulation of carbohydrate was lower in the transformants, reflecting reduced photosynthetic rate, but partitioning of this carbon between the carbohydrates was similar.

Amounts of Rubisco protein measured with the CABP-binding assay (Figure 3b) and confirmed with an ELISA method (data not presented), and soluble protein (Figure 3a) were not affected by a large reduction in PRK activity. Maximal activities of NADP malate dehydrogenase and fructose-1,6-bisphosphatase were not affected until amounts of PRK were greatly reduced (Figure 3c and d).

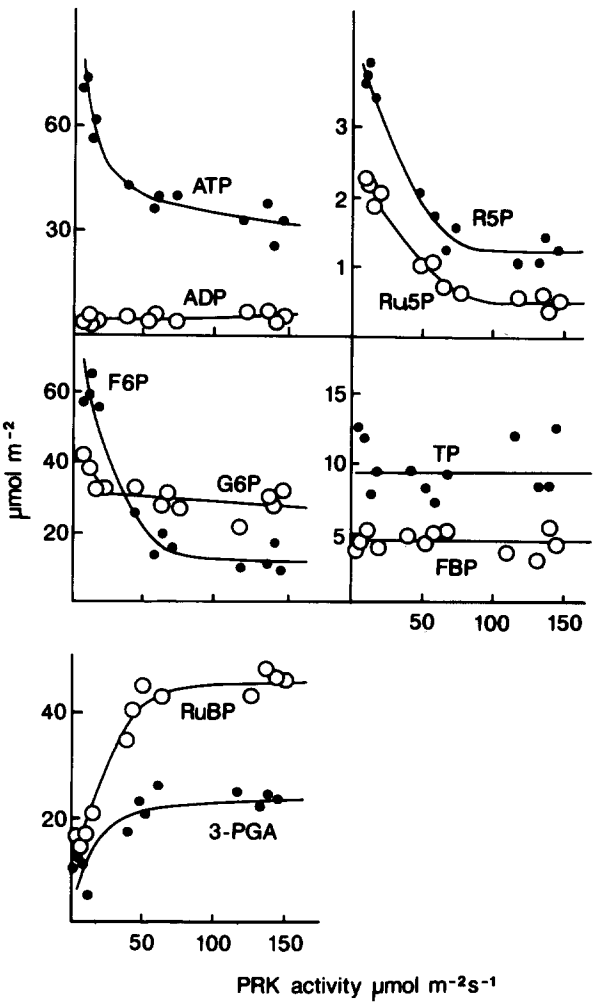

Figure 2. Response of metabolites (µmol m^{-2}) in source leaves in the light in the growing conditions to altered PRK activity.

Growth

Dry weights of shoots at flowering were similar over the whole population (Figure 4a). However, flowering was delayed in plants with the lowest PRK activities by up to 14 days compared with wild-type (Figure 4b), and relative growth rate of shoots between the seedling stage and the flowering stage was also lower (Figure 4c). There were no differences in numbers of leaves, specific leaf area or relative water content (data not presented).

Discussion

Transgenic tobacco with altered amounts of PRK have been used to disrupt CO_2 assimilation without large direct reductions in leaf protein content and to quantify the importance of PRK in photosynthesis. In plants grown in low light (330 µmol photons m^{-2} sec^{-1}) 85% of the enzyme can be removed before there is any effect on the rate of photosynthesis in these conditions or on shoot growth. Therefore, PRK has a flux-control coefficient of zero for CO_2 assimilation and growth in wild-type tobacco growing under these conditions. We conclude that PRK is in large excess in these conditions with only about 6% of maximal PRK activity required to sustain the rates of CO_2 assimilation observed in the wild-type. This accords with Gardemann *et al.* (1983) who demonstrated that when the light-activated enzyme from spinach was assayed in the presence of physiological concentrations of metabolites found in leaves in the light, PRK reached only 4% of its maximal activity. Our data also confirm the models of C_3 photosynthesis that predict that under these conditions PRK has a negligible effect on flux (Woodrow and Mott, 1993).

Table 1. Amounts of carbohydrates ± SE (mmol glucose equivalents m^{-2}) in leaves of wild-type plants (PRK activity 151 µmol m^{-2} sec^{-1}) and in plants expressing low PRK activity (10.7 ± 1.71 µmol m^{-2} sec^{-1}, 7% of wild-type)

Time into photoperiod	Wild-type				Low PRK			
	Glucose	Fructose	Sucrose	Starch	Glucose	Fructose	Sucrose	Starch
0	2.43±0.56	1.24±0.31	2.70±0.32	1.79±0.20	0.71±0.22	0.60±0.15	2.26±0.30	0.61±0.09
6	3.89±0.58	2.31±0.50	4.48±0.95	2.52±0.30	1.68±0.46	1.43±0.32	3.48±0.06	0.97±0.10

Measurements were made at the start of and 6 h into a photoperiod.

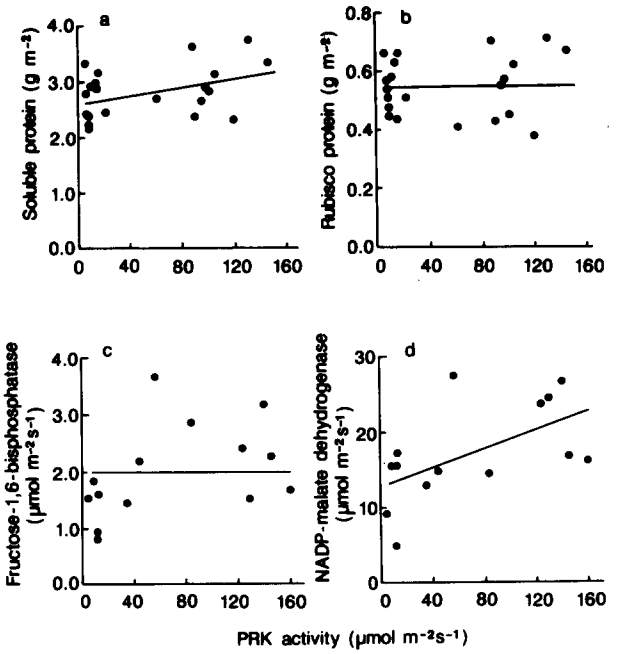

Figure 3. Response of protein and photosynthetic enzymes to altered PRK activity.
(a) Soluble protein; (b) Rubisco protein; (c) maximum activity of stromal fructose-1,6-bisphosphatase; (d) maximum activity of NADP-malate dehydrogenase.

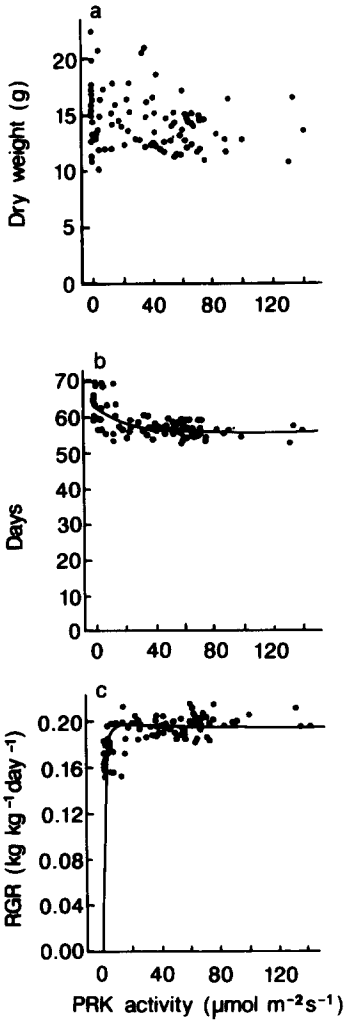

Figure 4. Response of shoot growth to PRK activity.
(a) Dry weight of shoots at flowering; (b) days from sowing to flowering; (c) relative growth rate of shoots between 2 weeks after sowing and flowering.

When PRK activity was below 20 μmol m^{-2} sec^{-1} there was a reduction in the rate of CO_2 fixation and in amounts of chlorophyll. The reduction in chlorophyll per unit leaf area had a small effect on the amount of light absorbed by leaves and would have accounted for a small part (about 10%) of the reduced assimilation. This agrees with the calculated effect of chlorophyll content on light absorption (Oquist *et al.*, 1992). When PRK activity was reduced to between 20 and 5 μmol m^{-2} sec^{-1} there was a reduction in assimilation rate with a flux-control coefficient of about 0.6. Clearly PRK is still not totally limiting for the rate of CO_2 assimilation because the flux-control coefficient is less than one. This can be explained from changes in metabolites observed: amounts of the substrates for PRK — Ru5P and ATP — were higher; levels of RuBP and 3-PGA which competitively inhibit with respect to Ru5P were lower, as were levels of ADP which inhibits with respect to ATP. It is estimated from kinetic data for PRK from Laing *et al.* (1981) and Gardemann *et al.* (1983) used in Woodrow and Mott (1993) to calculate carbon flux through PRK, that measured alterations in levels of metabolites would have maintained sufficient carbon flux through PRK to realise the CO_2 assimilation rates observed. The small discrepancies between the measured assimilation rates and calculated flux through PRK may be:

(i) due to the difficulties in relating whole leaf metabolite levels to concentrations in the stroma;

(ii) because we assume that stromal phosphate concentration is unchanged over the whole range of PRK activity;

(iii) due to the fact that the equation of Woodrow and Mott does not consider 6-phosphogluconate, which inhibits PRK (Gardemann *et al.*, 1983);

(iv) due to the possibility of control of PRK activity resulting from other metabolites or factors.

The combined effect of the changes in metabolites would have been to ensure that *in vivo* PRK activity was closer to its maximum potential activity as amounts of PRK protein were reduced. This fine control of PRK activity may be important for adjusting the rate of conversion of Ru5P to RuBP to the rate of conversion of 3-PGA to triose phosphates, reactions that both consume ATP. Feedback regulation of PRK by RuBP, ADP and 3-PGA is a very efficient way to avoid an excessive rate of PRK activity and

imbalances within the Calvin cycle which might otherwise occur because of PRK's low K_m for ATP, high maximal activity and irreversibility.

In comparison with wild-type, all measured metabolites before the FBP–triose phosphate interconversion were lower in plants with the lowest PRK activities and all measured metabolites after this reaction were higher. The amounts of FBP and triose phosphate were not affected. Since fructose-1,6-bisphosphatase catalyses an essentially irreversible reaction, this enzyme must be subject to increased regulation in plants with low PRK, presumably at least partly by inhibition by accumulated F6P (Gardemann *et al.*, 1986). Maintenance of triose phosphate levels may be important to avoid an inhibition of sucrose synthesis and would be facilitated by the elevated ATP:ADP ratio, despite lower 3-PGA. The rise in F6P in plants with the lowest PRK activity may be because reaction(s) downstream of F6P are inhibited due to alterations in amounts of metabolites caused by low PRK. The increase in F6P above the level of G6P is consistent with an effect of low PRK on the chloroplast pool of this ester, the slight increase in G6P at very low activities of PRK is expected as the 3-PGA concentration also becomes low (Dietz, 1985). Transketolase is thought to play an important role in regulating F6P levels (Murphy and Walker, 1982). Knowles (1985) proposed that feedback inhibition of transketolase could be of physiological significance in serving to restrict synthesis of RuBP. It is tempting to speculate that high levels of Ru5P inhibit transketolase serving to restrict flow of carbon through this reaction to avoid depleting carbon pools earlier on in the cycle which might depress amounts of triose phosphate and hence sucrose synthesis and depress photosynthetic rates even further. Alternatively, the relative concentrations of hexose-, pentose- and triose phosphates may be a consequence of the equilibria established in the complex of reactions catalysed by transketolase, ribose-5-phosphate isomerase, ribulose-5-phosphate, 3-epimerase and aldolase. This could be investigated further with knowledge of concentrations of other intermediates. The absence of a larger rise in G6P associated with the increase in F6P might indicate that the reaction catalysed by hexose-phosphate isomerase is not readily interacting with the Calvin cycle pool of F6P, perhaps providing further evidence for some degree of compartmentation within the chloroplast, perhaps due to the presence of Calvin cycle multi-enzyme complexes (Suss *et al.*, 1993).

Relative growth rate of shoots was reduced and time to flowering was delayed in plants with less than 85% of wild-type PRK, and it appears that time of flowering was related to development. There was no evidence for a change in specific leaf area (the amount of leaf area produced per unit of dry weight invested in leaves) in plants where rates of photosynthesis were reduced. This departs from the observations of Fichtner *et al.* (1993) where reduced rates of photosynthesis in plants with reduced amounts of Rubisco were correlated with increased specific leaf area. It was conjectured that a reduced rate of CO_2 assimilation but stable rates of transpiration in these plants lead to an imbalance between carbon and inorganic ions in the leaf and that increased leaf expansion could result from a change in the relation between cell wall pressure and osmotic pressure. Despite reduced water-use efficiency in PRK antisense plants (Figure 1d) specific leaf area remained unaltered. However, in contrast to Rubisco antisense plants, there were no large reductions in the sucrose content of leaves. It may be that the sucrose status of leaves is a more crucial factor in determining specific leaf area.

In conclusion, our data demonstrate that PRK is in excess in constant low light and that even when amounts of PRK are reduced 20-fold relative to wild-type, altered amounts of metabolites compensate for much of the reduction in PRK protein. Furthermore, we alter photosynthesis without large changes in leaf protein content and demonstrate that a reduction in the rate of photosynthesis and water-use efficiency are not enough to initiate a change in leaf geometry. Further work is being carried out to analyse the effect of the reduction in PRK on photosynthesis and growth and allocation in different environments.

Experimental procedures

Construction of PRK antisense RNA gene

A tobacco (*Nicotiana tabacum* var. Samsun) leaf cDNA library in λgt 11 (C. Smart, IPSR Cambridge Laboratory, Norwich) was screened using antibodies to spinach PRK protein (gift of N.H. Chua, Rockefeller University, New York) and one positive plaque was identified in 40 000 plaques screened. The positive plaque was purified by two more rounds of screening and the cDNA insert released with *Eco*RI was subcloned into *Eco*RI-digested pUBS to give pPRK3. Sequencing by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the double-stranded procedure described by Murphy and Kavanagh (1988) and comparison with PRK sequences from other higher plants (Michalowski *et al.*, 1992; Milanez and Mural, 1989; Raines *et al.*, 1989; Roesler and Ogren, 1988) indicated that the cDNA insert encoded the mature PRK protein, but was incomplete at the 5′ end and encoded only 42 amino acid residues of the chloroplast transit peptide in addition to the mature protein. A 1.3 kbp *Bam*HI-Asp718I restriction fragment from pPRK3 containing the tobacco PRK cDNA was subcloned into the binary vector pROK8, a derivative of pBIN19 (Bevan, 1984) with a tobacco *rbcS* promoter and *nos* terminator, to give the PRK antisense RNA construct.

Plant transformation and regeneration

The binary vector containing the PRK antisense construct was introduced into *Agrobacterium tumefaciens* LB4404 by electroporation (Shen and Forde, 1989). The antisense construct was then transferred to the tobacco genome using *Agrobacterium*-mediated leaf disc transformation (Horsch *et al.*, 1985). Kanamycin-

resistant primary transformants were transferred into compost (Levington, Fisons, UK) and grown in a greenhouse with supplementary artificial lighting (150 μmol photons m^{-2} sec^{-1}; 16 h photoperiod). The plants were allowed to flower, the florets covered to prevent cross pollination and seeds were collected.

Northern blot analysis of PRK antisense RNA transformants

Seeds from individual primary transformants were sown in trays of compost in a growth room (25°C; 50 μmol photons m^{-2} sec^{-1}; 16 h photoperiod) for 5–6 weeks. Total RNA was extracted from 1 g leaf tissue from pooled plants or 0.3 g leaf tissue from individual plants (0.3 g leaf ml^{-1} extraction buffer) by the procedure of Apel and Kloppstech (1978), except that the extraction buffer contained proteinase K at 1 mg ml^{-1} and 10 mM 2-mercaptoethanol. For Northern analysis 5 μg of total RNA were denatured in formamide, electrophoresed in a 1.2% agarose gel in Mops-formamide (Sambrook *et al.*, 1989) and transferred from 25 mM sodium phosphate buffer pH 6.4, to Hybond-N membrane (Amersham, UK). Prehybridization was in 50% formamide, 0.6 M NaCl 40 mM sodium phosphate pH 6.4, 20 mM Na_2EDTA, 7.5× Denhart's and 0.5 mg fish milt DNA ml^{-1} at 42°C for 6 h. Hybridization was for 16 h in the presence of these reagents with the addition of 10% dextran sulphate and the tobacco PRK cDNA labelled with [α-^{32}P]dATP by the random primer method (Feinberg and Vogelstein, 1983). The membrane was washed four times for 20 min in 0.1× SSC and 0.5% SDS at 42°C and exposed to film (Kodak X-Omat). The radiolabelled PRK cDNA was removed from the membrane according to the manufacturer's instructions (Amersham, UK) and the membrane was re-probed with a cDNA encoding the β subunit of the tobacco mitochondrial ATPase (Boutry and Chua, 1985) to quantify the amount of RNA loaded in each track. The amounts of PRK and ATPase mRNA were assessed by scanning the autoradiograph using a Molecular Dynamics 300S laser-scanning densitometer. There was a 20-fold variation in ratios of PRK/ β ATPase mRNA levels in the progeny of different primary transformants.

Assay for PRK maximum activity

PRK in leaves was extracted and assayed as in Kagawa (1982). Briefly, 3.68 cm^2 of leaf (3 discs) was extracted in 1 ml 200 mM Tris–HCl (pH 7.8) containing 200 mM KCl, 0.5 mM EDTA, 20 mM isoascorbate, 20 mM 2-mercaptoethanol in a Potter homogenizer at 4°C. Aliquots were diluted 20-fold in extraction buffer and assayed immediately at 25°C by coupling ADP formation to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase in the presence of 20 mM dithiothreitol to ensure full activation of PRK, exactly as described by Kagawa (1982). Activities of extracts assayed in this way were linear over the assay period (5–10 min) and with extract volume. There was a 20-fold range of maximum PRK activity in extracts from progeny of different primary transformants.

Experimental analysis of transgenic plants

Seeds from 11 lines over the 20-fold range of PRK activities and from plants transformed without the PRK cDNA were sown on moist filter paper in Petri dishes at 25°C. After germination they were transferred to loam-based compost supplemented with fertilizer in pots at 25°C, 330 μmol photons m^{-2} sec^{-1}, 14 h photoperiod, 70% relative humidity. Two weeks later plants were given liquid fertilizer three times a week (Vitafeeds, Vitax Ltd, UK). Care was taken to space plants out as they grew to prevent shading of neighbouring plants.

All measurements of photosynthesis (where data are presented), metabolites and enzymes were carried out on the most recently fully expanded leaves of 4- to 6-week-old plants. To calculate relative growth rate, growth measurements were carried out on 2-week-old seedlings and plants starting to flower (defined as when the colour of the first floret can be seen). For every data point presented, PRK activity was measured in the piece of leaf used for the measurement or from an adjacent piece of leaf. For plants used for growth analysis, the most recent large fully expanded leaf was sampled for PRK.

Lines were best fitted through data points as means, linear or exponential curves depending on whether the extra parameters were significant at 5%. The exponential equation used was $y = a + br^x$, where a and b are constrained to equal zero for cases where the line goes through the origin. Flux-control coefficients were calculated as the fractional change in steady-state flux through the pathway divided by the fractional decrease in amount of enzyme (Kacser and Porteous, 1987).

In order to estimate flux through PRK in wild-type and transgenic material, metabolite concentrations in the chloroplast stroma were calculated from measurements of metabolites in whole leaf extracts assuming a stromal volume of 30 μl mg^{-1} chlorophyll and correcting for stroma-cytosol distribution of metabolites according to Stitt *et al.* (1982). These values were then used in the following equation (Woodrow and Mott, 1993):

$$\text{Flux through PRK} = \frac{(\text{PRK } v_{max})\,[\text{Ru5P}]\,[\text{ATP}]}{[\text{ATP}]\,[\text{Ru5P}] + K_{ATP}K_{Ru5P} + K_{ATP}[\text{Ru5P}]f_4 + K_{Ru5P}[\text{ATP}]f_5}$$

where

$$f_4 = 1 + \frac{\text{ATP}}{K_{i41}}$$

and

$$f_5 = 1 + \frac{[\text{PGA}]}{K_{i42}} + \frac{[\text{RuBP}]}{K_{i43}} + \frac{[\text{P}_i]}{K_{i44}} + \frac{[\text{FBP}]}{K_{i45}}$$

where K_{ATP} = 0.053 mM; K_{Ru5P} = 0.048 mM; K_{i41} = 0.04 mM; K_{i42} = 2 mM; K_{i43} = 0.7 mM; K_{i44} = 4 mM; K_{i45} = 1 mM.

Owing to the difficulty in relating whole leaf phosphate measurements to concentrations of phosphate in the chloroplast stroma, phosphate concentrations are assumed to be the same over the whole population of plants.

Photosynthesis

Rates of net CO_2 fixation were measured in the laboratory under the conditions in which the plants had been grown (330 μmol quanta m^{-2} sec^{-1}; 350 μbar CO_2; 25°C; 70% relative humidity, 10 mbar vapour pressure deficit) using a six-chamber open-circuit gas exchange system with automatic data handling. CO_2 partial pressure was controlled by a gas blender (Signal Instruments Co., Croydon, UK) and measured with an infrared 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 in leaf chambers with forced ventilation. Flow rate of gas was 9 ml sec^{-1}. Stomatal conductance to water vapour, transpiration and CO_2 concentration within the leaf (Ci) was calculated as in von Caemmerer and Farquhar (1981). Leaves were allowed to equilibrate in chambers for 30 min prior to measurement. Measurements of the absorption of light at 330 μmol m^{-2} sec^{-1} by leaves were carried out in an integrating sphere as described by Rackham and Wilson (1968).

Metabolites, enzymes and carbohydrates

Discs were cut from leaves of plants still in the growth cabinets during the middle of the photoperiod (also at the start of the photoperiod for carbohydrate measurements), immersed in liquid nitrogen whilst still in the light and then stored in liquid nitrogen until analysis. For metabolite assays, leaf material was extracted in trichloroacetic acid (3.68 cm^2 ml^{-1}) as in Paul and Stitt (1993). Assays of metabolites were performed using a dual-wavelength spectrophotometer and established procedures as in Stitt *et al.* (1989). Levels of Ru5P and R5P were estimated using a modified version of the assay for PRK. To measure Ru5P, the reaction was initiated with purified PRK (Sigma). R5P was measured after completion of this reaction by adding purified R5P isomerase (Sigma). Rubisco was extracted and amounts determined by a [^{14}C]-CABP binding method as in Parry *et al.* (1993). Maximum activity of NADP-malate dehydrogenase was extracted and assayed as in Scheibe and Stitt (1988) and that of stromal fructose-1,6-bisphosphatase as in Stitt *et al.* (1982). Extractions were carried out at 4°C, assays at 25°C. To measure carbohydrates, leaf discs were rapidly homogenized in 50 mM Hepes/KOH buffer pH 7.4 in a Potter homogenizer at 4°C and then immediately heated to 95°C. Amounts of hexoses and sucrose were quantified from the supernatant as in Jones *et al.* (1977) and starch from the pellet as in Paul *et al.* (1993). Protein was measured as in Bradford (1976), chlorophyll as in Arnon (1949).

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