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Low sink demand limits photosynthesis under P_i deficiency

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Abstract

The role of the demand for carbon assimilates (the 'sink') in regulating photosynthetic carbon assimilation (P_n: the 'source') in response to phosphate (P_i) deficiency was examined in tobacco (*Nicotiana tabacum* L.). P_i supply was maintained or withdrawn from plants, and in both treatments the source/sink ratio was decreased in some plants by darkening all but two source leaves (partially darkened plants). The remaining plants were kept fully illuminated. P_i-sufficient plants showed little variation in rate of P_n, amounts of P_i or phosphorylated intermediates. Withdrawal of P_i decreased P_n by 75% under the growing conditions and at both low and high internal CO_2 concentration. Concomitantly, P_i, phosphorylated intermediates and ATP contents decreased and starch increased. RuBP and activity of phosphoribulokinase closely matched the changes in P_n, but Rubisco activity remained high. Partial darkening P_i-deficient plants delayed the loss of photosynthetic activity; Rubisco and phosphoribulokinase activities and amounts of sucrose and metabolites, particularly RuBP and G6P, were higher than in fully illuminated Pi-deficient plants. Rates of sucrose export from leaves were more than 2-fold greater than in fully illuminated P_i-deficient plants. Greater sucrose synthesis, facilitated by increased G6P content, an activator of SPS, would recycle P_i from the cytosol back to the chloroplast, maintaining ATP, RuBP and hence P_n. It is concluded that low sink strength imposes the primary limitation on photosynthesis in P_i-deficient plants which restricts sucrose export and sucrose synthesis imposing an end-product synthesis limitation of photosynthesis.

Key words: Phosphate, photosynthesis, sink strength, source/sink ratio, sucrose export.

Introduction

Phosphate deficiency decreases photosynthetic CO_2 assimilation rate due to decreases in RuBP pool size, for example, in *Glycine max* (Fredeen *et al*., 1989) and *Helianthus annuus* (Jacob and Lawlor, 1992) caused by insufficient ATP in *H. annuus* and *Zea mays* (Jacob and Lawlor, 1992). Despite decreased CO_2 assimilation, leaves contain large amounts of starch due to low availability of P_i for the triose phosphate translocator; carbon is retained in chloroplasts where starch synthesis is facilitated through activation of ADP-glucose pyrophosphorylase by low Pi : 3-PGA and increased expression of ADP-glucose pyrophosphorylase in *Nicotiana tabacum* (Nielsen *et al*., 1998). Starch also accumulates as a consequence of decreased demand from growth and respiration. Additionally, the effects of P_i supply depend not only on the amount but timing. With P_i deficiency from early growth, total leaf area, for example, decreases markedly, resulting from a reduction in the rate of leaf expansion and the number of leaves produced (Fredeen *et al*., 1989) as well as decreasing photosynthesis. In P_i withdrawal experiments, when P_i supply is restricted after early growth with adequate P_i, relative growth rate of a range of C_3, C_3–C_4 intermediate, C_4 annual and perennial monocot and dicot plants decreases before any significant effect on photosynthesis (Halsted and Lynch, 1996), suggesting that low P_i limits growth processes before carbon fixation and assimilation.

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Abbreviations: F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; P_i, inorganic phosphate; P_n, rate of net photosynthesis; PPF, photosynthetic photon flux; PRK, phosphoribulokinase; R5P, ribose-5-phosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose bisphosphate; S7P, sedoheptulose-7-phosphate; SPS, sucrose phosphate synthase; TFA, trifluoroacetic acid.

Many studies have emphasized the role of source/sink relations in regulating the rate of photosynthesis. Evidence from experiments involving the application of elevated CO_2 (for reviews see Lawlor and Mitchell, 1991; Stitt, 1991; Drake *et al*., 1997), low temperatures (Paul *et al*., 1992), preventing sucrose export by cold-girdling source leaves (Krapp and Stitt, 1995), and nitrogen (N) deficiency (Paul and Driscoll, 1997), suggests that sink strength, i.e. the demand for photoassimilate, can regulate the rate of photosynthesis. Mechanisms proposed to explain sink regulation of photosynthesis include changes in the rates of sucrose and starch synthesis (end-product synthesis) which recycle P_i back to the reactions of photosynthesis. End-product limitation of photosynthesis can be demonstrated following short-term changes in environmental conditions (for example, by exposure to saturating light and CO_2, and to low temperature) to which plants are not acclimated (Stitt, 1986; Sharkey *et al*., 1986; Labate and Leegood, 1988; Stitt and Grosse, 1988) and by supplying inhibitors of sucrose synthesis and P_i-sequestering agents (Stitt and Quick, 1989). However, there is little evidence that end-product synthesis limitation has physiological relevance in sink regulation of photosynthesis in the conditions under which the plants are actually growing (Stitt, 1991). An alternative mechanism, which has gained prominence in recent years, is that carbohydrates, specifically hexoses, can repress photosynthetic gene expression, particularly of the nuclear-encoded small sub-unit of Rubisco (Sheen, 1994; van Oosten and Besford, 1996), thus decreasing Rubisco content and P_n. However, P_i deficiency generally has little effect either on Rubisco content or activity (Brooks, 1986; Paul and Stitt, 1993; Nielsen *et al*., 1998) even though carbohydrates accumulate, suggesting that sugar repression is not the mechanism of sink limitation.

It is therefore clear that there is substantial interaction between sink size and demand for assimilates and CO_2 assimilation which will be affected by P_i supply. The question addressed in this paper is: does low sink demand impact on photosynthesis and, if so, by what mechanism? Firstly, the aim was to establish whether the sink exerts the main control of P_n during P_i deficiency and, secondly, if control does come from the sink, what was the mechanism through which it operates. It was hypothesized that decreasing P_i supply to plants grown with sufficient P_i would decrease ATP and RuBP in source leaves and that assimilation would decrease as a consequence, but that demand for carbohydrates from the sink would decrease more, and carbohydrates therefore accumulate. Darkening would increase demand for carbohydrates, so decreasing the accumulation of carbohydrates and diminishing the impact of low sink demand. It was further hypothesized that maintenance of sink strength by shading could overcome the effects of P_i deficiency on P_n by removing carbohydrates. Measurements of photosynthetic metabolism would provide an insight into the mechanism through which this operated. To test these ideas, tobacco (*N. tabacum* L.) plants were grown with sufficient P_i for growth, then some of the plants were deprived of P_i and contrasted with those continuing to receive sufficient P_i. To assess the importance of sink demand in limiting P_n under low P_i the source/sink ratio was altered by darkening all leaves except two mature source leaves per plant in comparison to plants that were fully illuminated. The experimental approach was designed to understand the significance of the sink in the control of photosynthesis under low P_i.

Materials and methods

Plant material and experimental design

Seeds of tobacco (*Nicotiana tabacum* L. var. Samsun) were germinated in a Petri dish and 10 d later seedlings were transferred to pots (1.0 l volume) containing washed silver sand. Plants were grown throughout the experiment in a controlled environment. Photosynthetic photon flux (PPF) at the top of the canopy was 300–350 μmol m^{-2} s^{-1}, supplied by a combination of fluorescent and incandescent lamps, photoperiod was 14 h, temperature 25 ± 2 °C and relative humidity $70 \pm 5\%$. On transfer to pots of sand, all seedlings were supplied daily for 10 d with nutrient solution containing sufficient P_i and other nutrients: 1.0 mol m^{-3} P_i as KH_2PO_4, 5.0 mol m^{-3} $Ca(NO_3)_2$, 3.0 mol m^{-3} KCl, 1.0 mol m^{-3} $MgSO_4$, 20.0 mol m^{-3} EDTA-Fe, 0.1 mol m^{-3} H_3BO_3, 0.02 mol m^{-3} $MnCl_2$, 2.0 mmol m^{-3} $ZnCl_2$, 2.0 mmol m^{-3} $CuCl_2$, and 1.0 mmol m^{-3} Na_2MoO_4. A preliminary experiment established that this allowed rapid growth without accumulation of excess Pi. After 10 d, pots were flushed with deionized water and then supplied daily with nutrient solution containing either 0.0 or 4.0 mol m^{-3} KH_2PO_4 to compare P_i deficiency and P_i sufficiency. The higher concentration of P_i (4 mol m^{-3} compared to 1 mol m^{-3}) was used to ensure there was no question of P_i deficiency in the P_i-sufficient plants as they grew larger. All other elements were at the same concentration and not limiting to growth. Twenty days after applying the nutrient treatment, the source/sink ratio was decreased for half of the plants of each P_i treatment by enclosing the lower three leaves with aluminium foil, leaving only the fourth (most recently fully expanded leaf) and fifth leaves (not yet fully expanded) exposed to light. Measurements of P_n under the growth conditions, P_i, ATP, carbohydrates, and phosphorylated metabolites, together with Rubisco and PRK activities were made on fourth leaves until day 30. After day 30 the fifth leaf had become the most recently fully expanded leaf and from that point on measurements were carried out on the fifth leaf in exactly the same way as they had been on the fourth leaf. In a preliminary experiment, measurements of gas exchange, metabolites and enzyme activity showed that leaves of different insertion had very similar metabolism. Also, it was established that the darkening and phosphate treatments did not alter the time at which the fifth leaf became mature (fully expanded) or the development of the plants. Phosphate deficiency did decrease the final size of leaves beyond the fifth leaf and slowed and decreased later development and growth of the plant.

After the preliminary experiment, the experiment was performed twice. The results from all three experiments were very similar and data from the third experiment are presented. Four

replicate plants were measured and sampled for each treatment at each sampling. A single leaf on each plant was placed in the gas exchange system and the leaf area (10 cm^2) freeze-clamped and stored for metabolite analysis. Samples were taken for P_i (1.2 cm^2 leaf), enzyme activities (1.2 cm^2 leaf) and carbohydrates (1.2 cm^2 leaf). Leaves were sampled for biochemical analysis 7 h into the photoperiod.

Photosynthesis measurements

Exchange of CO_2 was measured on 10 cm^2 areas of whole leaves attached to the plant, using an automated multi-chamber open-circuit gas exchange system. The system comprized an infrared gas analyser (IRGA) (WA-225-MK3, ADC, Hoddesdon, UK) for CO_2 concentration measurements, a gas handling unit (WA-161 2K, ADC, Hoddesdon, UK) and a mode switching unit (WA-357-MK3, ADC, Hoddesdon, UK). The O_2 concentration in the gas phase was measured with a gas analyser (Series 80, Ox-An Systems, Huddersfield, UK). The CO_2 and O_2 composition of the air entering the leaf chambers was regulated by a gas blender (Signal Instruments Co., Croydon, UK); the flow rate was 9 $cm^3\ s^{-1}$, regulated by mass-flow meters and controllers (Bronkhorst Hi-Tech bv, Holland). Temperature and relative humidity of the air in the chambers was regulated by bubbling the gas stream through water at 25 °C and then through a condenser set to the required dew point. Leaf temperature was calculated from the energy balance (Ehleringer, 1989). Chamber temperature was measured with thermocouples. Water content of the air before and after passing through the leaf chamber was measured by a capacitance humidity sensor (Vaisala, Helsinki, Finland). Photosynthetic photon flux was supplied by metal-halide lamps (Wotan, Phillips, Holland) and measured with selenium sensors (Megatron, London, UK). Photosynthetic rate was measured under the growing conditions and at different C_i, by varying C_a with the gas blender at a PPF of 800 ± 35 μmol $m^{-2}\ s^{-1}$; all other conditions were as stated above. Calculations of P_n and C_i were according to von Caemmerer and Farquhar (von Caemmerer and Farquhar, 1981).

Carbohydrate determination

Leaf samples were kept in liquid nitrogen until analysis. They were extracted in 0.5 ml of 80% ethanol (v/v) for 20 min at 70 °C, and glucose, fructose and sucrose determined (Stitt *et al.*, 1989). Starch was extracted from the residue by grinding in 0.5 ml of 50 mol m^{-3} sodium acetate (pH 4.8) containing α-amylase (10 U) and amyloglucosidase (6 U). The extract was incubated for 36 h at 37 °C to convert starch to glucose, which was determined as above.

Estimation of export from leaves

Export of carbon from leaves (μmol C $m^{-2}\ s^{-1}$) is estimated as in Paul and Driscoll (Paul and Driscoll, 1997) from data in Figs 1 and 3. Export of carbon from leaves is: cumulative CO_2 fixation over 7 h (μmol C $m^{-2}\ s^{-1}$) minus starch, sucrose and hexoses accumulated in 7 h (μmol C $m^{-2}\ s^{-1}$) from Paul and Driscoll (Paul and Driscoll, 1997).

P_i determination

P_i was extracted from the leaf discs by plunging them into Eppendorf tubes containing 2 ml of water at 100 °C for 5 min; they were kept overnight in a water bath at 70 °C. P_i was determined by the method described by Saheki *et al.* (Saheki *et al.*, 1985). Two hundred microlitres of sample was added to 1.5 ml of a mixture containing 100 mol m^{-3} zinc acetate, 15 mol m^{-3} ammonium molybdate and 5.67 mmol m^{-3} L-ascorbic acid. The samples were incubated for 30 min at 50 °C and the absorbance read at 850 nm in a spectrophotometer (ULTROSPEC II, LKB-Biochrom). The P_i content in the samples was determined by comparison against standards made with KH_2PO_4 ranging from 0.01 to 0.4 μmol.

Phosphorylated intermediates

Leaf samples kept in liquid nitrogen were ground to a fine powder using a pestle and mortar under liquid nitrogen and 1 ml of 500 mol m^{-3} TFA containing 10 mol m^{-3} 8-hydroxyquinoline was added and grinding continued. Once thawed, extracts were kept on ice for 20 min and then spun at 16 000 *g* and 4 °C for 5 min. The supernatant was passed through a column containing 1 g of Mega-Bond Elut C18 (Varian, UK), then the column was washed with 3 ml of water; the two eluants were pooled and dried *in vacuo* over sodium hydroxide pellets and anhydrous calcium chloride. The samples were redissolved in 1 ml of water and passed through a second column containing 2 ml of Dowex-50 (H^+) cation exchange resin. The column was then washed and the two eluants pooled and dried *in vacuo* as before. The samples were re-dissolved in 250 μl of water, filtered through a nylon membrane (Whatman Kent, UK) of 0.2 μm pore size and analysed. Recoveries of metabolites added to leaf material in physiological concentrations during extraction were in excess of 90%.

G6P, F6P and S7P were separated by HPLC. One hundred microlitres of sample was injected onto a column of Carbopac PA1 (Dionex), 250 mm long and 4 mm internal diameter (id), with a guard column (Carbopac PA1, Dionex) 50 mm long and

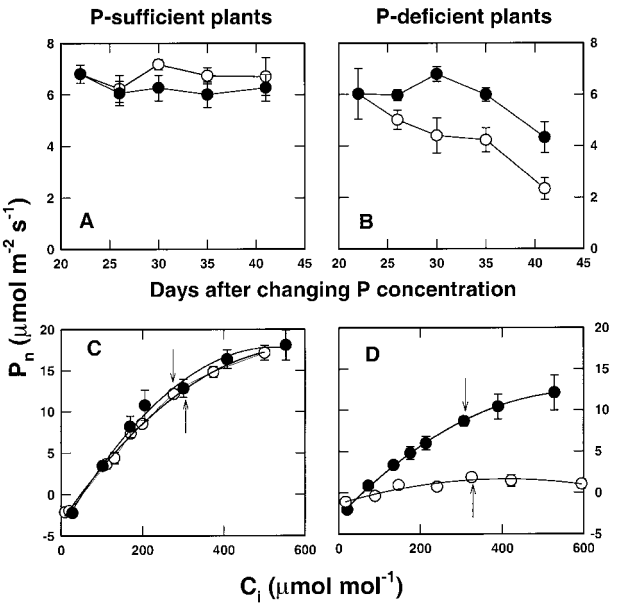

Fig. 1. Photosynthetic rate (P_n) of leaves of plants grown with either 4.0 (left panel) or 0.0 mol m^{-3} P_i (right panel). Plants were either fully illuminated (○) or the lower leaves were darkened (●). Photosynthesis was measured under the growth conditions (A, B) and at saturating PPF as a function of changing C_i on day 41 (C, D). Bars represent standard error of the mean of four replicates. Arrows indicate C_i under growth CO_2.

4 mm id, of an HPLC system (DX500, Dionex). The system consisted of a gradient pump (GP40, Dionex), an autosampler (AS3500, Dionex) and an electrochemical detector (ED40, Dionex) operating in the amperometric mode, connected to an electrode. Solvents were degassed with helium for 20 min and pumped at a flow rate of 1 ml min^{-1} at a pressure of 10 MPa. The eluant consisted of a combination of sodium acetate, sodium hydroxide and water. Concentrations of sodium acetate and sodium hydroxide were 100 mol m^{-3} and 10 mol m^{-3}, respectively, for the first 5 min. Then the sodium acetate concentration was increased linearly to 800 mol m^{-3} over 25 min while sodium hydroxide was kept at 10 mol m^{-3}; both were maintained for 5 min. The column was re-equilibrated for 15 min at the initial conditions before the next injection. Peak areas were measured using the Peak-Net© software (Dionex, UK) and concentrations of metabolites were calculated from calibration curves constructed with pure standards ranging from 1 to 8 nmol.

RuBP concentration was determined in the same extract as G6P, F6P and S7P, using Rubisco to convert RuBP to 3-PGA in the presence of ^{14}[C]sodium bicarbonate (Stitt *et al*., 1989). Purified Rubisco was activated at a concentration of 1 mg ml^{-1} in 100 mol m^{-3} Bicine (pH 8.2) containing 20 mol m^{-3} $MgCl_2$ and 10 mol m^{-3} sodium bicarbonate for 25 min at 40 °C. Forty microlitres of sample was added to 0.5 ml of 100 mol m^{-3} Bicine (pH 8.2) containing 5 μg of Rubisco, 20 mol m^{-3} $MgCl_2$ and 10 mol m^{-3} ^{14}[C]sodium bicarbonate at a specific activity of 35 kBq mol^{-1}. The assay mixture was incubated at 25 °C for 1 h and 100 μl of 1×10^4 mol m^{-3} formic acid was added to stop the reaction. The mixture was dried overnight at 70 °C and re-dissolved in 0.4 ml of water; 3.6 ml of scintillation cocktail (Ultima Gold Packard) was then added and the samples were counted in a liquid scintillation analyser (2500 TR Packard). RuBP concentration in the samples was calculated by comparison with a calibration curve constructed with 1–8 nmol pure RuBP which was synthesized enzymatically in the laboratory from AMP following the procedure of Wong *et al*. (Wong *et al*., 1980).

ATP was determined enzymatically according to Stitt *et al*. (Stitt *et al*., 1989) with modifications. An aliquot of the sample (40 μl) was added to 0.55 ml of assay buffer 50 mol m^{-3} HEPES/KOH (pH 7.0) containing 5 mol m^{-3} $MgCl_2$, 15 mol m^{-3} NADP, 50 mol m^{-3} D-glucose, PGI (4 U) and G6PDH (1.4 U) in a quartz cuvette. After 10 min, 2.5 U of hexokinase was added to start the reaction. The assay was carried out at 25 °C. ATP concentration was calculated from the change in absorbance at 340 nm in a dual-wavelength spectrophotometer (Varian Cary 210, Australia).

Rubisco and PRK activities

Activities of Rubisco and PRK were determined in four separate leaf samples (total area 1.2 cm^2) taken from leaves exposed to light for 7 h in the normal photoperiod under the growth conditions. Leaf samples were stored in liquid nitrogen and then extracted in 0.5 ml of ice-cold 100 mol m^{-3} HEPES/KOH buffer (pH 8) containing 10 mol m^{-3} $MgCl_2$, 1.0 mol m^{-3} EDTA-Na_2, 0.1% Triton X-100 and 15 mol m^{-3} mercaptoethanol.

Initial Rubisco activity was measured as described previously (Paul and Driscoll, 1997). Twenty microlitres of extract was added to 870 μl of extraction buffer containing 100 mol m^{-3} ^{14}[C]sodium bicarbonate at a specific activity of 0.35 kBq mol^{-1} (assay buffer). To start the reaction, 10 μl of 33 mol m^{-3} RuBP was added and the reaction was allowed to proceed for 1 min at 25 °C before being stopped with 100 μl of 1×10^4 mol m^{-3} formic acid. RuBP was synthesized in the laboratory from AMP following the procedure of Wong *et al*. (Wong *et al*., 1980). Total Rubisco activity was measured as for initial activity, but 20 μl of leaf extract was incubated in 870 μl of assay buffer for 3 min at 25 °C to allow total activation of the enzyme. Samples were evaporated overnight at 70 °C and re-dissolved in 0.4 ml of water; 3.6 ml of scintillation cocktail (Ultima Gold Packard) was added and the ^{14}C counted in a liquid scintillation analyser (2500 TR Packard).

Maximum PRK activity was measured as in Kagawa (Kagawa, 1982). Twenty microlitres of 20-fold diluted extract was added to 860 ml of 50 mol m^{-3} HEPES/KOH buffer (pH 7.8) containing 40 mol m^{-3} KCl, 8 mol m^{-3} $MgCl_2$, 20 mol m^{-3} DTT, 1.0 mol m^{-3} PEP, 1.0 mol m^{-3} ATP, 0.25 mol m^{-3} NADH, pyruvate kinase (1.7 U), lactate dehydrogenase (1.7 U), and R5P-isomerase (4.3 U). After 5 min, 20 μl of 250 mol m^{-3} R5P was added and the change in absorbance at 340 nm followed for another 5 min in a dual-wavelength spectrophotometer (Varian Cary 210, Australia). Assays were linear over time and with extract volume.

Data are presented as means and standard errors. Analyses of variance were done and the statistical significance of the differences given as probabilities in the text (not significant, $P > 0.05$).

Results

Photosynthetic rate

Decreasing the source/sink ratio by darkening some leaves had no significant effect on P_n in P-sufficient tobacco plants (Fig. 1A). However, partial darkening delayed the decrease in photosynthetic rate in P_i-deficient plants (Fig. 1B), with P_n remaining above 6 μmol m^{-2} s^{-1} until day 36 in these plants, after which P_n decreased by 25%. In contrast, P_n in fully illuminated plants but deprived of P_i was significantly decreased ($P < 0.05$) by day 26, and by day 41, values were approximately a third of those in P_i-sufficient plants. To examine the regulation of the photosynthetic response to P_i deficiency, rates of P_n were measured as a function of the internal CO_2 concentration at saturating PPF (1200 μmol m^{-2} s^{-1}) on day 41 (Fig. 1C, D). When grown with sufficient P_i, darkened plants compared to fully illuminated plants differed little in the photosynthetic response to C_i with similar maximum rates of P_n and carboxylation efficiencies. In contrast, fully illuminated P_i-deficient plants showed significant ($P < 0.05$) inhibition of P_n throughout the range of C_i, with the inhibition reaching 90% at high C_i. In darkened P_i-deficient plants maximum P_n was decreased by less than 30% compared to P_i-sufficient plants.

P_i content

P_i-deficient plants contained significantly ($P < 0.01$) smaller amounts of P_i per unit of leaf area than plants grown with 4.0 mol m^{-3} P (Fig. 2A, B). Darkening had no effect on P_i content.

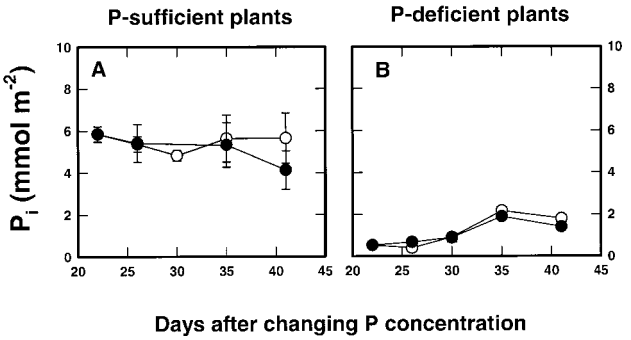

Fig. 2. P_i content of leaves of plants grown under either 4.0 (A) or 0.0 mol m^{-3} P_i (B). Plants were either fully illuminated to light (○) or the lower leaves were darkened (●). Samples were taken 7 h into the photoperiod. Bars represent standard error of the mean of four replicates.

Carbohydrate contents

The total carbohydrate content (measured in the middle of the photoperiod) of fully illuminated P_i-sufficient plants increased with time (Fig. 3A) but partial darkening prevented this increase. Darkening P_i-deficient plants resulted in smaller contents of total carbohydrate relative to fully illuminated P_i-deficient plants through the experiment (Fig. 3B). In P_i-sufficient plants, darkening significantly decreased glucose and fructose content (Fig. 3C, E) compared to fully illuminated P_i-sufficient plants, in which the glucose content per unit of leaf area increased from day 35. P_i deficiency did not increase glucose or fructose content per unit of leaf area (Fig. 3D, F) and decreasing the source/sink ratio in P_i-deficient plants had little effect on glucose or fructose content.

Sucrose content was greater in P_i-sufficient than in P_i-deficient plants, irrespective of darkening when compared at the same time, and increased with time in all treatments (Fig. 3G, H). The effect of decreasing the source/sink ratio by darkening the plants depended on P_i supply. Plants grown with 4.0 mol m^{-3} P_i had less sucrose when darkened than undarkened P_i-sufficient plants; in contrast, darkened P_i-deficient plants had more sucrose than undarkened P_i-deficient plants ($P<0.05$), towards the end of the experiment.

Starch content (Fig. 3I, J) was lower in P_i-sufficient plants than P_i-deficient plants ($P<0.05$). Darkening P_i-sufficient plants had little effect on starch content, but significantly decreased starch content ($P<0.05$) in P_i-deficient plants. Therefore, there was a large effect of darkening on sucrose/starch ratio in P_i-deficient plants.

Carbon export from source leaves was restricted by P_i deficiency by more than 4-fold compared to P_i-sufficient plants by the end of the experiment (Table 1). Darkening P_i-deficient plants prevented the decrease in export by up to 30 d with values similar to those of P_i-sufficient plants and more than 2-fold higher than in undarkened

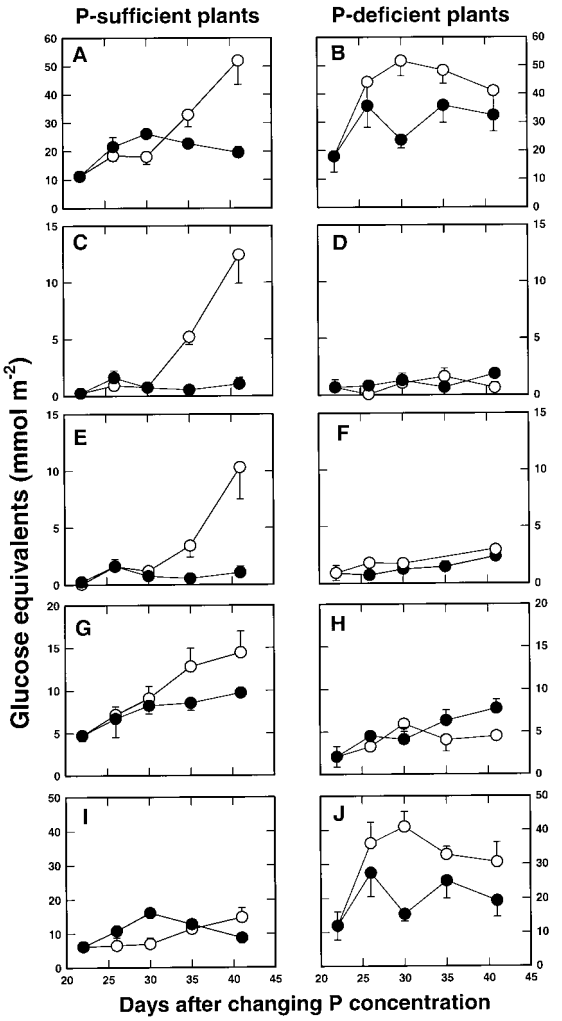

Fig. 3. Total non-structural carbohydrate (A, B), glucose (C, D), fructose (E, F), sucrose (G, H), and starch (I, J) contents expressed as mmol m^{-2} of glucose equivalents in leaves of plants grown under either 4.0 (left panel) or 0.0 mol m^{-3} P_i (right panel). Plants were either fully illuminated (○) or the lower leaves were darkened (●). Samples were taken 7 h into the photoperiod. Bars represent standard error of the mean of four replicates.

P_i-deficient plants. By the end of the experiment the export of carbon in all plants had decreased compared to the start of the experiment, but darkening P_i-deficient plants maintained carbon export more than 2-fold higher than in fully illuminated P_i-deficient plants. There was no strong effect of darkening on export of carbon from leaves of P_i-sufficient plants.

Rubisco and PRK activities

To investigate the mechanisms involved in the sink limitation of photosynthesis in P_i-deficient plants, the activities of Rubisco (initial and maximum) and PRK (maximum) were determined. Initial Rubisco activities showed little variation with P_i supply or darkening (Fig. 4A, B). Values

Table 1. *Carbon export (μmol C m^{-2} s^{-1}) from source leaves of P_i-sufficient and P_i-deficient plants where plants were fully illuminated and in plants where lower leaves were darkened*

Export of carbon from leaves is estimated from data in Figs 1 and 3 (as in Paul and Driscoll, 1997). Statistical analysis was performed on data from Figs 1 and 3 and shading had a statistically significant impact on photosynthesis and carbohydrate content in P_i-deficient plants. Estimates of export derived from these data are also assumed to be significantly different at $P<0.05$*. Export from source leaves is estimated as: cumulative CO_2 fixation over 7 h (μmol C m^{-2} s^{-1}) minus starch, sucrose and hexoses accumulated in 7 h (μmol C m^{-2} s^{-1}).

Days	+P		–P	
	No shading	Shading	No shading	Shading
21	6.30	6.30	5.58	5.58
30	6.46	5.20	2.67*	6.12*
41	4.82	5.76	1.20*	3.35*

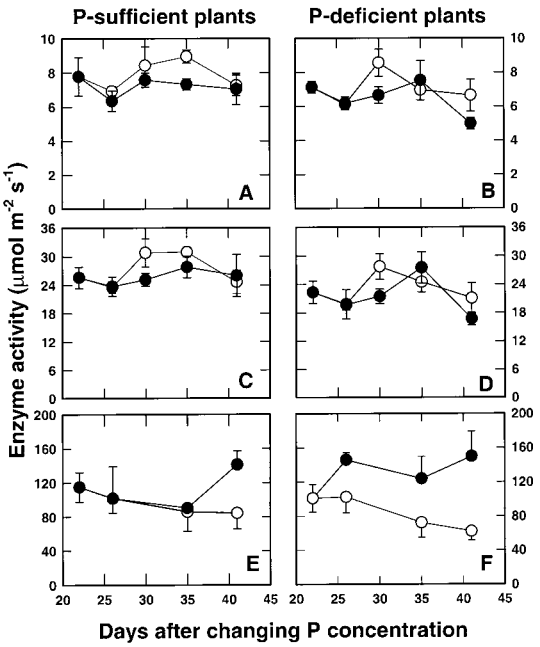

Fig. 4. Rubisco and PRK activities. Rubisco initial (A, B), maximum (C, D) and PRK maximum activity (E, F) in leaves of plants grown under either 4.0 (left panel) or 0.0 mol m^{-3} P_i (right panel). Plants were either fully illuminated (○) or the lower leaves were darkened (●). Samples were taken 7 h into the photoperiod. Bars represent standard error of the mean of four replicates.

were typically between 6 and 9 μmol m^{-2} s^{-1} throughout the experiment. Maximum Rubisco activities (Fig. 4C, D) exhibited similar trends to initial activities, with little variation due to either P_i deficiency or the source/sink ratio. Accordingly, activation state of Rubisco was unaffected either by P_i supply or by darkening (not shown).

Maximum activity of PRK (Fig. 4E, F) was similar in P_i-sufficient and P_i-deficient plants when fully illuminated and was little affected by darkening in P_i-sufficient plants, except on day 41 when the activity was greater ($P<0.05$) than in undarkened P_i-sufficient plants (Fig. 4E). In contrast, darkened P_i-deficient plants had much higher PRK activity ($P<0.05$) than undarkened P_i-deficient plants (Fig. 4F).

Metabolite contents

Plants grown with 4.0 mol m^{-3} P_i had 3–4-fold greater ($P<0.05$) RuBP concentrations than P_i-deficient plants (Fig. 5A, B). Darkening decreased RuBP content in P_i-sufficient plants towards the end of the experiment. In contrast, darkened P_i-deficient plants had up to 2-fold larger RuBP content than undarkened P_i-deficient plants ($P<0.05$, days 30, 35). The G6P content of P_i-sufficient plants was 4–5-fold greater than P_i-deficient plants ($P<0.05$), regardless of whether they were darkened or fully illuminated (Fig. 5C, D). P_i-sufficient plants maintained fairly stable G6P concentrations throughout the experimental period when fully illuminated, whereas darkened P_i-sufficient plants contained less G6P towards the end of the experiment. In P_i-deficient plants, darkening increased G6P content compared to illuminated plants ($P<0.05$, day 30). The content of F6P (Fig. 5E, F) in plants grown with 4.0 mol m^{-3} P_i, was almost twice that of P_i-deficient plants ($P<0.05$). Darkening had no consistent effect in either P_i treatment. The S7P content of P_i-sufficient plants was 2–3-fold greater ($P<0.05$) than that of P_i-deficient plants (Fig. 5G, H). The effect of darkening depended on P_i supply: in P_i-sufficient plants it decreased S7P towards the end of the experimental period, whereas it increased greatly in darkened P_i-deficient plants. ATP content of leaves of P_i-sufficient plants was between 16 and 20 μmol m^{-2} ATP throughout the experimental period. (Fig. 5I, J). In contrast, ATP content of fully illuminated P_i-deficient plants was less than 50% of that of P_i-sufficient plants after 41 d of P_i deficiency. Darkening P_i-deficient plants increased ATP content to values comparable to P_i-sufficient plants and restricted the decrease in ATP concentration observed on day 41.

The relationships between the rate of photosynthesis measured under the growth conditions and the contents of RuBP and G6P (Fig. 6A, B, respectively), are described by hyperbolic functions. P_n increased with increasing RuBP content up to about 30 μmol m^{-2} (corresponding to P_i-deficient plants), but did not increase further with larger RuBP contents (corresponding to P_i-sufficient plants) ($r^2=0.83$, $P<0.001$). P_n was also correlated hyperbolically with G6P but less strongly than with RuBP, increasing as G6P content rose from *c.* 2–5 μmol m^{-2} (corresponding to the P_i-deficient treatments) and reaching a plateau between 5 and 10 μmol m^{-2} G6P, the larger contents corresponding to P_i-sufficient plants. The relationships between P_n and ATP content, and between RuBP and ATP, were linear

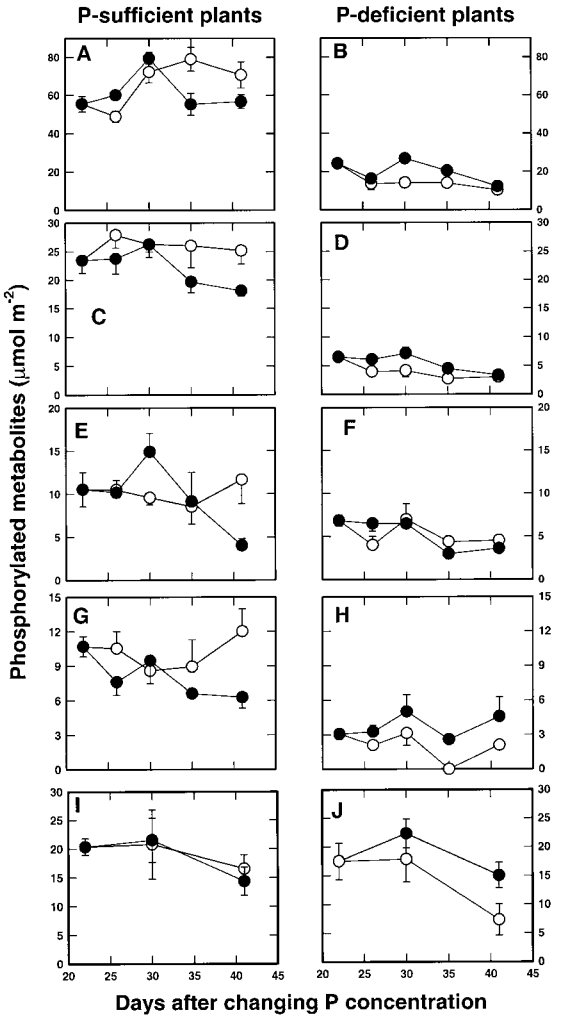

Fig. 5. Contents of RuBP (A, B), G6P (C, D), F6P (E, F), S7P (G, H), and ATP (I, J) in leaves of plants grown under either 4.0 (left panel) or 0.0 mol m^{-3} P_i (right panel). Plants were either fully illuminated (○) or the lower leaves were darkened (●). Samples were taken 7 h into the photoperiod. Bars represent standard error of the mean of four replicates.

(Fig. 6C, D), but weak with r^2 of 0.66 ($P<0.005$) and 0.23 (not significant), respectively.

Discussion

The data show that during the development of P_i deficiency, photosynthesis is regulated by sink strength. A decrease in source/sink ratio by darkening some of the leaves of P_i-deficient plants delayed the decrease in P_n relative to P_i-deficient plants fully exposed to light (Fig. 1). With abundant P_i, P_n was not affected by change in source/sink balance by darkening. This indicates that the response of photosynthesis to P_i is a 'whole plant' one, which depends on the dynamic interaction of sinks and sources; thus increasing the sink delayed, for some time, the decrease in P_n caused by the P_i deficiency. The effect

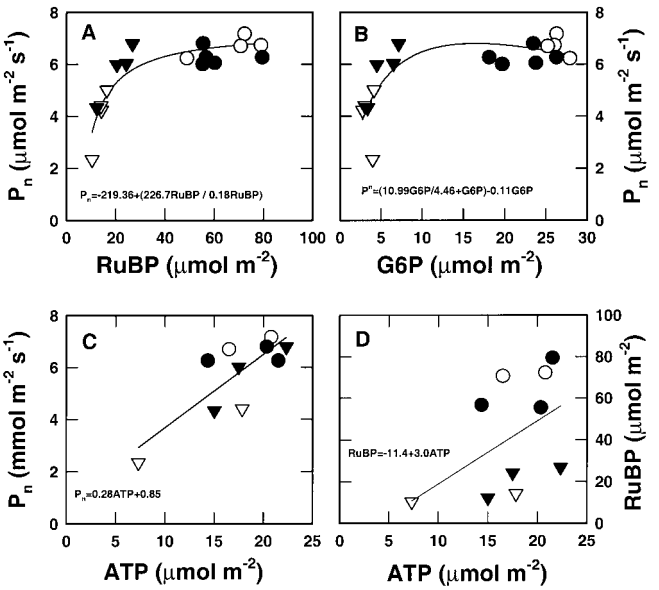

Fig. 6. Regression analysis of the relation between P_n, measured at growth conditions and RuBP (A), G6P (B), ATP (C) and the relationship between RuBP and ATP contents (D) in P_i-deficient (▽,▼) and P_i-sufficient (○,●) leaves of plants that were either fully illuminated (▽,○) or the lower leaves were darkened (▼,●).

of changing the source/sink balance occurred despite the observation that darkening P_i-deficient plants did not change the P_i content of the source leaf compared to those fully exposed to light (Fig. 2). Thus, it is not a function of the bulk P_i content of the leaves: however, it could reflect decreased flux of P_i into the chloroplast. This leads to the question: what is the mechanism of sink control? Paul and Driscoll reported similar results under N deficiency, where N-deprived plants with a decreased source/sink ratio had rates of photosynthesis the same as N-sufficient plants (Paul and Driscoll, 1997). In that work, the sink component of photosynthesis acted by altering carbohydrate status within the plant. Darkening prevented the accumulation of glucose and loss of Rubisco protein, whereas Rubisco decreased and glucose increased in N-deficient plants, providing strong support for sugar repression of photosynthesis under N-deficient conditions.

The results of this study show sink regulation of photosynthesis under P_i deficiency. However, this is not mediated through decreased Rubisco. In P_i-deficient and P_i-sufficient plants, Rubisco maximum activity remained unchanged in all plants despite contrasting amounts of hexose in leaves. Hexose did not increase with P_i deficiency. These results imply that sugar-mediated regulation of photosynthesis by repression of Rubisco protein is not a universal mechanism, as has been pointed out previously (Sheen, 1994; Paul and Stitt, 1993; Nielsen *et al.*, 1998). However, changes in Rubisco activity can play a role in the photosynthetic response to P_i deficiency. Activity of Rubisco decreased under P_i deficiency (Lauer *et al.*, 1989). The early deficiency of P_i prevented the

synthesis of Rubisco and decreased the activity, probably due to the low ratio of RuBP to RuBP binding sites of Rubisco (Jacob and Lawlor, 1992). The latter suggests that the capacity to regenerate RuBP is the rate-limiting factor in P_n of P_i-deficient plants, and that the decreased activity of Rubisco is a secondary effect. These results corroborate this view as P_i deficiency drastically decreased RuBP content (Fig. 5A, B) as reported by others (Rao *et al*., 1989; Jacob and Lawlor, 1992). Furthermore, P_n and RuBP exhibited a strong non-linear relationship in both P_i-deficient and P_i-sufficient plants either darkened or fully illuminated, with a hyperbolic function yielding the best fit between photosynthetic rate and any of the phosphorylated metabolites analysed (Fig. 6A). A plateau was reached at RuBP contents of about 20–40 μmol m^{-2} (80–90% of saturated rate of photosynthesis) providing further support for the limitation of photosynthesis by RuBP under P_i deficiency. The observed sink limitation of photosynthesis under P_i deficiency also operates through the regeneration of RuBP because RuBP content was increased in darkened plants compared to illuminated P_i-deficient plants. Current understanding of the photosynthetic mechanisms is that P_i is required for synthesis of ATP, which then determines the synthesis of RuBP together with other factors that contribute to sophisticated fine control of PRK activity (Paul *et al*., 1995). However, the relationship between P_n and ATP (Fig. 6C) content is very weak and of RuBP and ATP not significant (Fig. 6D). Figure 6D indicates a large RuBP content per unit area with adequate P_i in plants both fully illuminated and with lower leaves darkened, but much smaller RuBP with deficient P_i. However, there is only a small decrease in ATP of the bulk tissue despite the change in P_i and substantially decreased RuBP. Absence of correlation between ATP and RuBP may be due to scatter in the data which reflect the multiple pools and sites of ATP synthesis and consumption in the cell. This may obscure the relationship between ATP and RuBP. Given the strongly hyperbolic dependence of P_n on RuBP (Fig. 6A) and the relationship of RuBP on ATP it is, therefore, unsurprising that there is a poor correlation of P_n with ATP. Despite the lack of clarity regarding the role of ATP it is considered that the increased availability of P_i to the chloroplast as a consequence of the smaller source/sink ratio, would increase the P_i sufficiently to allow extra ATP synthesis and RuBP production, thus maintaining P_n. Also, S7P and G6P would increase as a consequence of the increased photosynthesis.

How might sink demand alter RuBP content? P_i deficiency resulted in a 4-fold decrease in carbon export from source leaves compared to P_i-sufficient plants (Table 1) and more starch and less sucrose than P_i-sufficient plants (Fig. 3G–J). Darkening P_i-deficient plants maintained carbon export at rates around those of P_i-sufficient plants for much of the experiment and more than 2-fold higher than in fully illuminated P_i-deficient plants (Table 1). Darkening also changed carbohydrate partitioning between starch and sucrose, with starch content lower and sucrose higher than in undarkened P_i-deficient plants (Fig. 3G–J). Higher G6P in darkened P_i-deficient plants (Fig. 6D) would activate SPS (Doehlert and Huber, 1983) facilitating the higher rate of sucrose synthesis necessary to support greater export from leaves. Increased rate of sucrose synthesis would recycle more P_i from the cytosol into the chloroplast and replenish ATP and hence RuBP pools giving faster rates of photosynthesis. End-product limitation of photosynthesis has previously been observed under rather artificial conditions (saturating light and CO_2 and particularly at low temperatures) to which plants are not acclimated (Stitt, 1986; Sharkey *et al*., 1986; Labate and Leegood, 1988; Stitt and Grosse, 1988) and was considered of limited physiological importance (Stitt, 1991). However, these data show that rates of sucrose synthesis (calculated from rates of photosynthesis and carbohydrate accumulation, Table 1) can limit photosynthesis under the conditions in which plants are growing and that end-product limitation is of physiological relevance under P_i deficiency. Towards the end of this experiment the rate of photosynthesis declined in darkened P_i-deficient plants (Fig. 1B). This may be a consequence of full expansion of the fifth leaf so that the whole plant source/sink balance had increased, overcoming the effects of the shading treatment.

Intriguingly, maximum activity of PRK was greater in darkened P_i-deficient plants than in fully illuminated P_i-deficient plants, indicating an effect of sink demand under low P_i on the expression of this enzyme. This would need to be confirmed by determination of PRK protein and mRNA. Such a differential response of Calvin cycle enzymes to P_i has been observed before, particularly for fructose 1,6-bisphosphatase, which shows enhanced expression compared to other Calvin cycle enzymes under low Pi (Rao and Terry, 1989; Freeden *et al*., 1990). The data from this study for enzyme activities demonstrate that, as well as direct effects of P_i on gene expression, there is also a sink demand component. Hence, like photosynthesis, to understand the effects of P_i deficiency on gene expression a whole plant view needs to be taken.

It is concluded that low sink strength imposes the primary limitation on photosynthesis during the development of P_i deficiency. This mechanism is one of end-product limitation in intact plants under the growing conditions, with decreased sucrose synthesis, due to low demand from the rest of the plant, restricting recycling of P_i to the chloroplast, thus limiting ATP synthesis and RuBP regeneration and, hence, the rate of photosynthesis. The experiments illustrate how integrated the source/sink activities of plants are, and the importance of taking a whole plant view to understand the limitations on photosynthesis imposed by P_i deficiency.

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