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Control of photosynthate partitioning in spinach leaves

Analysis of the interaction between feedforward and feedback regulation of sucrose synthesis

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Abstract. Experiments were carried out to estimate the elasticity coefficients and thence the distribution of control of sucrose synthesis and photosynthate partitioning between cytosolic fructose-1,6-bisphosphatase and sucrose-phosphate synthase (SPS), by applying the dual-modulation method of Kacser and Burns (1979, Biochem. Soc. Trans. 7, 1149–1161). Leaf discs of spinach (*Spinacia oleracea* L.) were harvested at the beginning and end of the photoperiod and illuminated at five different irradiances to alter (i) the extent of feedback inhibition and (ii) the rate of photosynthesis. The rate of CO_2 fixation, sucrose synthesis and starch synthesis were measured and compared with the activation of SPS, and the levels of fructose-2,6-bisphosphate (Fru2,6bisP) and metabolites. Sucrose synthesis increased progressively with increasing irradiance, accompanied by relatively large changes of SPS activity and Fru2,6bisP, and relatively small changes of metabolites. At each irradiance, leaf discs harvested at the end of the photoperiod had (compared with leaf discs harvested at the beginning of the photoperiod) a decreased rate of sucrose synthesis, increased starch synthesis, decreased SPS activity, increased Fru2,6bisP, a relatively small (20%) increase of most metabolites, no change of the glycerate-3-phosphate: triose-phosphate ratio, a small increase of NADP-malate dehydrogenase activation, but no inhibition of photosynthesis. The changes of sucrose and starch synthesis were largest in low light, while the changes of SPS and Fru2,6bisP were as large, or even larger, in high light. It is discussed how these results provide evidence that the control of sucrose synthesis is shared between SPS and fructose-1,6-bisphosphatase, and provide information about the in-vivo response of these enzymes to changes in the levels of their substrates and effectors. At low fluxes, feedback regulation is very effective at altering partitioning. In high light, changes of SPS activation and Fru2,6bisP can be readily overriden by increasing levels of metabolites.

Key words: Fructose-1-6-bisphosphatase – Photosynthate partitioning – *Spinacia* (photosynthate partitioning) – Sucrose phosphate synthase – Sucrose synthesis

Abbreviations: Fru1,6Pase = fructose-1,6-bisphosphatase; Fru2,6-bisP = fructose-2,6-bisphosphate; Fru6P = fructose-6-phosphate; Glc6P = glucose-6-phosphate; hexose-P = the sum of Fru6P, Glc6P and Glc1P; PGA = glycerate-3-phosphate; Pi = inorganic phosphate; SPS = sucrose-phosphate synthase; triose-P = the sum of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate; UDPGlc = uridine 5′-diphosphoglucose

Introduction

The following experiments were carried out to quantify the impact of sucrose-phosphate synthase (SPS) and fructose-2,6-bisphosphate (Fru2,6bisP) on the rate of sucrose synthesis and the partitioning of photosynthate, and to show how and why their impact varies, depending on the conditions.

During photosynthesis, triose-phosphates (triose-P) are exported to the cytosol and converted to sucrose. Inorganic phosphate (Pi) is released and returns to the chloroplast via a strict counterexchange with triose-P, catalysed by the phosphate translocator, to support further CO_2 fixation (Stitt et al. 1987b). The rate of sucrose synthesis is strongly regulated by feedforward mechanisms, which coordinate it with the rate of photosynthesis. It is also regulated by feedback mechanisms which allow the rate of sucrose synthesis to be decreased, so more photosynthate is stored in the chloroplast as starch. Several enzymes are involved, including the cytosolic fructose-1,6-bisphosphatase (Fru1,6Pase) and SPS (Stitt et al. 1987b).

The cytosolic Fru1,6Pase is regulated (inhibited) by the signal metabolite, Fru2,6bisP (Stitt et al. 1987a). The concentration of Fru2,6bisP is modulated by various metabolites which inhibit or activate fructose-6-phosphate,2-kinase (Fru6P,2-kinase) and fructose-2,6-bisphosphatase (Fru2,6Pase) (Stitt et al. 1984c; Larondelle et al. 1986; MacDonald et al. 1989). Rising rates of photosynthesis are signalled by an increasing glycerate-3-phosphate (PGA): Pi ratio. This drives the Fru2,6bisP concentration downwards, and activates the

cytosolic Fru1,6Pase (Stitt et al. 1984a; Neuhaus and Stitt 1989). An increased supply of photosynthate also stimulates Fru1,6Pase activity by generating an increase of triose-P and, thence, of Fru1,6bisP (Herzog et al. 1984). This feedforward regulation is counteracted by a feedback loop. When fructose-6-phosphate (Fru6P) increases in the cytosol, the Fru2,6bisP concentration rises and the cytosolic Fru1,6Pase is reinhibited (Stitt et al. 1984b; Neuhaus et al. 1989).

The activity of SPS is regulated in an analogous manner. Increasing rates of photosynthesis lead to a "light-activation" of SPS (Sicher and Kremer 1984; Pollock and Housley 1985; Kalt-Torres et al. 1987; Stitt et al. 1988). This involves protein dephosphorylation (Huber et al. 1989; Siegl and Stitt 1990), although the signal sequence has not yet been elucidated. An increased supply of photosynthate can also stimulate SPS via an increase of the glucose-6-phosphate (Glc6P): Pi ratio (Doehlert and Huber 1983, 1984; Stitt et al. 1988). This feedforward regulation is counteracted when sucrose accumulates in the leaf. In these conditions SPS is deactivated (Stitt et al. 1988) by a mechanism which may again involve protein phosphorylation (Huber et al. 1989).

In addition to information about the *kind* of regulatory mechanisms, we also need to know how *effective* they are in altering the flux through the pathway. This kind of information is essential if we are to evaluate how the feedforward and feedback loops interact, or decide which enzymes actually control the rate of sucrose synthesis in vivo. Specifically, we need to know (i) how sensitively an enzyme responds to changes in the concentration of each of its effectors in vivo and (ii) whether a change in the activity of that particular enzyme actually leads to a change in the flux through the entire pathway. These responses can be formalised as the elasticity coefficient and flux-control coefficient, respectively (Kacser and Porteous 1987).

One way to study the distribution of control is to use mutants with a partial reduction in the levels of a selected enzyme. This approach has already been applied to mutants of *Clarkia xantiana* with decreased activity of cytosolic phosphoglucose isomerase. The results showed that the effectiveness of the Fru2,6bisP feedback loop varies, depending on the conditions, and also indicated that control may be shared between SPS and the cytosolic Fru1,6-Pase (Neuhaus et al. 1989). The following experiments use an alternative approach to investigate the control of sucrose synthesis, which is based on the 'dual modulation' method proposed by Kacser and Burns (1979). In this approach, the pathway is perturbed in two independent ways, and the resulting changes of steady-state flux and metabolites levels are measured. The results can then be analysed to provide qualitative and quantitative insights into the contribution of SPS and Fru2,6bisP to the control of flux during photosynthesis.

Material and methods

Spinach (*Spinacia oleracea* U.S. Hybrid 424; Ferry Morse Seed Co., Modesto, Calif., USA) was grown as in Gerhardt et al. (1987).

Evolution of O_2 and $^{14}CO_2$ fixation was carried out in saturating CO_2 for 20 min in a leaf-disc O_2 electrode at 20° C as in Stitt and Große (1988). The light was from projectors, and the intensity was varied using neutral-density filters. Material was frozen in liquid N_2, and then separated into insoluble, neutral, acidic and basic fractions as in Quick et al. (1989). Recovery of ^{14}C during the analysis was greater than 90%. The neutral fraction was shown to be sucrose by paper chromatography (pyridine:ethyl acetate:water, 8:2:1; by vol.).

Leaf material for metabolite and enzyme assays was illuminated in a leaf-disc O_2 electrode for 12 min, and then quenched in liquid N_2 under continued illumination. Extracts were prepared and assayed for Fru2,6bisP, metabolites and adenine nucleotides as in Stitt and Große (1988), for SPS as in Stitt et al. (1988) using 2 mM Fru6P, 10 mM Glc6P, 5 mM Pi and 3 mM uridine 5'-diphosphoglucose (UDPGlc) in the assay, for Fru1,6Pase as in Stitt and Große (1988) and for NADP-malate dehydrogenase as in Scheibe and Stitt (1988).

Theory

The quantitative treatment of the results in this article is based on the following theorems, which were developed by Kacser and coworkers (see Kacser and Porteous 1987). The flux-control coefficient, C, describes the ability of an enzyme to control the flux through a pathway and is defined as

$$C = \frac{dJ}{J} \Big/ \frac{dE}{E}, \qquad \text{Eq. (1)}$$

where dE/E is a fractional change in the activity of the enzyme, and dJ/J is the resulting fractional change in the steady-state flux through the whole pathway. The elasticity coefficient, ε, describes the response of the enzyme to a change in one of its effectors when the enzyme is isolated from the rest of the pathway. It is defined as

$$\varepsilon = \frac{dv}{v} \Big/ \frac{ds}{s}, \qquad \text{Eq. (2)}$$

where dv/v is the fractional change in the activity of the enzyme which results from a change, ds/s, in the concentration of one of its effectors, when all the other effectors are kept constant (Kacser and Porteous 1987). The flux-control coefficients of two adjacent enzymes in a pathway are related by the connectivity theorem, which states that

$$\frac{C_{E1}}{C_{E2}} = -\frac{\varepsilon_{s_x}^{E2}}{\varepsilon_{s_x}^{E1}} \qquad \text{Eq. (3)}$$

where C_{E1} and C_{E2} are the flux-control coefficients of two 'adjacent' enzymes. $E1$, $E2$, and $\varepsilon_{s_x}^{E1}$ and $\varepsilon_{s_x}^{E2}$ are their elasticity coefficients for a shared substrate, s_x. The summation theory,

$$C_{E1} + C_{E2} \ldots C_{En} = 1 \qquad \text{Eq. (4)}$$

states that the flux-control coefficients of all the enzymes in a pathway sum up to unity. It follows that the flux-control coefficients for enzymes can be estimated via the connectivity and summation theorems, provided we are able to measure the in-vivo elasticity coefficients for their substrates and major effectors.

One way of estimating in-vivo values for elasticity coefficients is to use the dual-modulation approach (Kacser and Burns 1979). If we take the simplest case of an enzyme with two effectors, s_1 and s_2, the change in activity, dv/v, after perturbing the cell and allowing a new steady state to be reached can be expressed as

$$\frac{dv}{v} = \frac{dJ}{J} = \varepsilon_{s_1} \frac{ds_1}{s_1} + \varepsilon_{s_2} \frac{ds_2}{s_2} \qquad \text{Eq. (5)}$$

where dJ/J is the fractional change of the steady-state flux, ds_1/s_1 and ds_2/s_2 are the fractional changes in the steady-state levels of the metabolites s_1 and s_2, and ε_{s_1} and ε_{s_2} are the elasticity coefficients of the enzyme for s_1 and s_2, respectively. Empirical values can be obtained for dJ/J, ds_1/s_1 and ds_2/s_2 if we measure the steady-state flux and metabolite levels before and after metabolism is perturbed, while ε_{s_1} and ε_{s_2} will be unknowns. If two independent perturbations are carried out, two simultaneous equations can be written and solved for ε_{s_1} and ε_{s_2}.

This approach can be applied to sucrose synthesis, using a simplified model to allow suitable equations to be written for the cytosolic Fru1,6Pase and SPS (Stitt 1989). It should be noted that this approach makes no assumption about the kinetic properties or maximum activities of these enzymes; it merely assumes that the most important regulation properties are known. The response of Fru1,6Pase activities to a change in steady-state conditions is described as

$$\frac{dv}{v} = \varepsilon^F_{\text{triose-P}} \cdot \frac{d\,\text{triose-P}}{\text{triose-P}} + \varepsilon^F_{\text{Fru2,6bisP}} \cdot \frac{d\,\text{Fru2,6bisP}}{\text{Fru2,6bisP}} \qquad \text{Eq. (6)}$$

where $\varepsilon^F_{\text{triose-P}}$ and $\varepsilon^F_{\text{Fru2,6bisP}}$ are the elasticity coefficients of the Fru-1,6Pase for triose-P and Fru2,6bisP, respectively. The term $\varepsilon^F_{\text{triose-P}}$ implicitly includes the response to changes of Pi. Triose-P are used instead of Fru1,6bisP, because it is technically difficult to measure the cytosolic Fru1,6bisP level (Gerhardt et al. 1987); in effect, this equation treats triose-P isomerase, aldolase and Fru1,6Pase as one block of enzymes. Since the reaction is irreversible (Gerhardt et al. 1987) and Fru6P does not inhibit under physiological conditions (Stitt et al. 1985) the term for the product is omitted. The equation, for simplicity, omits the effect of AMP.

The activity of SPS is described as

$$\frac{dv}{v} = \frac{d\,\text{SPS}}{\text{SPS}} + \varepsilon^s_{\text{hexose-P}} \cdot \frac{d\,\text{hexose-P}}{\text{hexose-P}} + \varepsilon^s_{\text{UDPGlc}} \cdot \frac{d\,\text{UDPGlc}}{\text{UDPGlc}} + \varepsilon^s_{\text{sucrose}} \cdot \frac{d\,\text{sucrose}}{\text{sucrose}} \qquad \text{Eq. (7)}$$

where dSPS/SPS is the fractional change in the activation of the enzyme, $\varepsilon^s_{\text{hexose-P}}$ and $\varepsilon^s_{\text{UDPGlc}}$ are elasticity coefficients of SPS for the combined hexose-P pool and UDPGlc respectively, and $\varepsilon^s_{\text{sucrose}}$ is a notional elasticity coefficient for sucrose. This equation assumes that the 'inactive' form of SPS is totally inactive in vivo (see below for further discussion). Changes of Pi are tacitly included in the term $\varepsilon^s_{\text{hexose-P}}$, which actually describes the response of SPS to changes of Glc6P, Fru6P and Pi. To solve this equation in the following article, we assume that $\varepsilon^s_{\text{UDPGlc}}$ is 0.5, as would be the case if SPS were half-saturated with UDPGlc. The UDPGlc concentration estimated in the cytosol is about 3–3.5 mM (see below) assuming a volume of about 20 µl mg chlorophyll^{-1} (Chl), and the K_m (UDPGlc) is about 7 mM (Siegl and Stitt 1990).

If each of these equations is substituted with two independent sets of empirical values for ds/s and dv/v ($=dJ/J$, the fractional change in the steady-state rate of sucrose synthesis), they can be solved to provide values for the various elasticity coefficients. A modified form of the connectivity theorem (Stitt 1989) can then be used to estimate flux-control coefficients for sucrose synthesis for the cytosolic Fru1,6Pase (C_F) and SPS (C_s). Hexose-P are taken as the shared pool between Fru1,6Pase and SPS. However, hexose-P do not exert a direct effect on Fru1,6Pase (Stitt et al. 1985); instead, they act on Fru1,6Pase indirectly, via changes of Fru2,6bisP (Stitt et al. 1984; Stitt and Neuhaus 1989). The response of Fru2,6-bisP to an increase of Fru6P is described by the empirical term, α, where

$$\alpha = \frac{d\,\text{Fru2,6bisP}}{\text{Fru2,6bisP}} \Big/ \frac{d\,\text{Fru6P}}{\text{Fru6P}} \qquad \text{Eq. (8)}$$

dFru6P/Fru6P is the fractional change of Fru6P, and dFru2,6bisP/Fru2,6bisP is the resulting fractional change of Fru2,6bisP after peturbing metabolism to increase the Fru6P level (Stitt 1989). The response of the Fru1,6Pase to a change of hexose-P can then be described as $\alpha \cdot \varepsilon^F_{\text{Fru2,6bisP}}$. It follows that a modified connectivity theorem can be written,

$$\frac{C_F}{C_s} = -\frac{\varepsilon^s_{\text{hexose-P}}}{\alpha \cdot \varepsilon^F_{\text{Fru2,6bisP}}}. \qquad \text{Eq. (9)}$$

This theorem yields *relative* flux-control coefficients, describing the distribution of control in the metabolic sequence between triose-P and sucrose.

The entire pathway of photosynthetic sucrose synthesis, of course, also includes the fixation of CO_2 in the chloroplast. The flux-control coefficients of Fru1,6Pase and SPS for the conversion of CO_2 to sucrose will retain the same relation, but their magnitude may change. They will both be decreased when enzymes in the thylakoids or the Calvin cycle exert control on the rate of triose-P formation, and they will be increased if the enzymes of starch synthesis have negative control coefficients for sucrose synthesis (this follows from the summation theory). It is, however, important to note that SPS and Fru1,6Pase will still have finite flux-control coefficients for the conversion of CO_2 to sucrose even when they exert no control over the rate of photosynthesis. This follows because a decreased rate of sucrose synthesis can be offset by an increased rate of starch synthesis.

Results

Experimental design. The flux to sucrose was decreased by either (i) decreasing the irradiance or (ii) increasing feedback on the pathway. Spinach plants were taken after 14–19 h darkness, when there is negligible sucrose in the leaf, or after 8–10 h illumination, when sucrose has accumulated in the leaf (Stitt et al. 1983; Gerhardt et al. 1987). It has already been shown that these two sets of leaf material differ in the activation of SPS, the levels of Fru2,6bisP and metabolites, and in the partitioning of photosynthate (Stitt et al. 1983; Gerhardt et al. 1987; Stitt et al. 1988) i.e. they are 'programmed' to exert weak and strong feedback regulation of sucrose synthesis, respectively. Leaf discs harvested from both sets of material were then illuminated at five different irradiances.

Fluxes. Fluxes were measured as ^{14}C-incorporation after illuminating the leaf discs for 20 min in saturating [^{14}C] bicarbonate in a leaf-disc O_2 electrode. It has been shown that the sucrose accumulating in the disc does not exert feedback control itself during this time (Stitt et al. 1984b). Both sets of leaf material had a similar light-response of CO_2 fixation (Fig. 1A) and O_2 evolution (data not shown). However, the leaf material harvested at the end of the photoperiod had a lower rate of sucrose synthesis (Fig. 1B) and a higher rate of starch synthesis (Fig. 1C).

This change of partitioning was apparent at all irradiances. However, the change was largest in low irradiance. This is illustrated in Fig. 1D, which compares the ratio of sucrose synthesis:starch synthesis in material harvested at the end of the night and the end of the day, and in Table 1, which summarises the fractional changes of sucrose (dJ^{su}/J^{su}) or starch (dJ^{st}/J^{st}) synthesis when these two groups of leaf material are compared

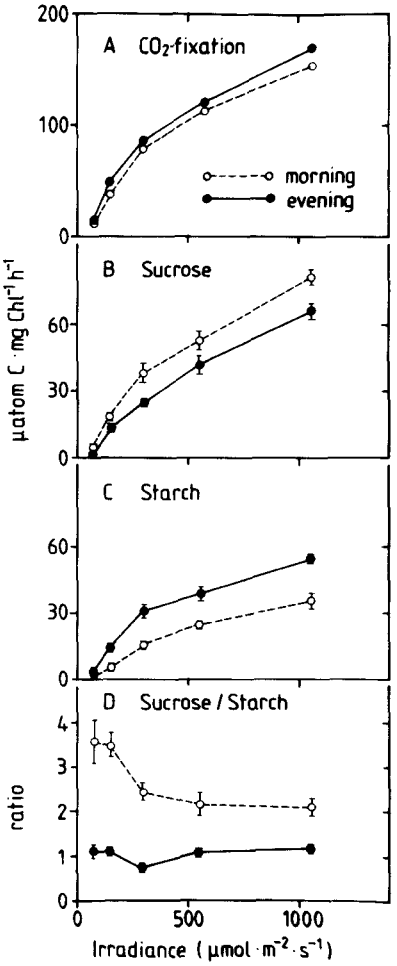

Fig. 1A–D. Response of fluxes to irradiance and feedback regulation. The light-responses of the fluxes were measured by ^{14}C incorporation in spinach leaf material harvested at the end of the dark period (○···○) and the end of the photoperiod (●–●). **A** CO_2 fixation; **B** sucrose synthesis; **C** starch synthesis; **D** ratio of sucrose synthesis:starch synthesis. Results are given as mean ±SE ($n=4$)

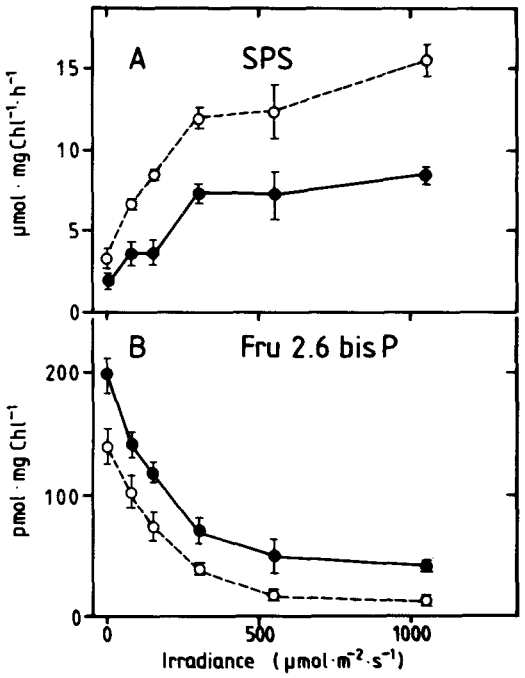

Fig. 2A, B. Activity of SPS and Fru2,6bisP levels. The light response of the parameters was measured in spinach leaf discs harvested at the end of the night (○···○) or the end of the photoperiod (●–●). **A** SPS, assayed in 2 mM Fru6P, 10 mM Glc6P, 5 mM Pi, 3 mM UDPGlc; **B** Fru2,6bisP. The results are the mean ±SE ($n=4$)

(see table legend for the calculation). There was a 56% reduction of sucrose synthesis ($dJ^{su}/J^{su}=-0.56$) in leaf material harvested at the end of the photoperiod if we compare the fluxes in low light. In contrast, sucrose synthesis was only reduced by 20% in high light. Starch synthesis was also stimulated more strongly in low irradiance than high irradiance (Table 1).

Activation of SPS, and Fru2,bisP levels. The 'coarse' regulation of SPS, and the changes of Fru2,6bisP were investigated to assess their contribution to these changes of flux. Extracts were rapidly prepared, and assayed for SPS in the presence of 2 mM Fru6P, 10 mM Glc6P, and 5 mM Pi. In these conditions, the 'active' form of SPS retains close to V_{max} activity, but the 'inactive' form has negligible activity (Stitt et al. 1988; Siegl and Stitt 1990). There was a progressive activation of SPS with increas-

Table 1. Fractional changes of fluxes, metabolites, Fru2,6bisP, and SPS activation during feedback inhibition of sucrose synthesis in spinach leaves: dependence on the steady-state irradiance. Leaf discs were harvested from plants at the end of the night or the end of the day, and were illuminated at five different irradiances. The fractional change (dX/X) in material harvested at the end of the photoperiod compared with the end of the dark period, was calculated at each irradiance using the formula $dX/X=(X^P-X^D)/X^D$, where X^P is the value for the parameter in material harvested at the end of the dark period, and X^D is the value in material harvested at the end of the photoperiod. The raw data used in the calculation are shown in Figs. 1–3

Irradiance	Fractional change in fluxes, metabolites, or activity between predarkened and 9-h-illuminated leaf material						
µmol · m^{-2} · s^{-1}	$\frac{dJ\text{ sucrose}}{J\text{ sucrose}}$	$\frac{dJ\text{ starch}}{J\text{ starch}}$	$\frac{d\text{ triose-P}}{\text{triose-P}}$	$\frac{d\text{ Fru2,6bisP}}{\text{Fru2,6bisP}}$	$\frac{d\text{ hexose-P}}{\text{hexose-P}}$	$\frac{d\text{ UDPGlc}}{\text{UDPGlc}}$	$\frac{d\text{ SPS}}{\text{SPS}}$
0	–	–	–	+0.70	+0.51	–	–
75	−0.56	(n.d.)	+0.23	+0.39	+0.19	+0.05	−0.46
150	−0.31	+1.60	0.00	+0.56	+0.12	+0.15	−0.56
302	−0.34	+0.95	0.00	+0.66	+0.09	+0.14	−0.40
576	−0.21	+0.57	+0.26	+1.68	+0.13	+0.27	−0.42
1050	−0.19	+0.54	+0.23	+1.68	+0.11	+0.17	−0.46

Table 2. Relation between the change in the steady-state Fru2,6bisP level or the activation of SPS, and the steady-state flux to sucrose and starch: dependence on the irradiance. The results are calculated from Table 1 for each irradiance shown (n.d. = not determined)

Irradiance	Pathway responses to Fru2,6bisP		Pathway responses to SPS activation	
($\mu mol \cdot m^{-2} \cdot s^{-1}$)	$\frac{dJ \text{ sucrose}/J \text{ sucrose}}{d\text{Fru2,6bisP}/\text{Fru2,6bisP}}$	$\frac{dJ \text{ starch}/J \text{ starch}}{d\text{Fru2,6bisP}/\text{Fru2,6bisP}}$	$\frac{dJ \text{ sucrose}/J \text{ sucrose}}{d\text{SPS}/\text{SPS}}$	$\frac{dJ \text{ starch}/J \text{ starch}}{d\text{SPS}/\text{SPS}}$
75	−1.43	n.d.	+1.21	n.d.
150	−0.56	+2.85	+0.55	−2.85
302	−0.51	+1.43	+0.97	−2.37
576	−0.12	+0.34	+0.88	−1.35
1050	−0.11	+0.32	+0.41	−1.17

ing irradiance (Fig. 2A). However, at a given irradiance, SPS activity was always lower in material harvested at the end of the photoperiod. Fructose-2,6-bisphosphate decreased continuously when the irradiance was increased (Fig. 2B), as previously seen (Stitt et al. 1984a). However, at each irradiance, there was more Fru2,6bisP in leaf material harvested at the end of the photoperiod (Fig. 2B).

We have already seen that the fluxes to sucrose and starch are more sensitive to feedback control in low irradiance (Fig. 1D, Table 1). In contrast, SPS activation was decreased by a fairly constant amount (40%–50%) at all the irradiances when material was harvested at the end of the photoperiod (Fig. 2A, Table 1). Fructose-2,6-bisphosphate actually increased more in high irradiance (170%) than low irradiance (30%–50%) when material was harvested at the end of the photoperiod (Table 1). The differing response of fluxes to changes of SPS or Fru2,6bisP at different irradiances is summarised in Table 2. For a given change of SPS or Fru2,6-bisP, there is a far larger relative change in the rate of sucrose synthesis in low irradiance than in high irradiance. Changes of SPS and Fru2,6bisP also stimulated starch synthesis more strongly in low irradiance than high irradiance (Table 2).

Metabolites. These changes of SPS activation and Fru2,6bisP will interact with changes in the concentrations of other effectors, e.g. substrates, to generate the observed changes of flux. We therefore measured the changes of some key metabolites involved in sucrose synthesis.

Increasing irradiance led to an increase of hexose-P (Fig. 3A) and triose-P (Fig. 3C), as previously seen (Badger et al. 1984; Stitt et al. 1984; Dietz and Heber 1986; Stitt and Große 1988). However, while flux increased 15-fold between 75 and 1050 $\mu mol \cdot m^{-2} \cdot s^{-1}$, hexose-P only rose by 20%–30% and triose-P only doubled. Uridine diphosphoglucose (UDPGlc) increased at the lowest irradiance used, and then stayed constant. Obviously, flux is increased with, relatively speaking, rather constant levels of metabolites when the irradiance is increased.

If leaf material harvested at the end of the photoperiod is compared with predarkened material, there is a general increase of all the intermediates. However, the increase is small, being only 20% or less. These changes are considerably smaller than the changes of flux, or the changes of SPS activation or Fru2,6bisP levels.

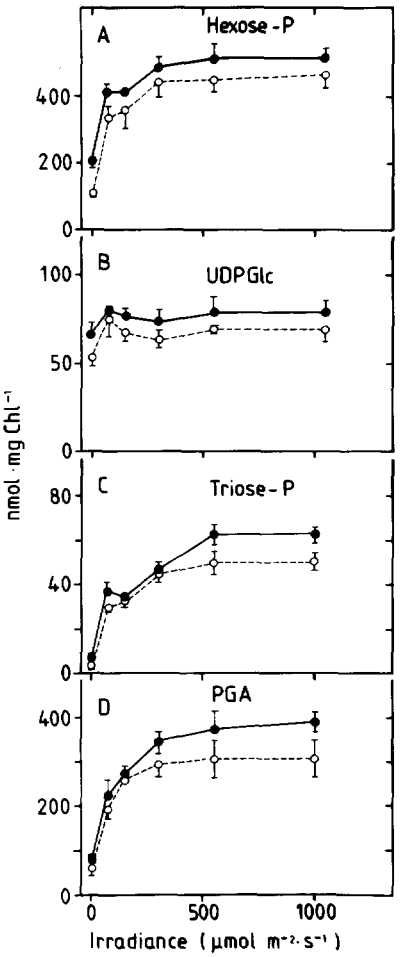

Fig. 3A–D. Metabolite levels. The light response was measured in spinach leaf discs harvested at the end of the night (○···○) or the end of the photoperiod (●–●). **A** Hexose-P, = sum of Glc6P + Fru6P + glucose-1-phosphat (Glc1P); **B** UDPGlc; **C** triose-P, = sum of dihydroxyacetone-P and glyceraldehyde-3-P; **D** PGA. The results are the mean ± SE ($n = 4$)

Interaction with the chloroplast. We also investigated some parameters which could provide information about

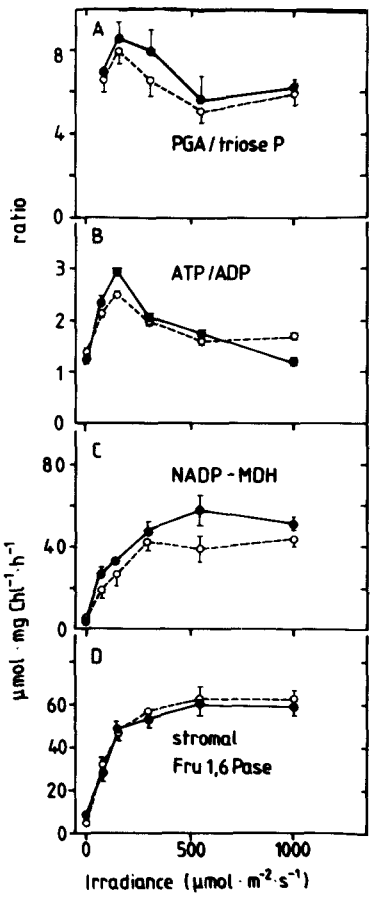

Fig. 4A–D. Energy status. The light response was measured in leaf discs harvested at the end of the dark period (○···○) or the end of the photoperiod (●–●). **A** Ratio of PGA:triose-P; **B** ratio of ATP/ADP; **C** activation of NADP-malate dehydrogenase (NADP-MDH), as metabolic indicator for the stromal NADPH system (Scheibe and Stitt 1988); **D** activation of the stromal Fru1,6Pase

the way in which the chloroplasts are responding to the decreased rate of sucrose synthesis in leaf discs harvested in the evening. A decreased rate of sucrose synthesis should decrease the supply of Pi to the chloroplast. In isolated chloroplasts, this leads to a decreased ATP/ADP ratio and a restriction of PGA reduction (Heldt et al. 1977). The resulting increase of the PGA: Pi ratio activates adenosine diphosphoglucose (ADPGlc) pyrophosphorylase (Preiss 1982) and starch synthesis is activated.

Discs harvested at the end of the photoperiod had an increased level of PGA at every irradiance used (Fig. 3D). The general increase of all the other metabolites indicates that the free Pi level would be lower in leaf material harvested at the end of the photoperiod. However, the metabolite pools do not increase by more than 20% at any particular irradiance, whereas starch synthesis is increased by 50%–160% (Table 1). Leaf material harvested at the end of the photoperiod (with decreased sucrose and increased starch synthesis) also only had a marginal increase of the PGA/triose-P ratio at any given irradiance (Fig. 4A).

Overall measurements of adenine-nucleotide levels did not reveal a consistent decrease of the ATP/ADP ratio (Fig. 4B). Although we cannot exclude the possibility that compartmentation may have concealed a decrease of the stromal ATP/ADP ratio, these results indicate that there is no large additional restriction of ATP synthesis due to low Pi during the operation of sink control in these conditions. Glycerate-3-phosphate reduction also requires NADPH. We measured the activation state of NADP-malate dehydrogenase, which can be used as a metabolic indicator for the stromal NADPH/NADP ratio (Scheibe and Stitt 1988). As seen before, NADP-malate dehydrogenase was activated as irradiance increased (Fig. 4C). However, the activation was consistently higher in leaf discs where feedback regulation was operating, indicating that the stromal NADPH system is more reduced in this leaf material. For comparison, the activation of the stromal Fru-1,6Pase was also measured. This enzyme is also activated by thioredoxin, but the activation is not modulated by the stromal NADPH/NADP system. In this case, there was no difference in the activation between plant material harvested at the beginning and the end of the photoperiod.

Discussion

We will first discuss these results qualitatively and will then show how they can be used to estimate elasticity coefficients for the cytosolic Fru1,6Pase and SPS, and provide quantitative insights into the control of photosynthetic sucrose synthesis.

Feedforward control. It is evident that decreasing Fru2,6bisP and activation of SPS provide an effective way of increasing the rate of sucrose synthesis in response to rising rates of photosynthesis. After each increase in irradiance, the fractional increase of flux is very large, while the steady-state metabolite levels only increase marginally. This sensitive response ensures that Pi is recycled more rapidly to the chloroplast, without a large increase in the levels of phosphorylated intermediates or a corresponding large depletion of the steady-state Pi concentration.

This sensitive response can be explained as follows. Firstly, decreasing Fru2,6bisP and activation of SPS increase the substrate affinity of the Fru1,6Pase (Herzog et al. 1984) and SPS (Stitt et al. 1988; Siegl and Stitt 1990). Secondly, Fru1,6Pase and SPS both respond in a sigmoidal manner to an increase in the pool of triose-P and hexose-P (Stitt et al. 1987a, b).

This sensitive response of flux to increasing rates of photosynthesis is found in the presence and in the absence of feedback control, i.e. in material harvested at the end of the night and at the end of the photoperiod, respectively. The feedback loops lead, in effect, to a shift of the 'threshold' concentration (Herzog et al. 1984; Stitt et al. 1987a) which is needed for flux at the Fru1,6Pase and SPS. This is illustrated in Fig. 5, in which the steady-state flux to sucrose is plotted against the concentration of triose-P (Fig. 5B) or hexose-P (Fig. 5A).

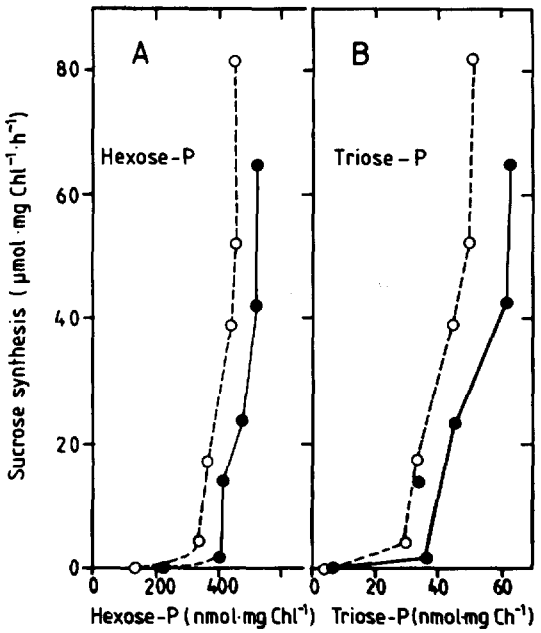

Fig. 5 A, B. Threshold response of fluxes to sucrose. **A** Relation between hexose-P pool and flux at SPS; **B** relation between triose-P pool and flux at the cytosolic Fru1,6Pase. The data are replotted from Figs. 1 and 3, and show the relation in leaves harvested at the end of the night (o···o), or at the end of the day (●–●)

Feedback regulation. Deactivation of SPS and increased Fru2,6bisP lead to an inhibition of sucrose synthesis and a stimulation of starch synthesis at all irradiances. However, the changes in metabolites which accompany this change of partitioning are relatively small. This has two important implications. Firstly, electron transport, ATP synthesis and the Calvin cycle will be able to adjust to these small changes of metabolite pools. In agreement, we observe only marginal changes of the PGA/triose-P ratio and a slight increase in the redox state of the chloroplast, and no loss of net photosynthetic flux. Secondly, these results show that ADPGlc pyrophosphorylase must be responding very sensitively in vivo to changes of the PGA/Pi ratio, as expected from its in-vitro properties (Preiss 1982). In this way, starch synthesis can be stimulated by small perturbations of the metabolite levels, which have no effect on the net rate of CO_2 fixation. An identical conclusion was reached using mutants of *Clarkia xantiana* with decreased cytosolic phosphoglucose isomerase (Neuhaus et al. 1989).

Interaction of feedback regulation with the supply of photosynthate. Feedback control has far more effect on partitioning at low rates of photosynthesis. This conclusion applies for feedback exerted via changes of SPS activation, and for feedback exerted at the Fru1,6Pase via changes of the Fru2,6bisP concentration.

A decreased activation of SPS has two- to three-fold more effect on the rate of sucrose synthesis at low irradiance than at high irradiance (Table 2). This implies that further factors can overcome the deactivation of SPS during rapid photosynthesis e.g. an increasing Glc6P/Pi ratio or increasing substrate concentrations.

Table 3. Response of Fru2,6bisP to a change in the Fru6P level: dependence on the irradiance. The levels of Fru2,6bisP and Fru6P were compared in material which was harvested at the end of the night, and the end of the day, and then left in the dark or illuminated at five difference irradiances. The fractional changes of Fru2,6bisP and Fru6P during feedback inhibition were calculated at each irradiance level (see Table 1). The response of Fru2,6bisP to a change of Fru6P, $=\alpha$ (Stitt 1989) was then calculated

Irradiance ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	$\alpha = \frac{d\ Fru2,6bisP}{Fru2,6bisP} / \frac{d\ Fru6P}{Fru6P}$
0	1.4
75	2.1
150	4.7
302	7.3
576	12.9
1050	11.2

It is possible that the "inactive" form of SPS shows some activity in saturating light and CO_2. It has a K_m(app) for Fru6P of about 2 mM in the presence of an equilibrium concentration of Glc6P (Siegl and Stitt 1990). For comparison, the levels of Fru6P found in leaf discs in high irradiance (150–170 nmol·mg Chl^{-1}) would be equivalent to a Fru6P concentration of about 4 mM in the cytosol (assuming about 50% of the Fru6P is in the cytosol, with a volume of 20 μl·mg Chl^{-1}, Gerhardt et al. 1987). The affinity for Fru6P will be lower in the presence of Pi but, nevertheless, this comparison indicates that the "inactive" form of SPS could show some activity in vivo in leaves which are carrying out rapid photosynthesis and generating high concentrations of cytosolic metabolites.

When SPS is inhibited, there is an increase of hexose-P, including Fru6P. This leads, in turn, to the increase of Fru2,6bisP (see *Introduction*). The response of Fru2,6bisP to rising Fru6P (α, see *Theory*, Eq. 8) is summarised in Table 4. A given change of the Fru6P pool is accompanied by a twofold increase of Fru2,6bisP at low irradiance, compared with a 10-fold increase at high irradiance (Table 3). This increased response of Fru2,6bisP at high irradiance has been predicted from the in-vitro properties of Fru6P,2-kinase and fructose-2,6-bisphosphatase (Fru2,6Pase) (Stitt 1989). At high irradiance, metabolite levels are higher and Pi will be lower (see above). Low Pi will increase the sensitivity of Fru6P,2-kinase and Fru2,6Pase to activation and inhibition by Fru6P (Stitt et al. 1984c; Larondelle et al. 1986).

Although Fru2,6bisP increases (relatively) more at high irradiance the relative change of flux is larger at low irradiance. Obviously, Fru2,6bisP is a more effective inhibitor of the Fru1,6Pase in the conditions found in vivo at low irradiance. A similar conclusion was obtained using *Clarkia xantiana* mutants with decreased cytosolic phosphoglucose isomerase to generate an increase of Fru6P and, thence, Fru2,6bisP (Neuhaus et al. 1989). This conclusion is fully consistent with the known properties of the cytosolic Fru1,6Pase. Fructose-2,6-bisphosphate inhibits competitively to the substrate, fructose-1,6-bisphosphate (Fru1,6bisP), and this inhibition is in-

Table 4. Fractional change of fluxes, metabolites, Fru2,6bisP and SPS activation after decreasing the irradiance: response to a change in the low- and high-irradiance range, and in the presence and absence of feedback inhibition of sucrose synthesis. The fractional changes (dY/Y) are calculated from the data in Figs. 1–3 using the formula $dY/Y=(Y^H-Y^L)\,Y^H$, where Y^H is the value of the parameter at the higher irradiance, and Y^L is the value at the lower irradiance. The high- and low-irradiance levels were changed from 302 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (limiting range), or 1050 to 302 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (high range). The fractional changes were calculated for each irradiance step in material harvested at the end of the night (no feedback inhibition) or the end of the photoperiod (with inhibition of sucrose synthesis)

Pretreatment of material	Irradiance change ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Fractional changes of fluxes, metabolites or activity in response to a adecrease of irradiance						
		$\frac{dJ\ sucrose}{J\ sucrose}$	$\frac{d\ triose\text{-}P}{triose\text{-}P}$	$\frac{d\ Fru2,6bisP}{Fru2,6bisP}$	$\frac{d\ hexose\text{-}P}{hexose\text{-}P}$	$\frac{d\ UDPGlc}{UDPGlc}$	$\frac{d\ SPS}{SPS}$	$\frac{dJ\ starch}{starch}$
16 h dark	302 → 150	−0.53	−0.27	+0.81	−0.19	+0.06	−0.29	−0.66
9 h light	302 → 150	−0.48	−0.40	+0.70	−0.16	+0.07	−0.05	−0.55
16 h dark	1050 → 302	−0.52	−0.12	+1.62	−0.04	−0.07	−0.25	−0.55
9 h light	1050 → 302	−0.61	−0.28	+0.62	−0.06	−0.09	−0.13	−0.56

creased at high Pi (Stitt et al. 1982; Herzog et al. 1984; Stitt et al. 1985). At high irradiance the levels of triose-P and Fru1,6bisP are higher (Fig. 3) and Pi is lower (see earlier discussion), so Fru2,6bisP will inhibit less effectively.

Control analysis. These qualitative conclusions can be made more precise by estimating the elasticity coefficients of the cytosolic Fru1,6Pase and SPS for selected substrates and regulatory metabolites. This reveals how sensitively the enzymes are responding to a change in the concentrations of their effectors in vivo and allows us to assess how control is distributed between these two enzymes.

Elasticity coefficients can be calculated using the dual-modulation method (see *Theory*). The data in Figs. 1–3 provide sets of empirical values for the fractional changes of fluxes and metabolites after two different perturbations, namely (i) an alteration of the light intensity or (ii) the imposition of feedback control. Values from both peturbations can be entered into the simplified equation describing the cytosolic Fru1,6Pase (Eq. 6). This yields two simultaneous equations, which can be solved for $\varepsilon^F_{Fru2,6bisP}$ and $E^F_{triose\text{-}P}$. A similar approach can be used to estimate the elasticity coefficients for SPS, (Eq. 7).

The elasticity coefficient of an enzyme varies, depending on the conditions e.g. upon whether it is substrate-limited or saturated. We have therefore treated the data in Figs. 1–3 as follows, to allow us to estimate the in-vivo elasticity coefficients for several different metabolic states. (i) The light response was divided into two sections (150 → 302 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 302 → 1050 $\mu mol \cdot m^{-2} \cdot s^{-1}$), corresponding to the ranges where CO_2-fixation is light-limited, or relatively insensitive to light, respectively. Table 4 summarises the fractional changes of metabolites and fluxes over each of these irradiance ranges for leaf material harvested at the end of the dark period, and for leaf material harvested at the end of the photoperiod. This provides empirical values for a first set of equations. They describe the response to perturbation of the irradiance in four different conditions, i.e. limiting irradiance without feedback, limiting irradiance with feedback, high irradiance without feedback, and high irradiance with feedback. (ii) The fractional changes of fluxes and metabolites between discs harvested at the end of the photoperiod and the end of the night (see Table 1) were averaged over the ranges 75 → 302 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and 302 → 1050 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This provides empirical values for a second set of equations. They describe the response of metabolism to feedback inhibition in two different conditions, i.e. in limiting irradiance and in high irradiance. By combining suitable equations from set (i) and set (ii), it is then possible to estimate elasticity coefficients for the Fru1,6Pase and SPS in four different conditions, i.e. limiting irradiance without feedback control, limiting irradiance with feedback control, high irradiance without feedback control, and high irradiance with feedback control. The estimated elasticity coefficients are summarised in Table 4.

The following picture emerges for SPS. In low irradiance the elasticity for hexose-P is already greater than unity. This reflects the sigmoidal response of SPS to increasing Fru6P (substrate) and Glc6P (activator) (Doehlert and Huber 1984; Stitt et al. 1988). These results indicate that the 'coarse' control of SPS is operating to maintain the hexose-P in a concentration range where SPS activity responds quite sensitively to changes of substrate availability, even in low irradiance when fluxes are low. In the high-irradiance range, the estimated elasticity coefficients for hexose-P are much higher. This could reflect a very sensitive response to a combination of rising hexose-P and falling Pi. However, the elasticity coefficients may be overestimated in these conditions. As discussed above, the inactive form of SPS may start to show some activity in vivo in conditions of very high hexose-P and low Pi. In this case, our simplified equation would lead to an overestimate of $\varepsilon^S_{hexose\text{-}P}$ in these conditions. The notational elasticity for sucrose is always negligible (see also Stitt 1989), which is fully consistent with the absence of any direct effect of sucrose on spinach leaf SPS in vitro (Stitt et al. 1987b).

For Fru1,6Pase, the following picture emerges. In low irradiance, the enzyme has a relatively low elasticity for triose-P and a relatively large (negative because it is an inhibitor) elasticity for Fru2,6bisP. This is the pattern expected if the Fru1,6Pase is operating in vivo with

Table 5. Calculated elasticity coefficients of Fru1,6Pase and SPS during photosynthesis at different irradiances, and in the presence and absence of feedback regulation. The elasticity coefficients are calculated using the equations given in the text and the fractional changes of metabolites summarised in Tables 2 and 4. The averaged values of α are calculated from Table 3

Irradiance range	Low irradiance		High irradiance	
Pretreatment of leaves	15 h dark	9 h light	15 h dark	9 h light
Feedback regulation	−	+	−	+
$\varepsilon^{Fru1,6Pase}_{triose\text{-}P}$	+0.18	+0.23	+0.68	+1.4
$\varepsilon^{Fru1,6Pase}_{Fru2,6bisP}$	−0.61	−0.62	−0.27	−0.36
α	(4.7)[a]		(12,1)[b]	
$\varepsilon^{Fru1,6Pase}_{Fru2,6bisP} \cdot \alpha$	(−2.86)	(−2.9)	(−3.26)	(−4.36)
$\varepsilon^{SPS}_{Hexose\text{-}P}$	+1.4	+2.5	+5.9	+7.2
$\varepsilon^{SPS}_{sucrose}$	+0.03	–	−0.03	–

[a] Averaged for the irradiances 75, 150 and 302 $\mu mol \cdot m^{-2} \cdot s^{-1}$
[b] Averaged for the irradiances 302, 576 and 1050 $\mu mol \cdot m^{-2} \cdot s^{-1}$

strongly limiting substrate concentration. It is fully consistent with the observations (i) that flux in these conditions is much lower than the V_{max} of Fru1,6Pase, and (ii) that the substrate concentration is low and the inhibitor (Fru2,6bisP) is high. In high irradiance a different picture emerges. The elasticity for triose-P is higher, and the elasticity for Fru2,6bisP is lower. This is the pattern expected if Fru1,6Pase is operating with substrate concentrations which are closer to saturating. These will decrease $\varepsilon^{F}_{Fru2,6bisP}$, because Fru2,6bisP is a competitive inhibitor (Herzog et al. 1984). The calculated elasticity coefficients in high irradiance resemble those estimated for *Clarkia xantiana* in moderate to high irradiance (Neuhaus et al. 1989). They are fully consistent with the observations that (i) the rate of sucrose synthesis is now about half of the effective V_{max} activity of the Fru1,6Pase (Stitt and Heldt 1985), and (ii) that triose-P have increased and Fru2,6bisP has decreased, compared to low irradiance.

The estimates of $\varepsilon^{s}_{hexose\text{-}P}$, $\varepsilon^{F}_{Fru2,6bisP}$ and α in Table 5 can then be used to solve Eq. (9), and estimate the *relative* flux-control coefficients for the blocks of reactions around Fru1,6Pase and around SPS. These describe the contribution of these enzymes to controlling flux from triose-P to sucrose.

In low irradiances, the block of reactions around Fru1,6Pase has a relative flux control coefficient of 0.52–0.68, and the block of reactions around SPS has a relative flux control coefficient of 0.32–0.48. Thus, control is distributed between the two enzymes. This is similar to the estimate of 0.52 for the flux-control coefficient of the Fru1,6Pase, obtained by applying the connectivity theorem to the distribution of control between Fru-1,6Pase and phosphoglucose isomerase in reduced-activity mutants of *Clarkia xantiana*, where the flux-control coefficient of the latter enzyme could be directly determined (Neuhaus et al. 1989). The previous discussion has emphasised how flux at the Fru1,6Pase will be primarily regulated by changes of Fru2,6bisP in these conditions, and it has earlier been shown that the Fru2,6bisP concentration itself is regulated via changes in the level of C-3 metabolites (especially PGA) and Pi (Stitt et al. 1987a; Neuhaus and Stitt 1989), and by changes of Fru6P (Stitt et al. 1987a, Neuhaus et al. 1989). This control pattern will ensure (i) that adequate amounts of triose-P are retained in the chloroplast for Calvin-cycle turnover and (ii) that a decreased demand for sucrose leads to an alteration of partitioning.

In high irradiance a quite different picture emerges. Owing to the uncertainties in our estimate of $\varepsilon^{s}_{hexose\text{-}P}$, we have not tried to estimate the precise distribution of control in these conditions. The values in Table 5 indicate that control is shared, but may underestimate the contribution of SPS. However, it is also obvious that Fru1,6Pase and SPS both become far more responsive to changes in the levels of their substrates in high irradiance. This means that an excess of photosynthate will be converted to sucrose anyway, even when feedback is operating (i.e. even though SPS is partially deactivated and Fru2,6bisP is increased). In these conditions, the triose-P concentration will be one of the dominant factors controlling the rate at which they are converted to sucrose, and will effectively override feedback regulation of SPS and the Fru1,6Pase.

In conclusion, these results confirm and extend the conclusions of Neuhaus et al. (1989). By using the dual-modulation approach, we have shown that control is normally shared between the Fru1,6Pase and SPS. Further, we have shown that the effectiveness of the feedback-regulation loop varies, depending on the conditions. In conditions allowing low or moderate photosynthetic fluxes, deactivation of SPS and rising Fru2,6bisP provides a very effective way of altering the partitioning of photosynthate, without large changes of metabolites or any inhibition of photosynthesis. In conditions of high photosynthetic flux, sucrose synthesis responds very sensitively to increasing metabolite levels. This ensures that high rates of sucrose synthesis are maintained, and minimises or avoids any inhibition of photosynthesis.

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