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Sucrose and Starch Synthesis in Spinach Plants Grown under Long and Short Photosynthetic Periods

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ABSTRACT

The flow of carbon into sucrose and starch was investigated in fully expanded primary leaves of spinach using the long to short day transition and partial defoliation as tools to manipulate sucrose/starch synthesis. Transfer from 12 hour to 7 hour photosynthetic periods resulted in a 4-fold increase in the initial rate of starch synthesis, a 50% increase in the initial rate of sucrose synthesis, a 30% increase in leaf sucrose, and a 40% decrease in fructose, 2,6-biphosphate. In addition, sucrose synthesis rates in cells isolated from shortened daylength plants are 80% higher than in cells isolated from control plants. These results show that, in spinach, an increase in the rates of both sucrose and starch synthesis can occur under short day conditions. In contrast, when short day plants are partially defoliated, starch levels remain high, fructose 2,6-biphosphate levels remain low, but the level of leaf sucrose drops by 50%. Thus, when demand exceeds supply, starch synthesis has priority over filling of leaf sucrose pools in the short day plant.

Sucrose and starch biosynthetic pathways are major consumers of fixed CO_2 in the photosynthetic cell. The regulation of these pathways, therefore, should be tightly coupled to the requirement for photosynthate by the rest of the plant. Previous work demonstrated that transfer of plants from long to shortened photosynthetic periods resulted in an increased rate of starch synthesis (2–5, 13, 14, 16, 19) and that this increased rate is maintained in chloroplasts isolated from SD[1] plants (16). These results show that starch synthesis rates can increase under conditions where daily assimilate production is reduced (SD) and that cytoplasmic metabolite regulation is not the only mechanism available for adjusting the rates of synthesis. Thus, starch synthesis is tightly coupled to the diurnal demand for assimilate and the process is not simply the result of an 'excess' supply of assimilate.

The effect of SD conditions on the rate of sucrose synthesis is not as well defined. As sucrose and starch synthesis are often inversely related (13, 24), it would be reasonable to conclude that in the SD plant, sucrose synthesis rates should decline. Such a decline has, in fact, been observed in cells isolated from SD soybean leaves (13). Yet under SD conditions, in order to fulfill a constant sink requirement, sucrose (and starch) synthesis would have to increase.

If sufficient fixed carbon were available, simultaneous increases in the rates of sucrose and starch synthesis could occur in response to the shortened photosynthetic period. In SD plants, less carbon is directed towards the 'residue' fraction (cell walls, protein) (2, 3, 19) and photosynthetic rates eventually increase (2, 5, 16), suggesting that some flexibility in partitioning may exist.

If the LD to SD transition does result in simultaneous increases in the rate of sucrose and starch synthesis, then this environmental manipulation would be useful for studying the mechanisms responsible for regulating the partitioning of fixed carbon into either sucrose or starch. In this paper we report on experiments designed to determine whether sucrose synthesis rates can increase simultaneously with starch synthesis in SD plants and, if so, under what metabolic conditions such increases occur.

MATERIALS AND METHODS

Plant Material. Spinach *(Spinacia oleracea* L. cv America) plants were grown as previously described (16). For leaf metabolite analysis, leaf disks (1 cm^2) were punched from fully expanded primary leaves and rapidly frozen in liquid N_2. Each sample consisted of disks from a single leaf and there were from four to six samples per time point. Dark samples were taken at a photon flux density of less than 0.5 $\mu E\ m^{-2}\ s^{-1}$. In the defoliation experiments, all leaves greater than 2 cm in length were removed, leaving only one fully expanded primary leaf per plant.

Leaf Extraction and Metabolite Assays. Leaf disks were extracted by a modification of the method of Stitt *et al.* (22). Three disks from each leaf were ground in a glass/glass homogenizer with 0.75 ml extraction solution (50 mM Tris-HCl (pH 8.2), 5 mM EDTA, 65% (v/v) methanol, and 25% (v/v) chloroform). The extract was centrifuged and the pellet re-extracted twice with 0.2 ml extraction solution. Supernatants were combined, the phases separated with 0.3 ml H_2O, and the methanol in the aqueous phase evaporated under N_2 gas. Starch, glucose, sucrose, G6P, and Chl were analyzed as in Robinson (16).

F2,6-P_2 was assayed essentially as in Van Schaftingen *et al.* (27). The reaction mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.15 mM NADH, 3 mM EDTA, 2 mM DTT, 0.25 units of aldolase, 5 units of triosephosphate isomerase, 0.5 units of glycerolphosphate dehydrogenase, 0.02 units of pyrophosphate:fructose-6-phosphate phosphotransferase, 0.5 mM pyrophosphate, and 1 mM F6P. Appropriate aliquots (0.5–3 pmol F2,6-P_2) of the sample were added and the rate of reaction compared to a standard curve. The standard curve was prepared using an acidified (0.1 N HCl, 15 min) and then neutralized (0.1 N NaOH) aliquot of the sample to which known quantities of F2,6-P_2 were added. An equivalent amount of NaCl was also added to the unknown. All reactions were run at 25°C. F2,6-P_2 recoveries using these extraction and assay procedures averaged 70%.

[1] Abbreviations: SD, short photosynthetic day (7 h); LD, long photosynthetic day (12 h); G6P, glucose 6-phosphate; F2,6-P_2, fructose 2,6-bisphosphate; G6P, fructose 6-phosphate; FBPase, fructose 1.6-bisphosphatase; SPS, sucrose phosphate synthase; F6P,2K, fructose 6-phosphate, 2-kinase; DAP, days after planting.

Leaf Photosynthesis. Photosynthetic rates of attached, fully expanded, primary leaves were measured in the chamber as previously described (16).

Cell Isolation, Labeling, and Analysis. Cells were isolated from individual leaves according to the following procedure. After 5 h in the light, a single fully expanded (1.0–1.5 g) primary leaf was chopped under isolation media (0.5 M sorbitol, 25 mM Mes-KOH [pH 5.7], 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM K_2SO_4, 2 mM DTT, 0.1% [w/v] BSA, and 0.2% [w/v] pectolyase Y-23) (Seishin Pharmaceuticals)[2], vacuum infiltrated, and incubated with gentle rocking for 2 h. The cell suspension was filtered through an 80 μm nylon mesh and spun at 100*g* for 15 s. The pellet was resuspended in assay media (0.5 M sorbitol, 50 mM Hepes-KOH [pH 7.8], 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM K_2SO_4), spun, resuspended, and spun again. The final preparations (six each for SD and LD plants) were resuspended in 0.3 ml assay media.

Cell suspensions were labeled in minivials in a shaking apparatus equipped with bottom illumination (300 μE m^{-2} s^{-1}, saturating with respect to photosynthesis) and water bath temperature control (25°C). [^{14}C]Bicarbonate (3.2 mCi/mmol) at a final concentration of 5 mM was added, the containers sealed, and the lights turned on. After 10, 20, and 30 min of illumination, 50 μl aliquots were removed by syringe and killed in 200 μl methanol containing 100 mM sucrose. Total time from leaf chopping to labeling was under 4 h.

Photosynthetic rates were measured as acid stable ^{14}C-incorporation; sucrose synthesis rates were calculated as follows. An aliquot (50 μl) of the methanol extract was passed through Amberlite MB3 mixed bed resin, dried down, and resuspended in 0.5 ml H_2O. The sugars in 50 μl of sample were separated via HPLC (21), the sucrose peak collected and then counted. Recovery of carrier sucrose averaged 80%.

RESULTS AND DISCUSSION

Development of the Short Day Response. Plant adjustment to the SD environment involves both short and long term adaptations. In the short term (1–4 d), foliar starch synthesis rates invariably increase (3, 4, 13, 14, 19), while sucrose levels show more variability, either increasing (19), decreasing (14), or showing no change (4). Photosynthetic rates are generally not affected at this stage (3, 14, 19). After several weeks at the shortened photoperiod, rates of starch synthesis remain high (2, 5, 16), while the amount of sucrose accumulated was found to increase in some studies (2, 16) but not in others (9). Photosynthetic rates, when expressed on a leaf area basis, are generally lower in the SD plants, but when expressed on a Chl or dry weight basis, rates are the same or higher (1, 2, 5, 16). This last observation is the result of the lower specific leaf weight of the SD plants (1, 2, 5, 16). SD plants, in addition, have thinner leaves with reduced mesophyll cell volume (1), show an increase in the shoot/root ratio (2, 9, 16), and a decrease in growth rates (2, 9, 16). Thus, the initial biochemical response to shortened days is clearly an increase in starch synthesis while the pool size of sucrose may or may not be affected. As adaptation continues, photosynthetic rates generally increase and the morphology of the plant changes (thinner leaves, increased shoot/root ratio) resulting in a better balance of source strength and sink requirements (16).

In order to use the LD to SD transition as a means to perturb sucrose/starch partitioning, short and long term response to the manipulation have to be clearly defined. To do so, plants were transferred from long (12 h) to shortened (7 h) photosynthetic periods for durations of from 1 to 8 d, and then leaf metabolite levels were measured. The results (Fig. 1) show that starch levels are elevated by the 1st d after transfer while several days are required before a major change in sucrose level occurs. Table I shows that, on the 7th SD, photosynthetic rates per unit leaf area are still unchanged although on a Chl basis they are slightly higher. However, a previous study (16) showed that by 15 d the SD rates on a leaf area basis will have dropped considerably. Therefore, the interval from 4 to 10 d after transfer was chosen to maximize the photoperiod effect on sucrose/starch partitioning but minimize the longer term effects on leaf morphology.

Metabolite Changes during the Photoperiod. Metabolite analysis of LD and SD plant leaves (Fig. 2) shows that, as with previous studies (2, 5, 9, 16), SD plants have a greater (0–5 h) rate of starch synthesis (12 μmol glu/mg Chl·h) than do LD plants (2.5 μmol glu/mg Chl·h). In addition, SD plants do not show a lag period in starch synthesis. Sucrose, glucose, and G6P levels are higher during the latter part of the day; however, F2,6-P_2 levels are lower (Fig. 2). When expressed on a leaf area basis, F2,6-P_2 levels are significantly (95%) lower in the SD plants over the entire day (data not shown).

Effect of Short Days on Sucrose Synthesis. Although sucrose levels are significantly higher in SD spinach (Figs. 1, 2), pool size measurements alone are not an adequate measure of synthesis rates as they reflect the balance between synthesis and export. To determine the effect of shortened photoperiods on the rate of sucrose synthesis, three approaches were used. The first is based on the observation that during the first few minutes of photosynthesis, export from the leaf is negligible (7), hence changes in the amount of leaf sucrose can be used to determine initial synthesis rates. This approach has been used to calculate rates of sucrose synthesis (from Fig. 2) during the first 15 min of photosynthesis with SD plants showing an increased rate (5.7 μmol sucrose/mg Chl·h) compared to LD plants (3.7 μmol sucrose/mg Chl·h). It

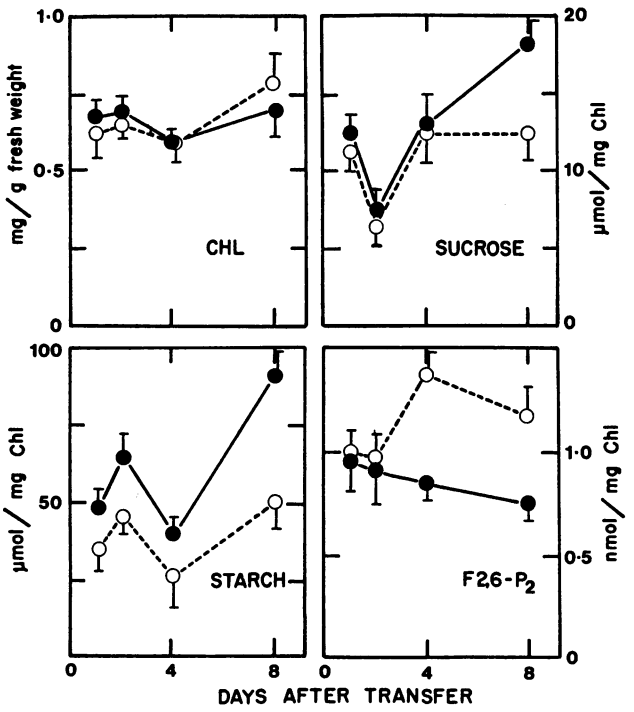

FIG. 1. Development of the short day response. Content of Chl, sucrose, starch, and F2,6-P_2 in leaves of long (○) and short (●) day plants as a function of number of days after transfer to the shortened photosynthetic period. Leaf disks were taken after 6 h of light. Values represent means ± SE of 4 plants. For clarity, only one side of the error bars is shown.

[2] Mention of a trademark, proprietary product, or vendor does not constitute guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Table I. *Net Photosynthetic Rates of Fully Expanded Primary Leaves*

The experiment was conducted at 22 DAP and SD plants had been in the shortened photosynthetic period for 7 d. Rates were measured between 5 and 7 h into the photosynthetic period. Values are means ± SE of 7 plants per treatment.

Treatment	Chl	Photosynthetic Rate	
	mg/dm^2	$\mu mol\ CO_2/dm^2 \cdot h$	$\mu mol\ CO_2/mg\ Chl \cdot h$
Long days	4.14 ± 0.49	519 ± 58	126 ± 13
Short days	3.34 ± 0.47	496 ± 47	150 ± 14

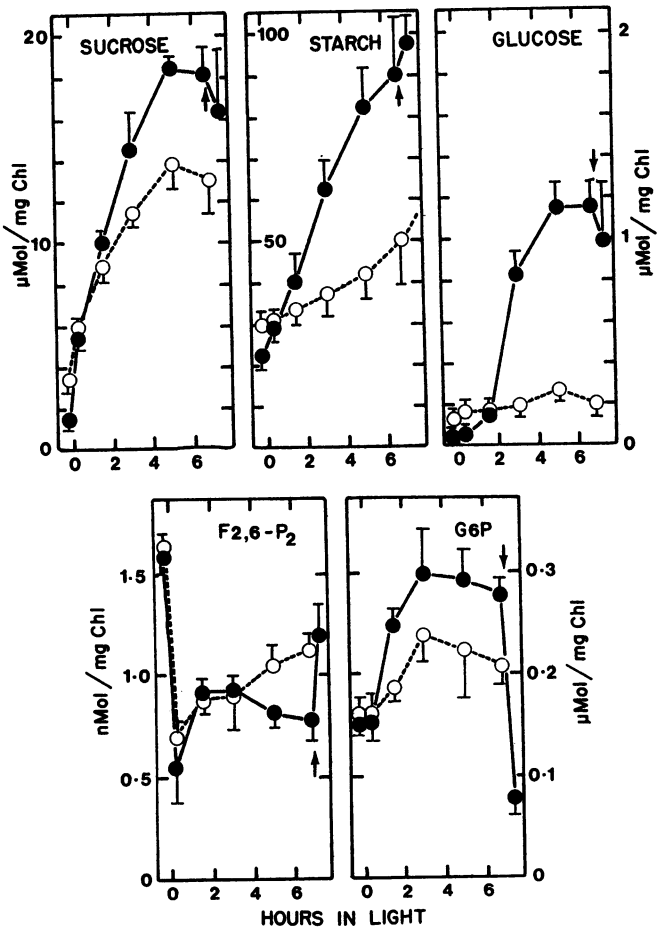

FIG. 2. Leaf metabolite transients during the day. Content of sucrose, starch, glucose, F2,6-P_2, and G6P in fully expanded primary leaves of short (●) and long (○) day plants. Values for starch are in µmol glucose equivalents/mg Chl. The experiment was conducted at 23 DAP and SD plants were in the shortened photosynthetic period for 8 d. In the SD treatment, the lights were turned off at 7 h (arrows). Values represent means ± SE of 4 plants per time point. For clarity, only one side of the error bars is shown.

should be emphasized, though, that this approach measures initial synthesis rates and extrapolation to later time points may not be justified.

In the second approach, the rates of sucrose synthesis in isolated mesophyll cells were measured. As shown in Table II, the rate of sucrose synthesis in cells isolated from SD plants (0.42 µmol sucrose/mg Chl·h) is about double the rate found in LD cells (0.23 µmol sucrose/mg Chl·h). Although these cells synthesize proportionally less sucrose than do intact leaves (10% *versus* 50% of net photosynthesis), we believe that the difference in sucrose synthesis rates between the LD and SD cells may reflect real differences between treatments.

The third approach relies on the postulated role of the regulatory metabolite F2,6-P_2 in controlling sucrose synthesis (12, 15, 22–24). This compound is a potent inhibitor of cytoplasmic FBPase (6, 11, 25), a key enzyme in sucrose biosynthesis, so a change in the level of F2,6-P_2 should tend to produce a reciprocal change in carbon flow through FBPase. Experimentally this has been shown to be the case. In spinach leaves for example, F2,6-P_2 levels are lower under conditions where sucrose synthesis rates are enhanced (24). In the present study, F2,6-P_2 levels in SD plants are reduced to about 60% of the level in LD plants (Figs 1, 2).

Collectively, these data (initial sucrose synthesis rates, Fig. 2; isolated cell sucrose synthesis rates, Table II; and F2,6-P_2 levels, Figs. 1, 2) suggest that the rate of sucrose synthesis is enhanced in spinach plants grown under SD. In contrast, Huber *et al.* observed a reduction in sucrose synthesis rates and SPS activity in isolated soybean mesophyll cells (13) and a decrease in soybean leaf sucrose levels (14) and SPS activity(13, 14) after 4 d at the shortened photoperiod. These differences are discussed in a later section.

Regulation of Sucrose Synthesis in Short Day Plants. Clearly some type of adaptation can occur in the regulatory mechanism(s) controlling sucrose synthesis since rates of sucrose synthesis were higher in cells isolated from SD plants (Table II). Although the adaptation observed in cells could be explained by assuming that the levels of metabolite effectors of the sucrose biosynthetic enzymes found in leaves are also maintained in isolated cells, the leaf metabolite data itself suggests that an additional regulatory mechanism may be operating in SD plants. Over much of the day, SD leaves have more sucrose, glucose, and G6P than the control leaves (Fig. 2). Higher levels of these compounds are often found under conditions where sucrose synthesis is likely to be reduced (22–24) and given the sensitivity of sucrose pathway enzymes to these as well as other pathway metabolites, feedback mechanisms controlling sucrose synthesis are clearly possible (for reviews, see Greiger [8], Herold [10], and Preiss [15]). Yet in SD leaves sucrose synthesis rates are enhanced, rather than reduced, in the presence of high levels of sucrose pathway metabolites. Similar results were reported by Rufty *et al.* (17, 18) who showed that, in defoliated soybean, SPS activity is not always inversely related to sucrose levels. They also concluded that factors other than feedback inhibition may regulate sucrose synthesis.

One possible explanation for the maintenance of high rates of sucrose synthesis in the presence of high sucrose, glucose and G6P levels lies in the observation that the level of F2,6-P_2 is reduced over much of the day (Fig. 2). As the level of this regulatory metabolite is within the range shown by Herzog *et al.* (11) to be effective in controlling FBPase, a reduction in F2,6-P_2 should tend to increase flow through sucrose biosynthesis (12, 22–24). Thus, given low F2,6-P_2 levels, carbon flow through FBPase could be maintained or enhanced in the presence of elevated levels of sucrose pathway metabolites.

The mechanism involved in lowering the level of F2,6-P_2 in SD plants is not entirely clear as F2,6-P_2 levels are generally enhanced in leaves with elevated levels of sucrose or G6P (22–

Table II. *Net Photosynthetic Rates, Sucrose Synthesis Rates, and Chl Content of Cells Isolated from Plants Grown under Short and Long Day Conditions*

SD plants had been in shortened photosynthetic periods for 10 d. Values are the means ± SE of 6 samples.

Treatments	Total Chl	Photosynthetic Rate	Sucrose Synthesis Rate
	μg	*μmol CO_2/ mg Chl·h*	*μmol sucrose/ mg Chl·h*
Long days	5.3 ± 2.1	35.2 ± 13.6	0.23 ± 0.11
Short days	5.9 ± 1.9	49.8 ± 12.6	0.42 ± 0.08

24). Our working hypothesis is that the decrease in F2,6-P_2 seen in SD plants is the result of a post-transitional modification of the enzyme synthesizing F2,6-P_2, F6P,2K, resulting in a change in the affinity of the enzyme towards its substrates or effectors. Although other explanations for our data are possible, it should be noted that spinach leaf F6P,2K can be phosphorylated *in vitro* and that phosphorylation results in a change in enzyme activity (C. Baysdorfer and J. M. Robinson, manuscript in preparation). SPS as well shows kinetic changes that may be the result of post-translational modification (20).

Sucrose/Starch Partitioning. Starch synthesis was originally thought to represent a means whereby fixed carbon in excess of current demand could be stored for future use. Although an inverse relationship is often found between rates of sucrose and starch synthesis (13, 24), environmental manipulations such as the LD to SD transition show that increases in starch synthesis also can occur at a time when the demand for sucrose should be increased (2–5, 13, 14, 16, 19). In addition, when plastids are isolated from SD leaves they retain the high rates of starch synthesis found in the intact leaves, implying that factors other than cytoplasmic metabolite levels also regulate starch synthesis (16). Thus, starch synthesis is coupled to the diurnal demand for assimilate and is not simply a means to store excess carbohydrate (2).

In this paper, we have shown that sucrose synthesis and starch synthesis can increase simultaneously in SD spinach plants. However, in the reports of Huber *et al* (13, 14), sucrose synthesis in soybean cells, SPS activity in cells and leaves, and leaf sucrose levels were all reduced under SD conditons while starch synthesis was increased. In the soybean studies of Huber *et al.* (13, 14), therefore, an inverse relationship holds, increased starch synthesis is accompanied by a decrease in sucrose synthesis. These findings raise the possibility that, if supplies of fixed carbon are limited, starch synthesis has priority over sucrose synthesis in the SD plants. If this were the case, then increasing the demand for assimilate beyond the capacity of the spinach leaf to supply it should result in preferential flow towards starch.

To answer this question, we used a combination of SD conditions and partial defoliation to create an excessive sink demand on the leaves, thus requiring a metabolic decision on which pathway to maintain. The data (Fig. 3) shows that, if SD conditions (4 d) alone are imposed, the expected increase in starch levels and decrease in F2,6-P_2 are observed. At this early stage of adaptation, sucrose and G6P levels are not significantly different between treatments.

When LD plants are defoliated (4 d), starch, sucrose, and G6P levels in the remaining leaf are unchanged; however, F2,6-P_2 levels decline (Fig. 3). Work in other laboratories has shown that sucrose levels increase and starch levels decrease upon defoliation or shading (17, 26) and it has been suggested (17) that, in soybean, defoliation induces an increase in sucrose synthesis at the expense of starch synthesis. In LD plant leaves, starch levels (6 h light) are maintained in the defoliated plants suggesting that starch synthesis is not reduced while the drop in F2,6-P_2 levels suggests

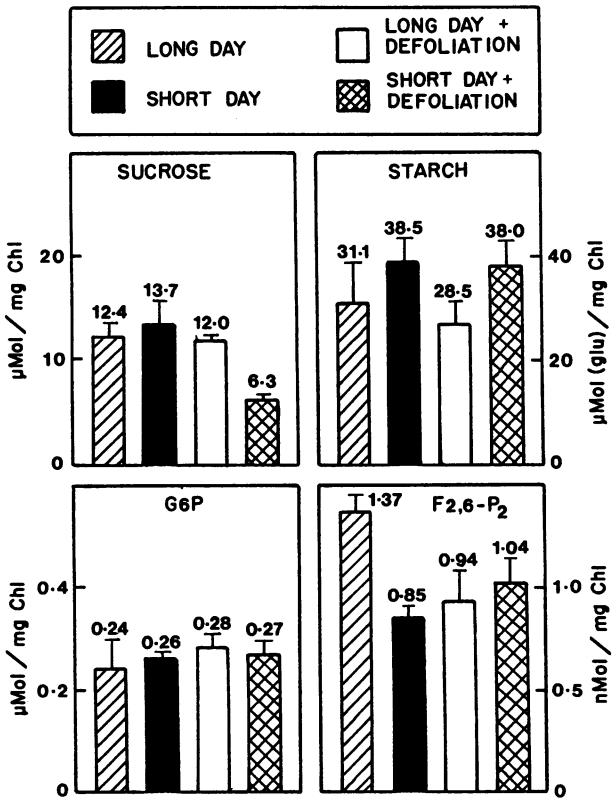

FIG. 3. Effect of SD and defoliation on leaf metabolite levels. Fully expanded primry leaves were sampled at 24 DAP. Disks were taken at 6 h into the photosynthetic period on the 4th d following transfer to SD conditions and/or partial defoliation. Values represent means ± SE of 4 plants per treatment.

that the rate of sucrose synthesis is enhanced in defoliated spinach. As with the long to short day transition, therefore, defoliation alone does not overtax the ability of the plant to increase carbon flow to one pathway while maintaining flow to the other.

In contrast, when plants are subjects to both SD conditions (4 d) and defoliation (4 d), a dramatic decrease in the level of leaf sucrose occurs. Starch levels, however, are maintained at the high level found in the SD plants and F2,6-P_2 levels are similar to the SD or defoliated treatments. These results show that when increased requirements for sucrose storage or export (SD, defoliation) and for starch storage (SD) exceed the supply capacity of the leaf, flow to starch is maintained while filling of the leaf sucrose pool is reduced. Thus, in SD conditions, when demand for both sucrose and starch is increased, carbon flow to both pathways will increase as long as supply is capable of meeting demand.

Redirection of Assimilate Flow in SD Plants. Increased flow

of carbon to leaf sucrose or starch could occur as a result of a decrease in flow to other cellular components, a decrease in export, or an increase in photosynthetic rate or by a combination of the above. In the present study, photosynthesis was increased by 20% in the SD plants, from 125 to 150 μmol CO_2/mg Chl·h (Table I). If daily (0–7 h) carbon flow to leaf starch and sucrose (excluding export) is calculated from Figure 2, fully expanded LD leaves invest 35 μmol CO_2/mg Chl·h (28%) in these compounds, while SD leaves invest 100 μmol CO_2/mg Chl·h (65%). Clearly the increasd flow of carbon to leaf sucrose and starch cannot come entirely from increased photosynthesis. A reduction in the rate of ^{14}C export from leaves was shown for maize, wheat, and pangola grass grown under SD conditions (19) and the decline in stem and root sucrose levels in SD spinach (16) suggests a similar reduction may occur in spinach as well. However, the magnitude of the increase in starch and sucrose synthesis rates suggests that, as was shown for other species (2, 3, 19), a reduction in carbon flow to other components (*i.e.* protein, cell walls, leaf thickness) may be a major source of the additional carbon in the SD plant.

CONCLUSIONS

Transfer from long to short photosynthetic days redirects the flow of carbon in the leaf towards a greater accumulation of storage carbohydrate. An increase in starch synthesis accounts for most of the stored carbon and has priority over increases in leaf sucrose. However, in spinach, sufficient fixed carbon is available to permit simultaneous increases in the rates of both sucrose and starch synthesis. In this species, therefore, sucrose and starch synthesis rates are not necessarily reciprocally related.

The increase in sucrose synthesis in the SD plants, which is accompanied by a drop in F2,6-P_2 levels, occurs in the presence of high levels of sucrose pathway metabolites. In part, this reflects the importance of F2,6-P_2 regulation at the level of FBPase. These data also show that an additional mechanism, other than metabolite regulation, is involved in the control of F2,6-P_2 levels and, thus, the flow of carbon into sucrose synthesis.

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