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Short Communication

Evidence for Control of Carbon Partitioning by Fructose 2,6-Bisphosphate in Spinach Leaves[1]

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STEVEN C. HUBER* AND D. MARK BICKETT
United States Department of Agriculture, Agricultural Research Service, Departments of Botany and Crop Science, North Carolina State University, Raleigh, North Carolina 27650

ABSTRACT

Excision of spinach (*Spinacia oleracea* L.) leaves had no effect on photosynthetic rates, but altered normal carbon partitioning to favor increased formation of starch and decreased formation of sucrose. The changes were evident within 2 hours after excision. Concurrently, leaf fructose-2,6-bisphosphate content increased about 5-fold (from 0.1 to 0.5 nanomoles per gram fresh weight). The activities of sucrose-P synthase and cytoplasmic fructose 1,6-bisphosphatase in leaf extracts remained constant during the time period tested. It is postulated that the rise in fructose 2,6-bisphosphate was responsible for the change in carbon partitioning.

Partitioning of photosynthetically fixed carbon between starch and sucrose can be altered by 'source-sink' manipulations (5). When 'sink demand' for sucrose was decreased by girdling (9), fruit removal (1), or leaf excision (8), photosynthetic rates were ultimately reduced while starch accumulation either increased or was similar to that in control plants. Hence, relative partitioning of carbon into starch was increased.

In short-term leaf excision experiments for soybean plants (8), the decreased rate of sucrose accumulation was attributed to a reduction in the maximum activity of SPS[2], the enzyme responsible for photosynthetic sucrose formation. Within 2.5 h after leaf excision, SPS activity was reduced to only 26% of the activity at the time of detachment (8).

In preliminary experiments with detached spinach leaves, changes in carbon partitioning were observed, but the maximum activity of SPS was not decreased. This is documented in the present report and, in addition, we show that the change in partitioning in spinach leaves was associated with a dramatic increase in F26BP content. F26BP is a potent inhibitor of cytoplasmic FBPase (2, 11), and the results obtained are consistent with a regulatory role for this metabolite in the sucrose formation pathway.

MATERIALS AND METHODS

Materials. Fresh beef liver for preparation of phosphofructokinase was obtained locally. All other reagents and chemicals were obtained from Sigma.

Plant Growth and Experimental Protocol. Spinach (*Spinacia oleracea* L.) plants were grown in the greenhouse during the months of April and May. Fully expanded leaves were excised at 0900 h on clear, sunny days. The petioles were recut while submerged in degassed distilled H_2O, placed in tubes of the same water, and positioned into full sunlight. Several leaves were harvested just after excision and at various times over the 4.5-h experimental period. Prior to each harvest, leaf CER was measured. After the CER measurement, four leaf punches (2.0 cm^2 total) were taken for starch and soluble sugar analysis. The midrib and petiole were removed from the leaf and the remaining tissue weighed and sliced. A subsample (0.5 g) of tissue was taken for F26BP determinations, and the rest was frozen at −80°C for SPS and FBPase measurements.

Photosynthesis Measurements. CER was measured using an Anarad[3] differential IR CO_2 analyzer equipped with a clamp-on plexiglass cuvette that enclosed 5 cm^2 of the upper and lower leaf surfaces. Ambient air was passed through the cuvette at a flow rate of 1.5 l/min, and the CO_2 differential of incoming and outgoing air was used to determine the CER.

Starch and Sugar Analysis. Leaf discs were extracted with hot 80% ethanol until the tissue was pigment free. The particulate fraction was analyzed for starch after digestion with amyloglucosidase (8). The supernatants were evaporated to dryness. The residues were resuspended in water and assayed for hexose and sucrose (8).

Leaf F26BP Determination. Immediately after harvest, 0.5 g of tissue was ground in a mortar with 2 ml of ice cold buffer containing 50 mM Hepes-NaOH (pH 7.5), 10 mM KF, 2.5 mM DTT, and 2 mM EDTA. The extract was placed in a boiling water bath for 2 min and then centrifuged at 32,000*g* for 5 min. The supernatant was used for F26BP analysis. Using these procedures, recovery of authentic F26BP added to the grind medium before homogenization of leaf tissue averaged 70%.

The determination of F26BP content was based on the ability of the extract to relieve the inhibition of phosphofructokinase by ATP. Phosphofructokinase was partially purified from fresh beef liver, and the F26BP assay was performed essentially as described by Furuya and Uyeda (4). The assay mixture contained 50 mM

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[2] Abbreviations: SPS, sucrose-P synthase; F26BP, fructose 2,6-bisphosphate; FBPase, fructose 1,6-bisphosphatase; CER, carbon exchange rate (mg CO_2/$dm^2 \cdot h$); FBP, fructose 1,6-bisphosphate.

[3] Mention of a trademark or proprietary product does not constitute a 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 that may also be suitable.

Hepes (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM NH_4Cl_2, 0.16 mM NADH, 2.5 mM DTT, 0.5 units aldolase, 5.8 units triose-P-isomerase, 0.5 units α-glycerol-P-dehydrogenase, 20 munits of phosphofructokinase, and 20 μl of spinach extract. After 2 min, the reaction was initiated by addition of 1.2 mM ATP and monitored by following the decrease in absorbance at A_{340}. All rate measurements were taken 4 min after initiation, and F26BP concentration was determined with respect to a standard curve. Acid treatment of samples (4) completely eliminated the apparent F26BP activity.

SPS and FBPase Assays. Frozen tissue was ground (1 g fresh wt/8 ml medium) with a mortar and pestle in 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.5% BSA. The extracts were filtered through eight layers of cheese cloth and clarified by centrifugation at 32,000*g* for 5 min. SPS was assayed by measuring fructose-6-P-dependent formation of sucrose (+ sucrose-P) from UDP-glucose as previously described (7). Cytoplasmic FBPase was assayed by a continuous spectrophotometric assay. The 1.0-ml reaction mixture contained 50 mM Hepes-NaOH (pH 7.0), 5 mM $MgCL_2$, 10 μM FBP, 0.2 mM NADP, 2 units each of phosphoglucoisomerase and glucose-6-P dehydrogenase, and an aliquot of leaf extract. Under these assay conditions, activity of chloroplast FBPase is neglible and there was virtually no activity in the absence of Mg^{2+} (*i.e.* nonspecific phosphatase).

RESULTS

Detached spinach leaves continued to photosynthesize at rates similar to those of attached (control) leaves during the 4-h test period. CER was constant at about 9 mg CO_2/g fresh wt·h. In the attached leaves, there was a gradual increase with time in leaf sucrose concentration (Fig. 1A). Excised leaves, in contrast, accumulated sucrose at an accelerated rate for the first 2 h. Thereafter, sucrose continued to accumulate, but at a reduced rate (Fig. 1A), which corresponded with a marked increase in the rate of starch accumulation (Fig. 1B). There was no accumulation of carbohydrates in the midrib or petioles of detached leaves (data not shown). Hence, translocation was effectively blocked by leaf excision. The results clearly indicated that, after 2 h, carbon partitioning was altered to favor increased formation of starch at the expense of sucrose formation.

The decrease in sucrose formation was not the result of changes in the maximum activities of SPS or cytoplasmic FBPase, as attached and detached leaves had similar enzyme activities in leaf extracts (Table I). However, leaf excision resulted in a dramatic increase in leaf F26BP content (Fig 2). Attached leaves maintained a relatively low and constant level of F26BP (about 0.15 nmol/g fresh weight). In detached leaves, the F26BP content increased rapidly so that, after 2 h, leaves contained about 0.5 nmol F26BP/g fresh weight.

DISCUSSION

Excision of spinach leaves resulted in an initial accumulation of sucrose that preceded the change in carbon partitioning. Similarly, Foyer *et al.* (3) reported that sucrose pretreatment of protoplasts from mature spinach leaves inhibited subsequent sucrose formation. However, with the protoplast system, the sucrose pretreatment also inhibited CO_2 fixation whereas, in the present study with intact leaves, the change in carbon partitioning occurred while CER remained constant.

It is unlikely that in excised leaves the reduction in sucrose accumulation after 2 h was caused by direct feedback inhibition by sucrose of enzymes in the sucrose pathway (3, 7). Similarly, the effect cannot be ascribed to reduced activities (measured *in vitro*) of SPS or cytoplasmic FBPase (Table I), as was the case with detached soybean leaves (8). It is likely that the increase in F26BP concentration (Fig. 2) was responsible for the reduction in sucrose formation. F26BP is a potent inhibitor of cytoplasmic FBPase (2, 11). Using conversion factors developed by Stitt *et al.* (12), and assuming strict compartmentation, the concentration of F26BP in the cytoplasm of detached leaves increased from about 1 μM to a maximum level of about 5 μM. This change in F26BP concentration would likely cause a substantial inhibition of FBPase activity (2).

It is intriguing to note that apparent species differences may exist in the mechanisms employed to reduce the rate of sucrose formation in response to a block in export. The results presented

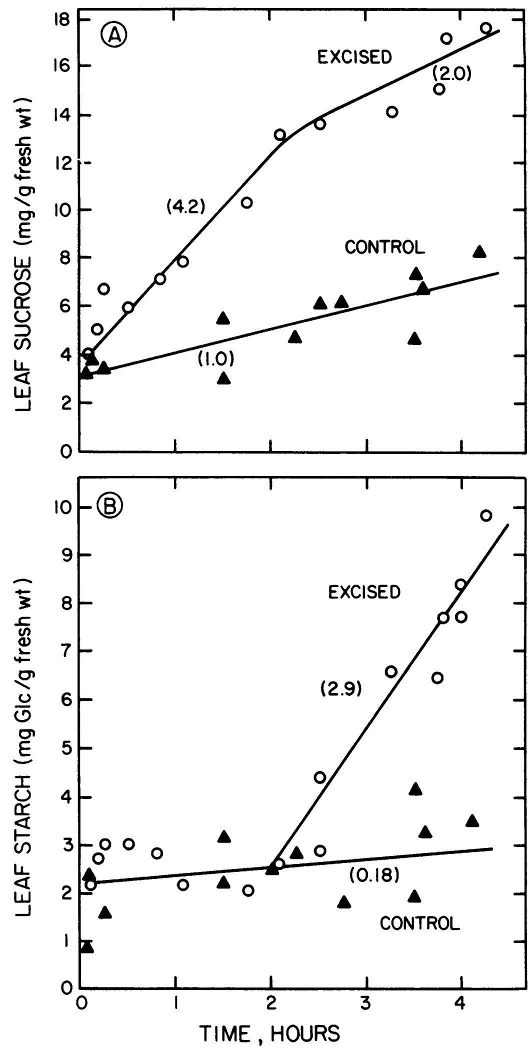

FIG. 1. Changes in (A) leaf sucrose content and (B) leaf starch accumulation of excised (O) and attached, control (▲) leaves of spinach plants. Rates of carbohydrate accumulation, expressed as mg/g fresh wt·h, are shown parenthetically.

Table I. *Effect of Excision on Activity of Certain Enzymes in Spinach Leaf Extracts*

Tissue	Elapsed Time[a]	Enzyme	
		SPS	cyto FBPase
	h	*µmol product/g fresh wt·h*	
Attached	0	16.1 ± 2.5	24.7 ± 1.6
Attached	4	13.8 ± 2.0	23.6 ± 1.5
Detached	4	14.7 ± 2.3	27.5 ± 3.4

[a] The experiment was started at time zero, which was about 0900 h, and lasted 4 h.

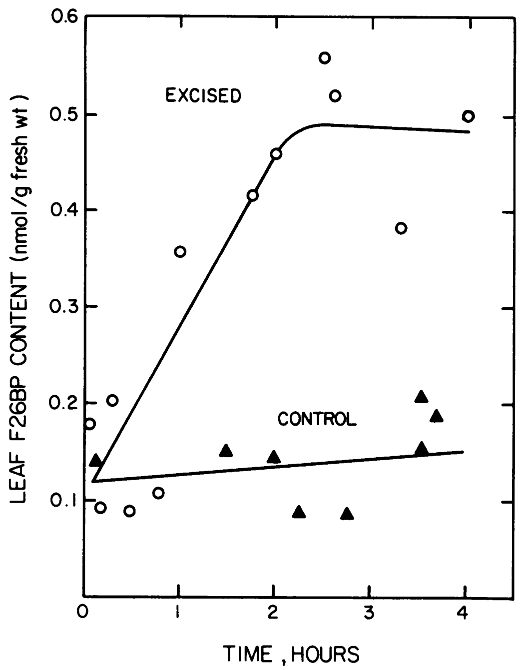

FIG. 2. Changes in F26BP content of excised (O) and attached, control (▲) leaves for spinach.

herein suggest that spinach plants modulate the level of the regulatory metabolite F26BP. In contrast, the maximum activity of SPS is regulated in soybean leaves (8). It is not known whether there are changes in the concentration of F26BP in soybean leaves under these conditions (we have encountered technical difficulties in extracting F26BP from soybean leaves).

F26BP plays an important role in regulating glycolysis and gluconeogenesis in the liver cell, and its concentration is hormonally controlled (6). It appears that F26BP may play a similar role in the leaf cell. To date, it is known that F26BP is present in leaves and that it is a potent inhibitor of cytoplasmic FBPase (2, 11). In addition, Stitt *et al.* (10) observed a slow accumulation of F26BP in attached spinach leaves during the course of the photoperiod that was correlated with accumulation of sucrose and increased starch formation. Floating leaf discs on solutions containing sucrose or glucose resulted in increased levels of F26BP (10), which suggested that the F26BP level may be regulated by the solbule carbohydrate pool. The results obtained in the present study show that F26BP increases in intact leaves in response to a decrease in export, which is consistent with a regulatory role for F26BP in carbon metabolism.

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