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Sucrose metabolic pathways in sweetgum and pecan seedlings

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Summary

Sucrose metabolism and glycolysis were studied in one- to two-year-old seedlings of sweetgum (*Liquidambar styraciflua* L.) and pecan (*Carya illinoinensis* (Wangenh.) C. Koch). The sucrose synthase pathway was identified as the dominant sucrose metabolic activity in sucrose sink tissues such as terminal buds and the root cambial zone. The sucrose synthase pathway was completely dependent on uridine diphosphate and pyrophosphate and it was activated by fructose 2,6-bisphosphate. Both acid and neutral invertases were less active than sucrose synthase in sucrose sink tissues. According to the magnitude of seasonal changes in activity, sucrose synthase, the pyrophosphate-dependent phosphofructokinase, and fructokinase were identified as adaptive enzymes, whereas neutral invertase, uridine diphosphate-glucopyrophosphorylase, phosphoglucomutase, and the nonspecific, nucleotide triphosphate-dependent phosphofructokinase were identified as maintenance enzymes. The periodically high activities of pyrophosphate-dependent phosphofructokinase indicate that pyrophosphate can serve as an energy source in trees. The observations support the hypothesis that sucrose glycolysis and gluconeogenesis in plants proceed by a network of alternative enzymes and substrates.

Introduction

Plant cells live and grow on sucrose. Sucrose (or one of its derivatives in the raffinose family, or both) is the most common sugar translocated in trees and sucrose often represents over 95% of the dry weight of translocated materials (Zimmermann and Brown 1971).

Sucrose metabolism in trees can be viewed simplistically in terms of the sink–source relationships developed for annual crop plants (Swanson 1959, Cronshaw et al. 1986). Thus a source is an active photosynthetic tissue and a sink is any rapidly growing or metabolizing tissue. Yet trees should be viewed differently because of their: (i) long life cycles; (ii) seasonal photosynthesis; (iii) periodic growth; and (iv) annual periodic, as well as long-term, accumulation and mobilization of reserves (Zimmermann and Brown 1971, Borchet 1973, Hanover 1980). Many of the metabolic activities of trees must therefore be episodic, and this provided, in part, our rationale for studying sucrose metabolism in trees.

Recently a number of new features of sucrose metabolism in plants have been discovered including: a new pathway for sucrose breakdown; new enzymes in glycolysis and gluconeogenesis; evidence of a pyrophosphate (PPi) pool in plants which can replace ATP with certain enzymes; the utilization of nucleotides other than

adenylates; the role of fructose 2,6-bisphosphate (Fru 2,6-P_2) as a regulator of hexose phosphate metabolism; and possibly, that sucrose is the starting point of glycolysis and the termination of gluconeogenesis in plants (Smyth and Black 1984*a*, Mustardy et al. 1986, Sung et al. 1986, Black et al. 1987, Sung et al. 1988).

The metabolism of sucrose has been the subject of many studies and its initial cleavage by either an acid or an alkaline invertase is now widely accepted. In contrast, the role of sucrose synthase has been much less certain because it has an equilibrium that theoretically allows both synthesis and breakdown. We know that plant cells must synthesize numerous polymers which are probably derived initially from UDP-glucose formed by sucrose synthase cleavage of sucrose (Turner and Turner 1980, Hawker 1985). Pyrophosphorolysis of UDP-glucose was not considered a feasible alternative until 1984 when a substrate level pool of PPi was measured in plants (Edwards et al. 1984, Smyth and Black 1984*b*). In 1986 Huber and Akazawa (1986) and Sung et al. (1986) proposed that a sucrose synthase pathway operates in plants. This pathway has several unique features including two internal cycles, one for forming UDP and the other for forming PPi. Overall the glycosidic bond energy of sucrose is conserved so that the pathway requires only one nucleotide triphosphate, whereas sucrose breakdown by invertase requires two nucleotide triphosphates for the production of two hexose phosphates. The UDP and PPi-dependent breakdown of sucrose was demonstrated in crop plants in 1986 by Xu et al.

Here our objective was to study sugar metabolism in sweetgum (*Liquidambar styraciflua* L.) and pecan (*Carya illinoinensis* (Wangenh.) C. Koch) seedlings with emphasis on sucrose metabolism as it relates to glycolysis. Specifically, we tested the following hypotheses: that trees contain three enzymes for metabolizing imported sucrose; that the sucrose synthase pathway (Huber and Akazawa 1986, Sung et al. 1986) operates in trees; that both adaptive- and maintenance-type enzymes (Mustardy et al. 1986, Black et al. 1987) function in glycolytic and gluconeogenic sugar metabolism; that PPi serves as an energy source in trees by replacing ATP in some enzyme-catalyzed reactions; that PPi can also drive the breakdown of sucrose; and that these biochemical activities exhibit seasonal changes in trees.

Materials and methods

Sweetgum (*Liquidambar styraciflua*) and pecan (*Carya illinoinensis*) seedlings were grown in nursery beds as described by Kormanik (1986). The procedures for seed collection from mother trees, nursery bed preparation, VAM fungi inoculation, assessing root infection, making spore counts, determining soil fertility, as well as details about planting times, and nursery cultural practice have been reported earlier (Kormanik 1986). The studies were conducted over the last four years during both the first and second years of seedling growth. Each study was repeated at least three times, the data presented are from a single experiment; similar results were obtained in experiments conducted in different years.

Tissue preparation

Terminal buds were freshly harvested, bud scales removed, and the meristems extracted. Usually eight to ten terminal buds were combined for each enzyme extraction. The root vascular cambium was obtained by peeling the bark from the main tap root and scraping off the inner cambium tissue with a razor. The tissue was quickly weighed and placed in liquid N_2. Usually tap roots of eight to ten seedlings were scraped to obtain each sample of vascular cambial tissue. To ensure comparability of results in spite of any diurnal variation in enzymatic activity, all tissues were harvested and extracted between 1000 and 1200 h.

Enzyme extracts

Plant tissues were frozen in liquid N_2 and powdered with a pestle and mortar. The frozen tissue powder was homogenized in extraction solution containing 200 mM Hepes/NaOH (pH 7.5), 3 mM Mg acetate, 5 mM dithiothreitol, 10% (v/v) glycerol, and 2% (w/v) insoluble polyvinyl pyrrolidone. The ratio of tissue fresh weight (g) to extraction solution (ml) was either 1/5 or 1/3. The homogenate was passed through one layer of Miracloth and centrifuged at 34 000 *g* for 20 min at 4 °C. The supernatant was fractionated with 30 to 70% $(NH_4)_2SO_4$. The 70% $(NH_4)_2SO_4$ pellet was resuspended in a solution of 10 mM Hepes/NaOH (pH 7.5) containing 2 mM DTT, and 2 mM Mg acetate and desalted on a Sephadex G-25 column. Recovery from the $(NH_4)_2SO_4$ fractionation step was between 85 and 100% for all enzyme activities.

Sucrose synthase was assayed by the multi-enzyme method described by Xu et al. (1986). The 1-ml reaction mixture contained 100 mM Hepes/NaOH (pH 7.5), 3 mM Mg acetate, 5 mM DTT, 0.02 mM glucose 1,6-P_2, and 0.5 mM NAD. Sucrose (50 mM), UDP (1 mM) and PPi (1 mM) were added to start the reactions. The glucose 1-P produced was coupled by phosphoglucomutase (2U) and *Leuconostoc* glucose 6-P dehydrogenase (2 U) to yield 6-phosphogluconate and NADH. The production of NADH was monitored spectrophotometrically at 340 nm with a Beckman DU-7. Because UDP-glucopyrophosphorylase activities were usually over 1 U mg^{-1} protein in the extracts, the limiting activity in this coupled reaction was sucrose synthase. If UDP-glucophyrophosphorylase activity was less than 1 U in an extract, 1 U of a commercial enzyme preparation was added.

Acid and neutral invertases were assayed at pH 5.0 and 7.0, respectively. Sucrose, 50 mM, was added to the incubation buffer (70 mM K_2HPO_4/40 mM citrate for acid invertase and 160 mM K_2HPO_4/20 mM citrate for neutral invertase) containing plant extracts to start the reaction. The standard incubation time was 15 min at 25 °C and reactions were stopped by boiling. Aliquots of incubation mixture were added to a hexose assay mixture consisting of: 100 mM Hepes/NaOH (pH 7.5), 3 mM Mg acetate, 5 mM DTT, 0.02 mM glucose 1,6-P_2, 0.5 mM NAD, with hexokinase (2 U) and *Leuconostoc* glucose 6-P dehydrogenase (2 U) as coupling enzymes. One mM of ATP was added to start the reaction and the reaction was completed when there was no more absorbance increase at 340 nm. All other enzyme assays have been described in detail earlier (Simcox et al. 1977, Wu et al. 1983, Smyth et al. 1984, Wu

et al. 1984, Xu et al. 1986). Nucleotide triphosphate-dependent phosphofructokinase was assayed with either 1 mM UTP or ATP (Smyth et al. 1984). Fructokinase was assayed with either 1 mM UTP or ATP (Turner and Copeland 1981). Protein was determined by the Bradford procedure with bovine serum albumin as the standard protein.

Results and discussion

Sucrose synthase, acid invertase and neutral invertase activities were detected in sucrose importing and exporting tissues and in quiescent winter root tissues of both pecan and sweetgum seedlings (Table 1). High activities of sucrose synthase and acid invertase were found in sucrose importing tissues, but these activities were lower in quiescent and sucrose exporting tissues. Neutral invertase activity was consistently low and showed little seasonal change.

Figure 1 shows that extracts from developing pecan buds, a sucrose sink tissue, cleaved sucrose in a process dependent on UDP and PPi. Omission of sucrose or UDP or PPi resulted in no reaction. Uridine diphosphate could not be replaced with UTP, ATP, ADP, GDP, CDP, TDP, or IDP nor could PPi be replaced with NTP, PPPi, or Pi. Similar results were obtained with extracts prepared from many other sucrose importing tissues of sweetgum and pecan.

In previous studies we found a PPi-dependent phosphofructokinase (PPi-PFK) (Carnal and Black 1979) that could be activated by a new regulatory molecule,

Table 1. Specific activities of enzymes that cleave sucrose in various tissues of pecan and sweetgum. For comparison some lima bean data are included.

Plant tissues	Sucrose synthase	Acid invertase	Neutral invertase
	nmol min^{-1} mg protein^{-1}		
Sucrose importing tissues			
Developing, 170 mg lima bean seed	74	1	1
Pecan buds at spring break	46	52	6
Partially unfolded pecan leaf	73	39	6
Pecan lateral root in October	56	35	nd[1]
Partially unfolded sweetgum leaf	29	36	5
Sweetgum lateral root in October	95	11	2
Quiescent tissues			
Mature, dry limabean seed	1	nd	1
Pecan lateral root in February	17	nd	6
Sweetgum lateral root in February	23	0.1	5
Sucrose exporting tissues			
Two-day-old limabean cotyledon	2	1	2
Pecan lateral root in April	18	10	5
Sweetgum lateral root in April	18	40	19

[1] nd = Not detectable.

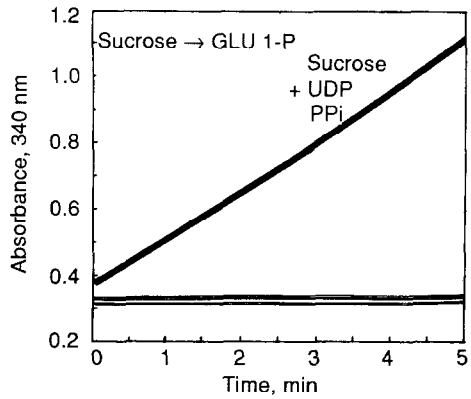

Figure 1. Sucrose breakdown by the sucrose synthase pathway to glucose 1-P catalyzed by a soluble extract from developing pecan buds. The reaction was followed by monitoring NAD reduction at 340 nm. The specific activity is 196 nmoles of G 1-P formed min^{-1} mg $protein^{-1}$. The lower tracings show no reaction when any substrate or combination of substrates was omitted.

fructose 2,6-bisphosphate (Fru 2,6-P_2) (Smyth and Black 1984*a*). Therefore we reasoned that the overall process of sucrose breakdown through to the formation of triose-P by means of the sucrose synthase pathway should be activated by Fru 2,6-P_2. As shown in Figure 2, the conversion of sucrose to triose-P was stimulated by 1 μM Fru 2,6-P_2 in an extract from the root cambial zone of pecan seedlings. Again, the omission of any substrate, e.g., sucrose or UDP or PPi, resulted in no reaction. We have obtained similar results from a variety of tissues that import sucrose (Sung et al. 1987*a* and 1987*b*). The results presented in Figures 1 and 2 support the conclusion that the breakdown of sucrose is initiated by sucrose synthase in a series of steps that

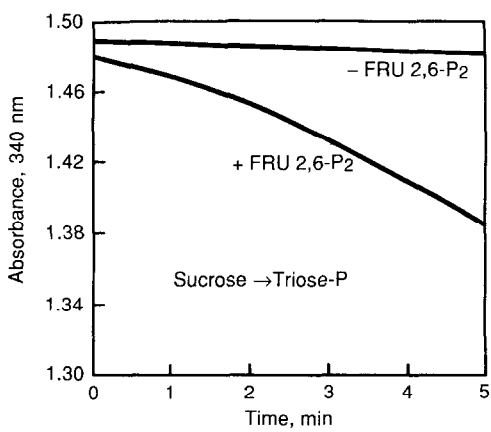

Figure 2. Fru 2,6-P_2 activation of sucrose breakdown to triose-P by the sucrose synthase pathway catalyzed by a soluble extract from the primary root cambial zone of pecan seedlings in June of their second growing season. Triose-P was assayed by glycerol 3-P dehydrogenase (Xu et al. 1986). The maximum specific activity is 23 nmoles of triose-P formed min^{-1} mg $protein^{-1}$.

are dependent on UDP and PPi and that can be activated by Fru 2,6-P_2. Thus, in trees the sucrose synthase pathway appears to provide an alternative to acid invertase and neutral invertase.

Pyrophosphate as an energy source in trees

Our studies on sucrose metabolism led us to reassess the pathways of glycolysis and gluconeogenesis in plants and particularly the biosynthetic role of PPi (Sung et al. 1988). In the present study we monitored the activities of two phosphofructokinases, PPi-PFK and ATP-PFK, in tree roots both with and without VAM fungal infection and grown at two levels of soil phosphate. Our aims were to determine whether the fungus was a sucrose sink and whether soil phosphate level (10 ppm *versus* 100 ppm) influences tissue PPi content and the relative activity of the two PFK enzymes.

The activities of PPi-PFK and ATP-PFK were followed in the lateral root system of sweetgum seedlings during their first growing season (Figures 3 and 4). Both PFK activities were present throughout the growing season. The PPi-dependent enzyme was slightly more active than the ATP-dependent enzyme, but the seasonal patterns of both PPi-PFK and ATP-PFK activity were unaffected by mycorrhizal infection or soil phosphate level. We conclude therefore that in the lateral root system of sweetgum seedlings both PFK enzymes have a role in converting Fru 6-P to Fru 1,6-P_2 and that PPi as well as ATP serve in the process as both a substrate and an energy source.

Enzymes of sucrose glycolytic metabolism in the cambial zone of tree roots

The cambial zone was chosen for a more detailed study of sucrose glycolytic metabolism because: (i) both xylem and phloem mother cells are present at the peeled surface; (ii) translocated sucrose from the phloem is metabolized here; (iii) the tissues

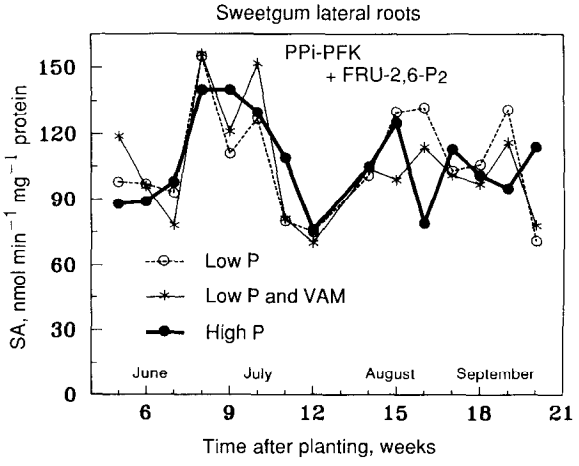

Figure 3. Seasonal changes in the activity of pyrophosphate-dependent phosphofructokinase in soluble extracts of lateral roots of sweetgum seedling. Fru 2,6-P_2 was added at 1 μM.

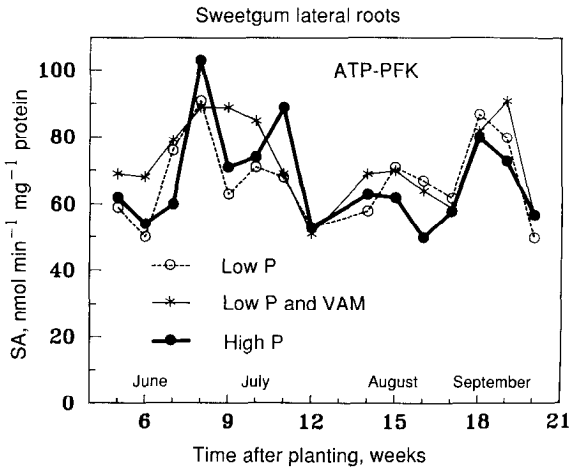

Figure 4. Seasonal changes in the activity of ATP-dependent phosphofructokinase in soluble extracts from lateral roots of sweetgum seedlings.

of this zone undergoes periodic alternations between cell division activity and quiescence; (iv) ray cells, which may store sucrose as starch, terminate here; and (v) seasonal storage and mobilization of carbohydrate reserves occurs in the cambial zone (Zimmermann and Brown 1971). In these studies we examined sucrose breakdown, hexose phosphorylation, hexose phosphate conversions, and Fru 6-P conversion to Fru 1,6-P_2.

Sucrose breakdown

As in whole root tissue (see Table 1), the three enzymes for cleaving sucrose were present in soluble extracts of the root cambial zone of sweetgum and pecan (Figures 5 and 6). Sucrose synthase, which exhibited marked seasonal changes, was the dominant activity, although periodically acid invertase also showed major activity, for example in sweetgum roots in December of the second winter (Figure 5). The activity of neutral invertase was low and showed little seasonal change, whereas acid invertase activity showed some seasonal change (Figures 5 and 6).

Hexose phosphorylation

When sucrose is cleaved by invertase, glucose and fructose are formed, whereas fructose is formed by sucrose synthase cleavage. Fructokinase activity dominates most of the year in both sweetgum and pecan (Figures 7 and 8). Glucokinase activity is generally low and constant; but in sweetgum it exhibited seasonal changes that tended to coincide with those of acid invertase (cf. Figures 5 and 7). Marked seasonal changes were observed also in fructokinase activities (Figures 7 and 8).

Hexose phosphate conversions

Two enzymes of hexose phosphate conversion were studied, namely, UDP-

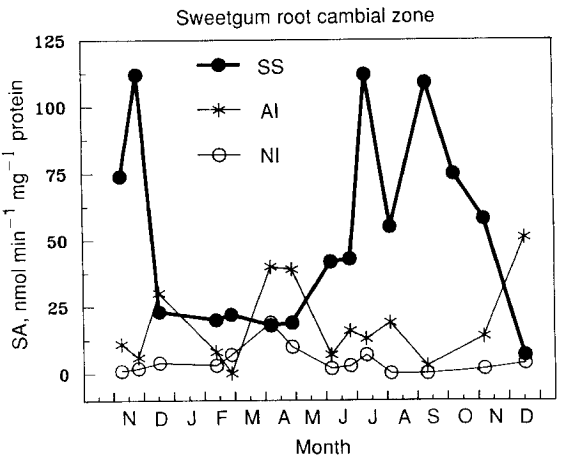

Figure 5. Seasonal changes in the specific activities of the sucrose breakdown enzymes sucrose synthase, acid invertase and neutral invertase in one- to two-year-old sweetgum seedlings planted in April. Soluble extracts of root cambial zone tissue were used in all assays.

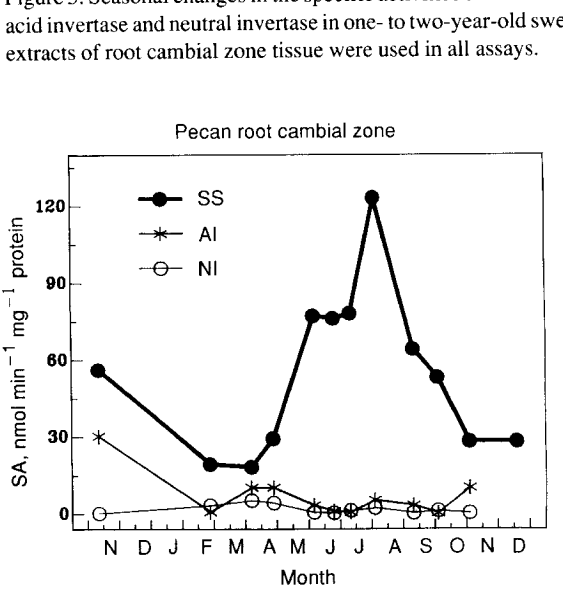

Figure 6. Seasonal changes in the specific activities of the sucrose breakdown enzymes sucrose synthase, acid invertase, and neutral invertase in soluble extracts of pecan seedling root cambial zone.

glucopyrophosphorylase (UDPG-PPilase) which forms glucose 1-P and phosphoglucomutase (PGM) which interconverts glucose 1-P and glucose 6-P. Both enzymes were very active and so it is unlikely that either enzyme limits glycolysis and gluconeogenesis. Phosphoglucomutase had a more constant seasonal activity than UDP-glucopyrophosphorylase, which exhibited a 2- to 3-fold variation in maximum activity during the year (Figures 9 and 10).

Fructose 6-P conversion to fructose 1,6-P_2

The interconversion of Fru 6-P and Fru 1,6-P_2 was thought to be a rate-limiting step

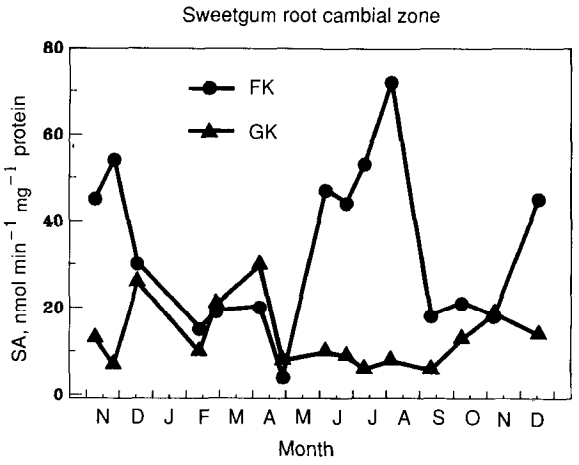

Figure 7. Seasonal changes in the specific activities of fructokinase and glucokinase in sweetgum root extracts.

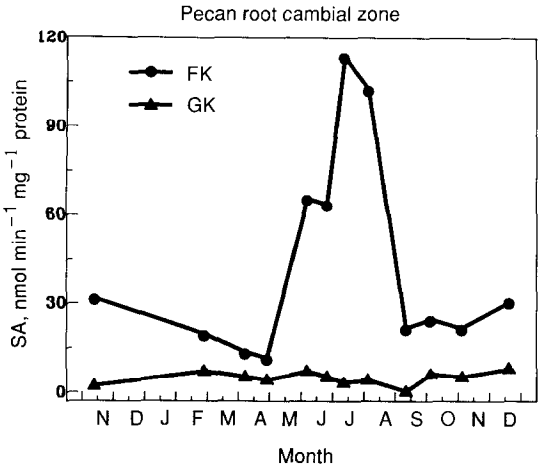

Figure 8. Seasonal changes in the specific activities of fructokinase and glucokinase in pecan root extracts.

in glycolysis and gluconeogenesis (Hawker 1985, Turner and Turner 1980) until the discovery of the alternative enzyme, the PPi-dependent PFK and its strong regulation by Fru 2,6-P_2 (Carnal and Black 1979, Smyth and Black 1984*a*, Sung et al. 1988). The results with both sweetgum and pecan (Figures 11 and 12) show that PPi-PFK was the dominant enzyme activity for converting Fru 6-P to Fru 1,6-P_2. Fru 2,6-P_2 at 1 μM activated the tree PPi-PFK in various assays from 2- to 10-fold.

The results in Figures 11 and 12 show that the nucleotide triphosphate-dependent PFK can utilize either ATP or UTP. Both activities were lower than that of PPi-PFK and fairly constant although UTP-PFK activity showed seasonal changes paralleling

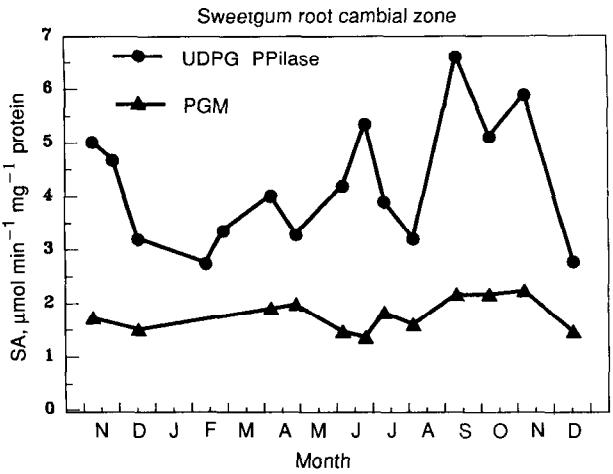

Figure 9. Seasonal changes in the specific activities of UDP-glucopyrophosphorylase and phosphoglucomutase in sweetgum root extracts.

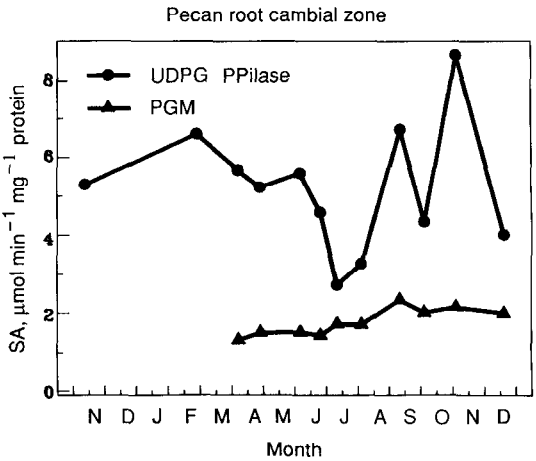

Figure 10. Seasonal changes in the specific activities of UDP-glucopyrophosphorylase and phosphoglucomutase in pecan root extracts.

those of PPi-PFK. Earlier we recognized that the nucleotide-dependent PFK was not specific for adenylates and proposed that it also utilized uridylates and even cycled UTP to produce UDP to operate the sucrose synthase pathway (Sung et al. 1986, Xu et al. 1986, Black et al. 1987). However PPi-PFK was the major activity for converting Fru 6-P to Fru 1,6-P_2 and it also showed marked seasonal changes in maximum activity (Figures 11 and 12).

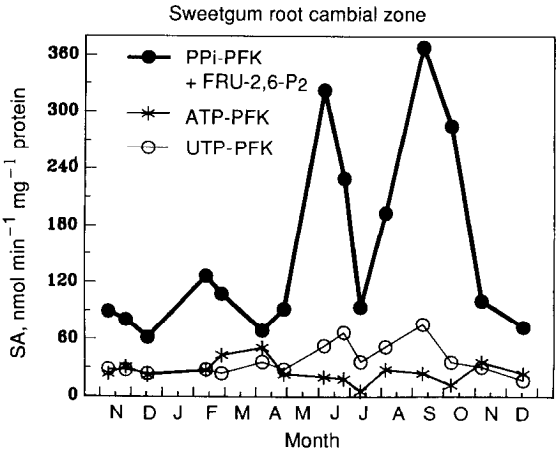

Figure 11. Seasonal changes in the activities of enzymes catalyzing the conversion of Fru 6-P to Fru 1,6-P_2 in sweetgum root extracts. These phosphofructokinase activities were dependent on either pyrophosphate or ATP or UTP.

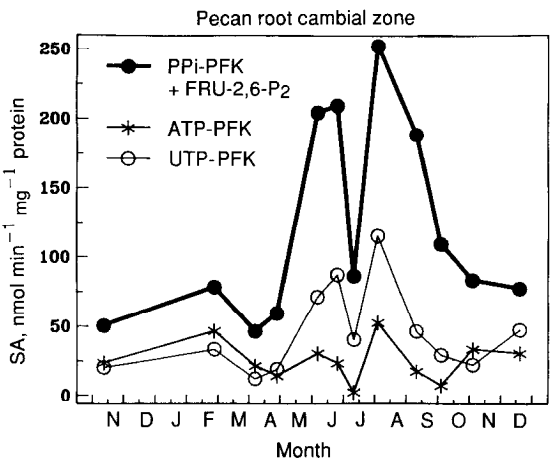

Figure 12. Seasonal changes in the activities of enzymes catalyzing the conversion of Fru 6-P to Fru 1,6-P_2 in pecan root extracts. These phosphofructokinase activities were dependent on either PPi or ATP or UTP.

The identification of adaptive and maintenance enzymes in trees during sucrose glycolytic metabolism

It has been reported that seasonal variation in activity is greater in some enzymes of plant sugar metabolism than in others. Much of the variation occurs either during plant development or in response to rapid, stressful environmental changes. Thus it was suggested that there are maintenance and adaptive pathways for glycolysis and

gluconeogenesis (Mustardy et al. 1986, Black et al. 1987). It has also been shown that alternative enzymes exist to utilize either nucleotide triphosphates or PPi and their relative activities vary with plant development and environmental conditions (Sung et al. 1988).

Adaptive enzymes are characterized by large (5- to 10-fold or more) and rapid changes in specific activity from very low levels in, for example, dry seeds or in the quiescent cambium during winter to high values in, for example, actively dividing cambium or germinating seeds. Maintenance enzymes are characterized by relatively small (no more than 3-fold) and slow changes in specific activity associated with development or in response to changing environmental conditions. Thus the specific activities of maintenance enzymes are sufficient to maintain appreciable metabolic activity, e.g., glycolytic and gluconeogenic sugar conversions, even in dry or dormant seeds or quiescent cambial tissue.

According to these criteria, we earlier identified PPi-PFK as an adaptive enzme in pea, corn, and lima bean; sucrose synthase in corn kernels, Johnson grass, onion bulbs, and lima bean; and acid pyrophosphatase, alkaline pyrophosphatase and acid invertase in lima bean. From the developmental results presented here with sweetgum and pecan we identify: sucrose synthase (Figures 5 and 6, Table 1); fructokinase (Figures 7 and 8); and PPi-PFK (Figures 11 and 12) as adaptive enzymes. Acid invertase (Figure 5) and glucokinase (Figure 6) also are more or less adaptive in sweetgum.

As maintenance enzymes, we earlier identified the ATP and UTP-dependent PFK, UDP-glucopyrophosphorylase, UDPase, phosphoglucomutase, neutral invertase, glucokinase, NTP-fructokinase and sucrose-P synthase in lima bean and pea seeds. The present study indicates that, in both sweetgum and pecan, maintenance enzymes include neutral invertase (Figures 5 and 6), glucokinase (Figures 7 and 8), UDP glucopyrophosphorylase and phosphoglucomutase (Figures 9 and 10) and ATP- and UTP-dependent PFK (Figures 11 and 12).

Conclusions

Numerous studies with trees have shown that sucrose is the major form of translocated carbon. However, there have been few studies to identify the enzymes responsible for sucrose metabolism, glycolysis, or gluconeogenesis in trees and none (to our knowledge) with sweetgum or pecan. The enzyme activities reported here are compatible with the published rates of sucrose translocation in trees. For example Webb (1975) found that, in one-year-old Douglas-fir seedlings, newly photoassimilated carbon partitioned strongly into sinks such as roots and new needles at rates of up to 7.98 and 5.39 mg day^{-1}, respectively. If the carbon translocated was sucrose, then sucrose must have reached the root at an average rate of 11–16 nmoles min^{-1} Similar values have been obtained with many crop species (Cronshaw et al. 1986). Most of the enzyme activities reported here are sufficient to metabolize sucrose translocated at these rates.

In their recent reassessment of sucrose glycolysis and gluconeogenesis, Sung et al. (1988) proposed alternative enzymes and substrates at numerous steps. The present work supports this hypothesis in that it demonstrates the presence of three enzymes of sucrose cleavage (Table 1, Figures 5 and 6). In the root cambial zone, particularly, sucrose synthase is the dominant enzyme (Figures 5 and 6). The UDP and PPi-dependent sucrose synthase pathway for sucrose metabolism functions in trees and is activated by Fru 2,6-P_2 (Figures 1, 2, 5 and 6), particularly in sucrose sink tissues, such as the developing bud or the primary root. Earlier we demonstrated Fru 2,6-P_2 in trees (Black et al. 1987) and here we demonstrate that it regulates PPi-PFK (Figures 11 and 12), which results also in the regulation of sucrose breakdown (Figure 2). Pyrophosphate serves as an energy source with at least two enzymes; the PPi-PFK and the UDP-glucopyrophosphorylase; hence PPi is an energy source in tree metabolism. Two phosphofructokinases are present in sweetgum and pecan trees, i.e., a pyrophosphate-dependent and a nucleotide triphosphate-dependent PFK (Figures 3,4,11 and 12). The NTP-dependent PFK may use either ATP or UTP (Figures 11 and 12).

We previously suggested that plants regulate some enzymes in the various pathways of sucrose metabolism but not others and we classified the enzymes accordingly as adaptive and maintenance enzymes (Mustardy et al. 1986, Black et al. 1987). Three adaptive enzymes in sweetgum and pecan are sucrose synthase, the PPi-PFK, and fructokinase. Of the three, the first provides a major entry point for sucrose imported into the metabolic pool, the second exerts a strong regulatory influence within the glycolytic–gluconeogenic network, and the third forms a hexose-P to allow a free sugar to enter plant sugar metabolism. As maintenance-type enzymes in sweetgum and pecan we have identified: neutral invertase, and tentatively, glucokinase, UDP-glucopyrophosphorylase, phosphoglucomutase, and the UTP and ATP-dependent PFK.

Acknowledgments

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