Alterations in Carbohydrate Intermediates in the Endosperm of Starch-Deficient Maize (Zea mays L.) Genotypes

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ABSTRACT

Metabolite levels in kernels of selected starch-deficient mutants of maize (Zea mays L.) were investigated to gain insight into partitioning of carbohydrate metabolism during kernel development. Several free sugars, hexose phosphates, triose phosphates, fructose-2,6-bisphosphate, and pyrophosphate were measured in normal, shrunken, shrunken-2, amylose extender dull waxy, and brittle genotypes, which were in a near-isogenic W64A background. These mutants were selected to include at least one lesion in both the cytosolic (shrunken) and amyloplastic (shrunken-2) compartments. All the starch-deficient genotypes contained elevated levels of fructose-2,6-bisphosphate and triose phosphates but reduced levels of pyrophosphate, indicating an enhanced glycolytic utilization of carbohydrates in response to the reduced utilization of sugars for starch synthesis. The shrunken kernels (sucrose synthase deficient) contained reduced levels of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate, and this reduction paralleled the reduction in starch accumulation, but levels of triose phosphates were elevated. In shrunken-2 kernels, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate were increased, but fructose-1,6-bisphosphate was lower. These findings support the view that hexose phosphate transport across the amyloplast envelope is more important for starch biosynthesis than transport of triose phosphates. The amylose extender dull waxy mutation showed less dramatic effects on hexose phosphates, but the triose phosphates were greatly increased. The brittle mutation, which has an unknown lesion, showed distinctly similar changes in metabolite levels with shrunken-2, suggesting that the lesion may be associated with the amyloplast.

For several decades, a number of maize (Zea mays L.) endosperm mutants that affect the quantity and quality of carbohydrates in the endosperm, as well as kernel development and morphology, have been identified and extensively studied (8). These mutant genotypes have been used in genetic studies and as biochemical tools in studying sugar and starch metabolism in kernels (6, 11). Many of the carbohydrate mutants condition the accumulation of sugars, particularly sucrose, accompanied by a marked decrease in starch deposition. The genetic lesions involved either a defective system for sugar breakdown leading to a low level of substrates (UDP-Glc and ADP-Glc) for starch synthesis (class 1 mutants) or a deficient enzyme system for the conversion of the sugar-nucleotides to starch (class 2 mutants) in the amyloplast (6, 11).

In nonphotosynthetic tissues, carbohydrate movement into the amyloplast is currently highly controversial (Fig. 1). Several investigators (12, 22, 24, 25) have proposed that triose phosphates are transported into the amyloplast via a phosphate translocator and, subsequently, these are utilized to form starch via gluconeogenesis requiring the activity of a plastid FBPase2 (Fig. 1). It was recently reported that the phosphate translocator in the amyloplast membrane, in contrast to that in the chloroplast membrane, also functions in the uptake of hexose phosphates (4). The preferred substrate for transfer (Glc-1-P or Glc-6-P) reportedly differs with the source of the amyloplasts (4, 32). The significance of the transfer of triose phosphate into amyloplasts has been questioned (14, 15, 19).

Two lines of evidence against the movement of triose phosphates and their use for starch synthesis were given. First, a key regulatory enzyme in gluconeogenesis, FBPase, was reported to be absent from amyloplasts isolated from non-green starch-storing tissues (15). Second, when specifically labeled hexoses were fed to developing kernels of wheat or maize (17, 19), potato tubers (17), and seeds (35), the pattern of label redistribution among the carbon positions of the hexoses in starch did not support a pathway utilizing triose phosphates as intermediates.

Very recently, it was reported that an adenylate transporter in the amyloplast envelope effectively transports ADP-Glc, providing a third possible route of carbon movement into amyloplasts (Fig. 1). Pozueta-Romero et al. (26) reported that the adenylate-specific carrier in purified and intact amyloplast from cultured sycamore cells took up ADP-Glc, which was an effective substrate for starch synthesis.

Dancer and ap Rees (9) measured changes in hexose phosphates and PPI during kernel development of sh and su

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2 Abbreviations: FBPase, fructose-1,6-bisphosphatase; PFPase, PPI-dependent phosphofructokinases; PFP(F), glycolytic activity of PPI-dependent phosphofructokinase; Fru-1,6-bisP, fructose-1,6-bisphosphate; Fru-2,6-bisP, fructose-2,6-bisphosphate; Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate; DPP, days postpollination; AM, ammonium molybdate; MG, malachite green.
mutant genotypes. The commercial sweet corn, Golden Bantam was used for the su mutant genotype and the sh kernels were from an unrelated genetic background. Although they (9) referred to the sweet corn as "wild type," their study did not contain a nonmutant (wild type) control for comparison. In an effort to better understand the in vivo starch biosynthetic pathway in maize kernels and its regulation, we determined the quantity of several key metabolites in developing kernels of selected starch-deficient maize endosperm mutants. All mutants were in a near-isogenic W64A background that was used as the normal counterpart. The mutants selected have known lesions affecting (a) an enzyme specific to the cytosol, shrunken (sh), (b) an enzyme specific to the amyloplast, shrunken-2 (sh2), and (c) multiple amyloplast-specific enzymes, amylase-extender dull waxy (ae du wx). The genetic lesion of brittle (bt), the fourth mutant genotype selected, is unknown.

The sh mutation is a structural lesion resulting in a 90% reduction in sucrose synthase activity (7), an enzyme localized in the cytosol (12). Starch content in sh mutants is 40% less than in normal kernels at 22 DPP and at maturity (7). The sh2 kernels lack, or have a very low, plastid ADP-Glc pyrophosphorylase (10). ADP-Glc pyrophosphorylase activity in sh2 is only 17% of that found in starchy maize endosperm (10).

The ae du wx triple-mutant combination contains a mixture of genetic lesions affecting amyloplast-specific enzymes, including branching enzyme and soluble- and granule-bound starch synthases. The ae mutation causes the absence of branching enzyme Iib (5). The du gene affects the activities of branching enzyme Ila and soluble starch synthase II, and the wx mutant lacks a starch granule-bound starch synthase (27).

MATERIALS AND METHODS

Plant Material

A normal inbred (W64A) and four starch-deficient maize (Zea mays L.) endosperm mutant genotypes were grown in the field during the summer months (June to August 1989). The mutants included bt, sh, sh2, and the triple-mutant combination ae du wx, all in a near-isogenic W64A background. The ears were hand pollinated and harvested at 21 DPP. The kernels were sliced longitudinally into two sections and the pericarp and embryonic tissues removed. The endosperm slices were frozen quickly in Freon-12 cooled to its freezing point with liquid nitrogen and lyophilized. The dried tissues were pulverized with a mortar and pestle and stored desiccated at −20°C until used.

Metabolite Extraction

Dry pulverized tissue (0.5 g) was homogenized with 3 mL of 5.5% (v/v) HClO4 in a Potter-Elvejem homogenizer and placed on ice for 60 min. The homogenate was centrifuged at 2000g for 5 min, and the supernatant was saved. The pellet was washed twice (2000g, 5 min) with 1 mL deionized water, and the resulting supernatants were combined with the initial supernatant. The combined supernatant was neutralized with 3.5 mM K2CO3 and placed on ice (10 min) to allow further precipitation of the potassium perchlorate, which interfered with the coupled enzymatic assay of metabolites. The neutralized extract was then centrifuged (2000g, 10 min), and the supernatant was lyophilized (24 h). The dried residue was suspended in 1.3 mL of deionized water, frozen at −80°C for 2 h, thawed, and centrifuged at 8000g for 5 min to remove additional potassium perchlorate. The clarified supernatant was used to assay all metabolites except Fru-2,6-bisP. All steps were carried out at 0 to 4°C. Duplicate samples of normal tissue with authentic standards of the different hexose phosphates and triose phosphates added to the extraction solution were included to determine the percentage of recovery of the different metabolites. The difference in the measured amounts between the samples with and without added standards was used as an estimate of the percentage of recovery. All data were corrected for loss during extraction.

Figure 1. Proposed scheme of carbohydrate metabolism in nonphotosynthetic tissues. Enzymes designated by numbers are as follows: 1, invertase; 2, sucrose synthase; 3, hexokinase; 4, hexose-6-phosphatase; 5, Glc-phosphate isomerase; 6, UDP-Glc pyrophosphorylase; 7, phosphoglucomutase; 8, FBPase; 9, PFKase; 10, PFPase; 11, aldolase; 12, triose phosphate isomerase; 13, triose phosphate/ADP translocator; 14, hexose phosphate translocator; 15, adenylate translocator; 16, ADP-Glc pyrophosphorylase; 17, pyrophosphatase; 18, soluble starch synthase; 19, starch-bound starch synthase; 20, phosphorylase; 21, branching enzyme.
Fru-2,6-bisP Extraction and Assay

The Fru-2,6-bisP extraction was based on a procedure suggested by P. S. Kerr (personal communication, 1988). Pulverized dry endosperm (0.25 g) was homogenized with 11 mL of 10 mM KOH, and the homogenate was centrifuged at 20,000g for 10 min. The pellet was reextracted with 5 mL of 10 mM KOH and centrifuged as above. The combined supernatants were used for the assay of Fru-2,6-bisP. Duplicate samples of normal W64A endosperm tissue containing a known amount of added Fru-2,6-bisP standard were extracted as above with KOH to determine the percentage of recovery. The assay for Fru-2,6-bisP utilized the ability of Fru-2,6-bisP to stimulate activity of PFPase (34). The assay mixture (1 mL total volume) contained the following (added in order): 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 1 mM Fru-6-P, 150 μM NADH, 2.5 units each of aldolase and glycerophosphate dehydrogenase, 5 units of triose phosphate isomerase, 10 to 40 μL of 10 mM KOH (depending on the sample size), 1 unit of partially purified potato PFPase, and 20 to 40 μL of tissue extract or standard Fru-2,6-bisP (0.5–2.0 pmol). The assay mixtures were preincubated for 2 to 3 min before 1 mM PPI was added to initiate the reaction. The change in A₅₄₀ nm per min was recorded for 5 to 10 min at 25°C. An active PFPase preparation was partially purified from potato (Solanum tuberosum L.) (34). To destroy any contaminating Fru-2,6-bisP in the commercial Fru-6-P before use, the Fru-6-P solution was acidified with HCl to pH 2.0 and held for 30 min at 25°C and then neutralized with NaOH. A control without added standard Fru-2,6-bisP or tissue extract was run concurrently to correct for any background activity. To be sure that extracts from mutant genotypes were free of compounds that may interfere with the assay, 0.5 pmol of standard Fru-2,6-bisP was added to a known volume of extracts from the different genotypes. The percentage of recovery of the added authentic Fru-2,6-bisP was the same in extracts from normal and mutant genotypes.

Starch Extraction

Starch in the pellets remaining after the HClO₄ and water extractions was solubilized with 20 mL of 90% DMSO for 2 h at 70°C. A 2-mL aliquot of the DMSO suspension was mixed with 6 mL of methanol, allowed to stand on ice for 2 h, and centrifuged (1000g, 10 min) to collect the partially solubilized starch. The supernatant was further precipitated by addition of KCl to a final concentration of 1% and centrifuged (1000g, 10 min). The combined pellets were suspended in 5 mL of 0.5 M sodium acetate, pH 4.8, and digested with 5 units/mL of glucoamylase overnight at 37°C. Following the incubation, the enzymic digest was heat denatured (boiling water bath for 5 min) and centrifuged (20,000g, 10 min), and the supernatant was used for starch quantification.

HPLC Analysis of Neutral Sugars and Starch

Following the resuspension of the lyophilized perchloric acid extract with deionized water and centrifugation, the soluble sugar extract and starch digest were analyzed by HPLC fitted with a Sugar-Pak I column maintained at 90°C with a column heater and temperature control modules. The samples were loaded in an autosampler (Waters WISP model 712), injected, eluted with 50 mg/L Na₂·Ca·EDTA, and monitored by a differential refractometer (Waters model R401). Glucose, fructose, and sucrose were identified by comparing retention times with those obtained for standards, and concentrations were determined from standard curves.

Metabolite Assays

DHAP, G-3-P, and Fru-1,6-bisP were assayed according to the method of Michal (23). The standard assay mixture in 1 mL contained 66 mM triethanolamine-HCl, 5 mM MgCl₂, 7.5 mM EDTA (pH 7.5), 225 μM NADH, and 10 to 300 μL of extract or standard mixture containing DHAP, G-3-P, and Fru-1,6-bisP (2.5–100 nmol). The samples were preincubated for 2 min, and 1 unit of glycerophosphate dehydrogenase, 4 units of triose phosphate isomerase, and 0.5 units of aldolase were added sequentially for the determination of DHAP, G-3-P, and Fru-1,6-bisP, respectively. The decrease in A₅₄₀ nm at 25°C was monitored to completion after each enzyme addition.

Glc-6-P, Glc-1-P, and Fru-6-P were measured according to the method of Michal (23). The assay mixture of 1 mL contained 66 mM triethanolamine-HCl, 5 mM MgCl₂, 7.5 mM EDTA (pH 7.5), 200 μM NADP, and 10 to 50 μL of extracts or standard mixture containing Glc-6-P, Glc-1-P, and Fru-6-P (2.5–50 nmol). The samples were preincubated for 2 min, and 0.5 units of Glc-6-P dehydrogenase, 4 units of phosphoglucomutase, and 2 units of phosphoglucoisomerase were added sequentially for the determination of Glc-6-P, Glc-1-P, and Fru-6-P, respectively. The complete increase in A₅₄₀ nm at 25°C was monitored after each enzyme addition.

The PPI assay was based on the consumption of PPI by PFPase activity toward the formation of Fru-1,6-bisP (13). The assay mixture (1 mL total volume) contained 0.15 M imidazole-HCl, 1 mM MgCl₂ (pH 7.4), 1 mM Fru-6-P, 150 μM NADH, 2.5 units each of aldolase and glycerophosphate dehydrogenase, 5 units of triose phosphate isomerase, and 20 to 50 μL of extract or standard PPI (2.5–10 nmol). Samples were preincubated for 2 to 3 min, and 0.01 unit of bacterial PFPase (Sigma) was added to initiate the reaction. The total decrease in A₅₄₀ nm at 25°C was recorded. The assay was tested for substrate specificity using other potential phosphoryl donors. ATP, ADP, AMP, CTP, GDP, CMP, UTP, UDP, UMP, GTP, GDP, and GMP at 20 nmol per assay did not significantly change the measured amount of 5 nmol of standard PPI or the amount of PPI in a tissue extract. To test whether the extracts from the different genotypes contained compounds that interfered with the assay, 5 nmol of standard PPI was added to a known volume of the different tissue extracts. None of the extracts from the different genotypes showed any effect on the percentage of recovery of standard PPI.

Pi was quantified by a colorimetric procedure (20). A solution containing three parts of 0.045% Mg in deionized water and one part of 4.2% AM in 4 N HCl was prepared and mixed for 20 min. The MG/AM solution was filtered through Whatman No. 5 filter paper and 100 μL of Sterox (Anderson Laboratories, Fort Worth, TX) was added for every 5 mL of MG/AM solution. The MG/AM/Sterox solution (800 μL) was added and mixed with 10 to 100 μL of sample extract or...
standard NaH₂PO₄ (2.5–20 nmol). After 1 min, 100 μL of a 34% sodium citrate solution was added and mixed. After setting for 30 min, the A₆₀₀nm was determined.

RESULTS

Metabolite Levels in Normal (W64A)

The normal endosperm contained similar amounts of glucose and fructose, but these concentrations represented <20% of that of sucrose and starch (Table I). Among the hexose phosphates, the amount of Glc-6-P was 16-fold higher than Glc-1-P, the hexose phosphate with the lowest amount (Table I). The levels of the triose phosphates, DHAP and G-3-P, were <30% of Glc-1-P (Table I). The endosperm PPI content (Table I) was severalfold higher than amounts reported in pea seedlings (13), corn seedlings (28), and in spinach leaves (36). Likewise, the concentration of Fru-2,6-bisP in maize endosperm (Table I) was severalfold higher than amounts reported in photosynthetic tissues (3, 18), in the nonphotosynthetic tissues of carrot roots, tubers of potato, and Jerusalem artichokes, and the developing club of Arum maculatum (2, 30, 33).

Metabolite Levels in the Endosperm of Starch-Deficient Genotypes

Endosperm of the sh2 mutant was significantly higher in hexoses and sucrose, but considerably lower in starch than normal endosperm (Fig. 2A). The sh endosperm also contained higher amounts of DHAP, G-3-P, Pi, and Fru-2,6-bisP, but was lower in the hexose monophosphates, Fru-1,6-bisP, and PPI than normal endosperm.

The sh2 endosperm contained higher amounts of neutral sugars, Glc-1-P, Glc-6-P, Fru-6-P, Fru-2,6-bisP, and triose phosphates as compared to that contained in normal endosperm (Fig. 2B). In contrast, starch, Fru-1,6-bisP, Pi, and PPI contents were reduced.

In ae du wx endosperm, neutral sugars, triose phosphates, and Fru-2,6-bisP were higher compared to normal endosperm (Fig. 2C). On the other hand, the amounts of starch, Glc-6-P, Pi, and PPI were lower.

Similar to the other starch-deficient genotypes, the bt endosperms contained higher levels of neutral sugars than those found in normal endosperms but greatly reduced amount of starch (Fig. 2D). The levels of Glc-1-P, triose phosphates, and Fru-2,6-bisP were also increased; Pi and PPI were decreased.

DISCUSSION

When compared to normal genotype, the reduction in the amounts of Glc-1-P, Glc-6-P, and Fru-6-P (33–48%) in the sh mutation resembles the reduction of the amount of starch accumulated (46%) in the amyloplast. In comparison, the Fru-1,6-bisP level was reduced by only 19%, and levels of the triose phosphates, DHAP and G-3-P, were 70 and 40% higher than normal, respectively. The similarity in the change of hexose phosphate pools and the amount of starch formed seemed to suggest that these hexose phosphates are directly associated with the pathway leading to the synthesis of starch in the amyloplast. If substrate production in the cytosol is limited by the sh mutation but starch synthesis in the amyloplast is not affected, then the carbohydrates moving into the amyloplast in support of starch synthesis should be low. Because the triose phosphates are higher rather than lower, in the normal genotype, it is unlikely that the triose phosphates are moving across the amyloplast envelope to provide substrates for starch synthesis. The DHAP to G-3-P ratio of 2 or less in the normal genotype (Table I) and the mutants (Fig. 2) is surprising because at equilibrium one would expect DHAP to be at least 10 times higher than G-3-P. This deviation from the expected may have been due to differences in the compartmentation of the two triose phosphates. This suggestion is supported by the results of Liu and Shannon (21) who reported that 27% of the cellular DHAP was recovered in the nonaqueously isolated amyloplast fraction but only 7% of the G-3-P was recovered in that fraction.

The reduced quantities of hexose phosphates may also indicate that sucrose mobilization for starch synthesis primarily occurs via the sucrose synthase pathway rather than the hydrolytic invertase pathway. The enzymes of the sucrose synthase pathway (sucrose synthase and UDP-Glc pyrophosphorylase), phosphoglucomutase, phosphoglucosomerase, and PFPase catalyze reactions near equilibrium *in vivo* (1, 8).

### Table I. Metabolite Levels in Normal (W64A) Endosperm and the Percentage of Recovery of Added Metabolites following Extraction

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Level</th>
<th>Metabolite</th>
<th>Level</th>
<th>Standard Added</th>
<th>Standard Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g fresh wt</td>
<td></td>
<td>μmol/g fresh wt</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.0 ± 0.4</td>
<td>Glc-1-P</td>
<td>140.5 ± 0.6</td>
<td>116</td>
<td>114.3 ± 4.3</td>
</tr>
<tr>
<td>Fructose</td>
<td>7.8 ± 0.3</td>
<td>Glc-6-P</td>
<td>2180.0 ± 50.0</td>
<td>872</td>
<td>96.3 ± 6.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>44.6 ± 0.5</td>
<td>Fru-6-P</td>
<td>558.9 ± 17.4</td>
<td>253</td>
<td>102.7 ± 11.0</td>
</tr>
<tr>
<td>Starch</td>
<td>934.7 ± 39.4*</td>
<td>Fru-1,6-bisP</td>
<td>467.4 ± 13.7</td>
<td>233</td>
<td>93.7 ± 7.5</td>
</tr>
<tr>
<td>Pi</td>
<td>4.2 ± 0.13</td>
<td>DHAP</td>
<td>37.8 ± 2.4</td>
<td>58</td>
<td>66.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G-3-P</td>
<td>24.8 ± 1.0</td>
<td>58</td>
<td>28.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPI</td>
<td>82.0 ± 3.4</td>
<td>58</td>
<td>88.4 ± 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fru-2,6-bisP</td>
<td>6.1 ± 0.4</td>
<td>6</td>
<td>97.8 ± 2.3</td>
</tr>
</tbody>
</table>

* μmol Glc equiv/g fresh weight.
Therefore, it is likely that the concentration of incoming sucrose will be reflected in the concentrations of the sugar phosphates in tissues utilizing the sucrose synthase pathway (9). It follows that blocking the pathway at the sucrose synthase site would result in the reduction of hexose phosphate levels. Such a reduction in hexose phosphates is less likely to occur when sucrose is broken down hydrolytically via the irreversible invertase reaction. A similar conclusion was reported by Dancer and ap Rees (9) based on their analysis of the sh maize mutant.

By restricting the hexose phosphate route with the sh lesion, an increased proportion of carbon from sucrose is probably diverted toward triose phosphate formation favoring glycolysis in the cytosol. This possibility is supported by the increased Fru-2,6-bisP, reduced PPI, and the increased triose phosphates of sh endosperm compared with normal. Increased maximum extractable activity of PFP(f) was observed in sh endosperm (31). Increased Fru-2,6-bisP and reduced PPI may signify increased consumption of PPI by PFPase activity in the glycolytic direction (16). The lower PPI in sh endosperms may also result from a reduction in PPI generated in biosynthetic reactions (e.g., starch synthesis) or due to increased consumption by other reactions.

The sh2 mutation, which is deficient in plastid ADP-Glc pyrophosphorylase activity, restricts the conversion of Glc-1-P in the amyloplast to ADP-Glc, the primary substrate of starch formation. Pozueta-Romero et al. (26) recently suggested that cytosol-generated ADP-Glc may be transferred into the amyloplasts via the adenylate translocator and that this ADP-Glc could serve as an important substrate for starch synthesis. However, if this is correct, then the sh2 lesion in the plastid ADP-Glc pyrophosphorylase should have little or no effect on starch synthesis. It is well established that sh2 kernels accumulate only about 20% as much starch as normal kernels. The dramatic reduction in starch and accumulation of Glc-1-P, and the other hexose phosphates, Glc-6-P and Fru-6-P, which are in equilibrium with Glc-1-P, strongly supports the conclusion that the cytosolic hexose phosphates are the more important direct source of sugar for starch synthesis. The sh2 results argue against the adenylate transporter as the sole or major transporter of substrate for starch synthesis. It is possible that cytosolic synthesized ADP-Glc transported into amyloplasts provided the substrate for the small amount of starch synthesized in sh2 kernels.

As with the sh endosperm, the increase in the levels of the triose phosphates, DHAP (160%) and G-3-P (140%), could be attributed to an enhanced glycolytic activity as supported by an increased Fru-2,6-bisP and increased utilization of PPI. Increased maximum catalytic activity of PFP(f) was also observed in sh2 endosperm (31). The endosperm mutants wx and ae are known to affect the final stages of starch synthesis; i.e. granule-bound starch synthase and branching enzyme IIb, respectively (5, 27). When these mutations were combined together along with du into a triple recessive genotype, high sugar/low starch kernels were produced.

Although starch accumulation is reduced by 73% in the ae du wx genotype compared to normal, the effects on hexose phosphates were less dramatic. Glc-1-P increased slightly, Glc-6-P and Pi declined, and Fru-6-P and Fru-1,6-bisP remained unchanged. Although the ADP-Glc content was not measured, we predict that it should be higher, which could contribute to the down regulation of the allosteric enzyme, ADP-Glc pyrophosphorylase, so that ADP-Glc production is matched to its utilization in starch synthesis. In this scenario, we assume that the elevated levels of DHAP and G-3-P, positive effectors of ADP-Glc pyrophosphorylase, are localized outside the amyloplasts. Similar to sh and sh2, ae du wx showed reduced a PPI level and considerably higher triose phosphates, indicating enhanced glycolysis.

The alterations in metabolite pools of bt, which has an unknown lesion, are closest to those of the sh2 mutation. In particular, the levels of the soluble sugars (glucose, fructose, and sucrose) and starch and the dry matter accumulation (31) are essentially identical with the levels in the sh2 endosperm. PPI levels of bt and sh2 are similarly reduced. The increase in the hexose phosphates, Glc-1-P and Glc-6-P, and decrease in Fru-1,6-bisP parallel the changes in the corresponding sugar phosphates found in sh2. Furthermore, bt and sh2 are the only low starch genotypes that have at least 1.8 times more Fru-2,6-bisP than normal. This striking similarity between bt and sh2 indicates that the bt lesion is probably associated with the amyloplast rather than the cytosol.

Changes in enzyme activities and metabolite levels associated with mutations of several starch biosynthetic enzymes

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**Figure 2.** Metabolite levels as a percentage of normal in sh (A), sh2 (B), ae du wx (C), and bt (D) genotypes. Values are means of two to six replications. Vertical lines, SE; where absent, SE is smaller than the symbol.

<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>sh</th>
<th>sh2</th>
<th>ae du wx</th>
<th>bt</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>fructose</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>starch</td>
<td>500</td>
<td>500</td>
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</table>

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have provided us with some insights concerning the pathway leading to starch biosynthesis. As shown in the preceding paper (31), the glycolytic activity of PFPase was increased and the gluconeogenic FBPase was decreased by the different mutations, but these changes were not as dramatic as the reduction in starch accumulation. As opposed to the known correlation of ADP-Glc pyrophosphorylase and starch synthesis activities with starch synthesis, activities of the enzymes supposedly regulated by Fru-2,6-bisP (PFPase and FBPase) exhibited no distinct correlation with starch accumulation (31). The quantity of Fru-2,6-bisP and PPI also showed no apparent correlation with starch accumulation.

Previous studies of maize endosperm, *Arum* clubs, potato tubers, several pea genotypes, and *Chenopodium* cultures indicated similar results (29). In vivo levels of triose phosphates were generally higher in genotypes that synthesized less starch. A simplistic explanation would indicate that triose phosphate could not be the limiting factor in starch synthesis. Collectively, these findings are interpreted as indirect evidence indicating that the allosteric enzymes regulating carbon flux via the glycolytic/gluconeogenic sequence of reactions are not in the direct pathway leading to starch synthesis in developing maize kernels. Thus, the present evidence does not substantiate the idea that the precursors for starch synthesis are triose phosphates crossing the amyloplast membrane.

On the other hand, the alterations in the levels of hexose phosphates, particularly in *sh* and *sh2* mutants, distinctly support the idea that cytosolic hexose phosphates transported across the amyloplast envelope provide the substrate for starch synthesis in situ. For example, when starch synthesis was blocked at a cytosolic site (*sh*), the hexose phosphate levels declined in a manner that is expected for intermediates in a pathway down-stream of the mutant enzyme lesion. When the lesion resulted in loss of activity of an amyloplast enzyme (*sh2*), the hexose phosphates increased, as would be expected of intermediates of a pathway up-stream of the enzyme lesion.

Further definitive evidence is needed to more completely elucidate the movement of carbon into the amyloplast. Feeding experiments with labeled hexose phosphates, triose phosphates, and ADP-Glc in purified and intact amyloplast and amyloplast membrane vesicles will provide additional insights into which sugar crosses the membrane and its metabolic fate.

**LITERATURE CITED**


