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EFFECTS OF SUGARY-1 AND SHRUNKEN-2 LOCI ON KERNEL CARBOHYDRATE CONTENTS, PHOSPHORYLASE AND BRANCHING ENZYME ACTIVITIES DURING MAIZE KERNEL ONTOGENY*

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Abstract—The changes in the contents of dry matter, reducing and non-reducing sugar, water-soluble polysaccharide, starch, and the activities of phosphorylase and the branching enzyme were determined during maize kernel ontogeny for the four possible combinations of the su_1 and sh_2 genes in a common genetic background. The increase in carbohydrate content expressed as percent of the kernel dry matter was greatest during the 12–18-day period following pollination for all genetic lines. By the 15th day after pollination, reducing sugar content was decreasing in all lines. This was also the time of maximum non-reducing sugar content for kernels of the full (Sh_2) lines; however, in the shrunken (sh_2) lines the non-reducing sugar content was present in large quantities throughout the remainder of the sampling period. Water-soluble polysaccharide occurred to an appreciable extent only in the sugary-full line. The phosphorylase activity of the shrunken kernels was over twice that of the full kernels, suggesting that phosphorylase may be influenced by the sh_2 gene. The branching enzyme activity of the sugary-full kernels was much higher during the early sampling dates than those of the other lines and the starch of the sugary-full line consisted almost entirely of amylopectin.

INTRODUCTION

THE EFFECTS of various genes upon endosperm carbohydrate composition in maize are well known,[1,2] but only in recent years have attempts been made to correlate enzyme activity with the resultant carbohydrate composition. Genotype–enzyme relations in maize which have been reported include the inability to demonstrate ADPglucose-α-glucan glucosyltransferase activity in waxy kernels[3,4] and the deficiency of the ADPglucose pyrophosphorylase system in shrunken-2 mutants.[5] Studies of this type, coupled with the judicious use of available maize mutants, offer an opportunity to gain an understanding of the mechanisms of genetic control of endosperm carbohydrate synthesis.

Phosphorylase (α-glucan phosphorylase) was selected for the initial study since the procedures for purification and assay are well established; however, the function of phosphorylase in plants is a matter of controversy. Since the discovery of ADPglucose

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[5] C. Y. TSAI and O. E. NELSON, *Science* **151**, 341 (1966).

glucosyltransferase in plants[6,7] and the demonstration of its capability to mediate starch formation from nucleoside-sugar donors, many workers have concluded that phosphorylase functions solely in starch degradation[8,9] while others still believe that it is involved in starch synthesis.[10–12] In either case, phosphorylase activity could affect the carbohydrate composition of the developing kernel. The branching enzyme (α-glucan-branching glucosyltransferase) is thought by some investigators to be involved with phosphorylase in the synthesis of amylopectin.[13] The importance of the branching enzyme in the formation of amylopectin in maize is not clear, since the pathway for the synthesis of this branched polysaccharide has yet to be determined.[14] Several genes in addition to the waxy (wx) have been associated with the proportions of amylose and amylopectin in starch, but the effects of the sugary-1 and shrunken-2 in a common genetic background have not been reported.[15]

As the first phase of a study of the possible mechanisms of control by the sugary-1 (su_1) and shrunken-2 (sh_2) loci, the activities of the enzymes phosphorylase and the branching enzyme were determined. Changes in kernel carbohydrate fractions during kernel ontogeny were also followed. Some information is available concerning the types and amounts of endosperm carbohydrates formed in the presence of these two genes[16] but much of it pertains only to the mature kernel[17] or was obtained with the mutants in dissimilar backgrounds.[15] If the results are to be unquestionably correlated with a specific gene, the mutants must have a common genetic background.

RESULTS

The dry matter contents of the four genetic lines are presented in Fig. 1. No differences between the genotypes were observed before the 21st day after pollination at which time the dry-matter content of the full lines became greater than that of the shrunken lines. Changes in the carbohydrate fractions during kernel ontogeny are presented on a dry weight basis for the four lines in Fig. 2. The reducing sugar contents of the four lines followed a similar trend throughout kernel ontogeny. The contents were greatest in the early stages of development, then decreased rapidly from the 12th to the 18th day after pollination.

The non-reducing sugar contents of all the lines were initially low but increased until midway of the sampling period. Beyond this time distinct differences between the full and shrunken lines were apparent in the accumulation of non-reducing sugar. The non-reducing sugar content decreased with each successive sampling in the full lines starting 15 days after pollination, whereas in the shrunken kernels the contents increased and remained at a high level for both the 18th and 21st day after pollination. This was followed by a slight decrease such that the non-reducing sugar contents of the shrunken lines were several-fold greater than those of the corresponding full lines at the end of the sampling period. The largest

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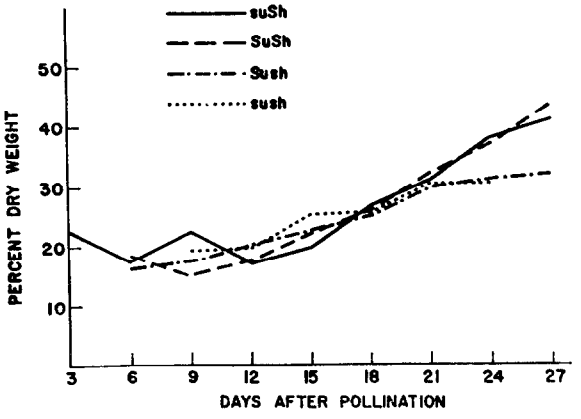

FIG. 1. DRY MATTER CONTENT DURING ONTOGENY OF MUTANT MAIZE KERNELS.

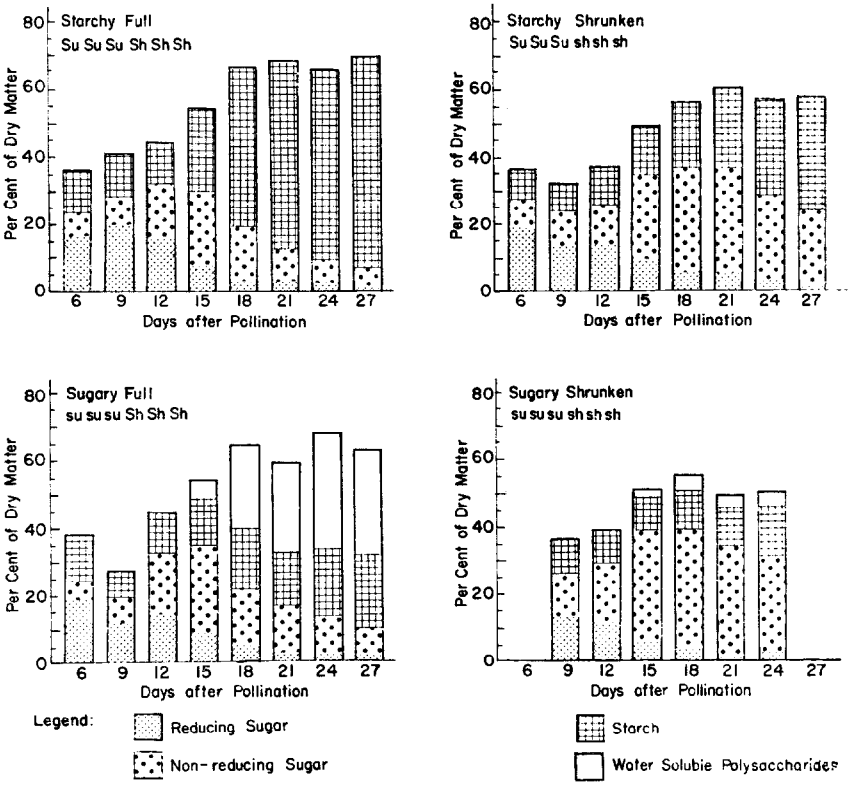

FIG. 2. CARBOHYDRATE CONTENTS OF MAIZE MUTANTS DURING KERNEL ONTOGENY.

amount of non-reducing sugar was accumulated by the doubly recessive or sugary-shrunken line (sush). Analyses of a number of sample extracts by paper chromatography revealed that glucose and fructose comprised the reducing sugar fraction and sucrose the non-reducing sugar fraction.

The water-soluble polysaccharide fraction, i.e. the branched, water-soluble glucose polymer related to amylopectin and similar in structure to glycogen, was present in large quantities only in the sugary-full line (suSh). Smaller amounts were present in the sugary-shrunken (sush) samples, and amounts too small for the graphic presentation in Fig. 2 (less than 0·5 per cent on a dry weight basis) were detected in the starchy samples. This polysaccharide was first detected in samples harvested 15 days after pollination. Starch was detected in all lines at every sampling date. Relatively similar amounts were present in the four genotypes through the 12th day after pollination. Accumulation then proceeded at a rapid rate in the starchy-full line (SuSh) so that by the 18th day after pollination over three-fourths of the maximum starch content was present. The other three lines accumulated starch in much smaller amounts during this period. As expected, starch was the predominant carbohydrate present in kernels of the starchy lines at the end of the sampling period; however, the starchy-full line contained over twice as much starch as the starchy-shrunken line (Sush). The per cent amylose present in the starch of samples harvested 24 days after pollination was 35 per cent for both of the starchy lines, 10 per cent for the sugary-shrunken and none in the starch of the sugary-full kernels. Analyses of normal dent (starchy-full) and sweet (sugary-full) corn gave the same results for the dent, but 5 per cent of the starch of the sweet corn was amylose.

With respect to total carbohydrate content, all the lines had one feature in common; regardless of the type or amounts of contributing components, the total carbohydrate content was maximal, or nearly so, by the 18th day after pollination. At the end of the sampling period the predominant carbohydrates of the full lines were the polysaccharides, with the condition of the sugary gene determining whether solely starch or a mixture of starch and water-soluble polysaccharide accumulated. In the shrunken lines, the starchy mutant accumulated both starch and some sucrose whereas in the sugary mutant sucrose was the predominant carbohydrate.

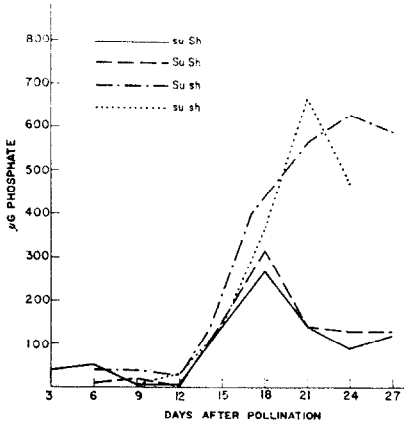

FIG. 3. PHOSPHORYLASE ACTIVITY DURING KERNEL ONTOGENY OF MAIZE MUTANTS. ACTIVITY IS EXPRESSED AS µg PHOSPHATE LIBERATED IN 20 min.

Only limited phosphorylase activity was detected in the four genetic lines through the first 12 days after pollination (Fig. 3). An increase then occurred in all lines and distinct

differences were observed in the time and amount of maximum activity. The peak phosphorylase activities of the shrunken lines were over twice those detected in the samples of the full lines. The decline in activity appears to have ceased by the end of the sampling period in the case of the full lines; however, the activities of the shrunken lines were still high but decreasing at this time.

The relative activity of the branching enzyme is shown in Table 1. Except for the first sampling date, the sugary-full line exhibited the greatest activity at any given sampling. Also, the increase in the branching enzyme activity of this line for successive samplings was greatest between the 14th and 16th day after pollination. After this date the activity either increased only slightly or decreased.

TABLE 1. RELATIVE ACTIVITY OF THE BRANCHING ENZYME DURING KERNEL ONTOGENY OF MAIZE MUTANTS. ACTIVITY IS EXPRESSED AS PER CENT DECREASE IN ABSORPTION

	% A decrease				
	Days after pollination				
Mutant	14	16	18	22	24
Starchy-full	15·0	23·4	19·4	42·4	30·0
Sugary-full	15·0	49·2	58·1	63·3	55·0
Starchy-shrunken	20·9	28·1	21·0	44·1	47·7
Sugary-shrunken	12·9	18·8	24·2	52·5	53·3

DISCUSSION

The carbohydrate compositional results found at the end of the sampling period differ in magnitude from those reported by Laughnan[17] for the same mutants but from dry, ground kernels, presumably far more mature than those utilized in this study. However, the qualitative differences were maintained and seen for the first time in relation to the ontogeny of the kernel. The significant aspects include the high content of water-soluble polysaccharide in the sugary-full line, the increased sucrose and decreased starch contents of the shrunken lines and the difference in phosphorylase activity between the full and shrunken lines. The branching enzyme also showed itself to be more active at an earlier stage in the sugary-full than in the other lines.

Sucrose has been established as the main sugar of transport[15, 18] and was identified as such in the vascular exudate of the mutants by paper chromatography. Presumably the glucose and fructose present in the early stages of kernel development are derived from the hydrolysis of translocated sucrose. Conversions are possible by the action of oligosaccharide hydrolyase enzymes including β-D-fructofuranoside fructohydrolyase (invertase) or the reversible sucrose synthetase system as suggested by Murata.[19] The rapid decline in the reducing sugar content that occurs in all four genetic lines at 15 days after pollination suggests that whatever system is operative functions mainly in the early phase of kernel ontogeny or that glucose utilization in the kernel increases at this time. The fact that sucrose or polysaccharide increases as the reducing sugars decrease tends to support this; however, additional

[18] N. P. BADENHUIZEN, in *Starch, Chemistry and Technology* (edited by R. L. WHISTLER and E. F. PASCHALL), p. 65, Academic Press, New York (1965).
[19] T. MURATA, T. SUGIYAMA, T. MINAMIKAWA and T. AKAZAWA, *Archs Biochem. Biophys.* **113**, 34 (1966).

information such as the content of hexose phosphates is needed before a detailed explanation can be given.

The highly branched water-soluble polysaccharide fraction accumulated to any extent only in the sugary-full line and to a much lesser degree in the sugary-shrunken. This indicates that if phosphorylase were involved in synthesis of higher polysaccharides, it is not involved in water-soluble polysaccharide synthesis.

The arguments against phosphorylase as a starch-synthesizing enzyme have included the less-favorable energy relations in comparison to the ADPglucose transferase system and the unfavorable inorganic/organic phosphate ratio found in plants.[9] However, if the findings of Tsai and Nelson[5] are corroborated and certain plants or tissues lack the ability to synthesize the substrate ADPglucose for the glucosyltransferase system, then phosphorylase may function as a synthesizer of starch in these tissues. Furthermore, the unfavorable inorganic/organic phosphate ratio of plant-tissue homogenates may not reflect the true ratio in the organelles where starch is actively being synthesized.[20] Although ADPglucose has been shown to be the preferred donor molecule in maize starch synthesis by the glucosyltransferase system, other nucleoside-sugars may also serve as glucose donors[8] and therefore the glucosyltransferase system may be able to account for the lower content of starch produced in the shrunken lines.

The results of this study indicate that the sh_2 locus either controls or influences indirectly the degree of phosphorylase activity in maize. If phosphorylase does not mediate starch synthesis *per se*, it may still be involved in the synthesis of a primer carbohydrate of low molecular weight for the reaction mediated by the glucosyltransferase system.[9,21] The high phosphorylase activity and sucrose content and the low starch content in the shrunken lines might indicate a role of starch degradation for phosphorylase in these genetic lines leading to sucrose accumulation. However, more information is needed before an adequate interpretation of the role of phosphorylase in the developing maize kernel can be made.

High-branching enzyme activity was detected in the sugary-full line by the 16th day after pollination but not until the 22nd day for the other three lines. The sugary-full line also contained starch that was almost entirely composed of the branched polysaccharide, amylopectin. However, unless the analyses are made near the 16th–18th day after pollination, the correlation between enzyme activity and amylopectin content would not be evident. The amylose and amylopectin contents reported in this study were determined only for kernels harvested 24 days after pollination and although they may reflect the early differences in the branching enzyme activity, it would be presumptuous to state that correlative results were obtained. Although distinct differences are evident among the lines with respect to the amounts of the various carbohydrate fractions present in the kernels near maturity, a similarity exists in the rate of total carbohydrate accumulation. In all lines, extensive carbohydrate synthesis occurs during the period of 12–18 days after pollination. This should be the optimum sampling period for studying the enzymes involved in carbohydrate synthesis.

EXPERIMENTAL

Plant Material

Seed for initial plantings of the four maize lines used in this study were obtained from the Maize Cooperative Genetics Stock Collection. The lines had an identical genetic background and were homozygous except as indicated:

[20] R. AIMI, T. MURAKAMI and K. FUJIMAKI, *Proc. Crop Sci. Japan* **25**, 124 (1956).
[21] I. IGAUE, *Agri. Biol. Chem.* **26**, 424 (1962).

Endosperm genotype	*Kernel phenotype*	*Designation*
$Su_1 Su_1 Su_1 Sh_2 Sh_2 Sh_2$	Starchy-full	SuSh
$Su_1 Su_1 Su_1 sh_2 sh_2 sh_2$	Starchy-shrunken	Sush
$su_1 su_1 su_1 Sh_2 Sh_2 Sh_2$	Sugary-full	suSh
$su_1 su_1 su_1 sh_2 sh_2 sh_2$	Sugary-shrunken	sush

Plants of each line were self-pollinated and the sampling regime initiated on the 3rd day after pollination and continued over a 27-day period, except for the sugary-shrunken line for which a limited number of plants were available because of poor germination. A minimum of three ears per line for each sampling date was harvested before 8:00 a.m., kernels removed and placed in chilled containers for subsequent sampling. The embryos were not removed as having been shown to have no effect on the electrophoretic pattern of proteins of the kernels.[22]

Sample Preparation

Subsamples were taken for dry matter, sugar analyses, starch and water-soluble polysaccharide determinations, and for acetone powder preparations. The sugar contents were determined by the dinitrosalicylic acid method[23] before and after acid hydrolysis. Starch was determined by the AOAC method[24] on the residue resulting from kernel homogenization and subsequent washing with 10% trichloroacetic acid. The supernatant and washings from this operation were combined and made 50% with respect to ethanol. After standing at 4° for 1 hr, the precipitated water-soluble polysaccharide was centrifuged, taken up in an appropriate volume of water and the carbohydrate content determined with anthrone reagent.[25] The amount of amylopectin in the starch was determined by the method of McCready *et al.*[25]

Enzyme Assays

For phosphorylase activity determinations, weighed portions of acetone powders adjusted to contain 0·012 g of nitrogen were extracted overnight at 4° with 0·004 N $NaHCO_3$, centrifuged, and the supernatant taken as the enzyme source. The crude bicarbonate extract was partially purified after the procedure reported by Fuwa.[14] The reaction mixture for the assay contained 0·5 ml of 0·6 M citrate buffer at pH 6·0, 0·2 ml of 0·625 M ammonium molybdate and 0·5 ml of the enzyme extract. The mixture was equilibrated for 10 min at 37° before addition of 0·5 ml of 4% soluble starch and the reaction initiated by the addition of 0·3 ml of 0·26 M glucose-1-phosphate. After 20 min the reaction was stopped by the addition of 5 ml of 10% trichloroacetic acid, neutralized with NaOH and the liberated phosphorus determined by a modification of the Fiske–Subbarow method.[26] Protein contents of the samples were determined by the micro-Kjehldahl procedure.

The branching enzyme activity was determined on extracts of freshly harvested kernels with the enzyme purified by the procedure of Barker *et al.*[27] The assay was a modified procedure of Igaue[21] and the results expressed as the per cent decrease in absorption of the iodine-stained amylose solution. The reaction mixture contained 1 ml of 0·2% amylose, 0·8 ml of 0·2 M citrate buffer, pH 7·0, and 1 ml of purified enzyme preparation. After a 20 min incubation period at 28°, an aliquot of 1 ml was taken, added to 0·5 ml of 1 M acetic acid and diluted to 30 ml with water. After adding 1 ml of 0·2% iodine in 2% potassium iodide solution the total volume was brought to 35 ml and the absorptivity recorded at 660 nm.

Acknowledgements—The authors are indebted to Dr. Patterson of the Maize Cooperative Genetics Stock Collection for the mutants used in this study and to H. Sox and E. Wilson for their excellent technical assistance.

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