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Development of wild type, *shrunken-1* and *shrunken-2* maize kernels grown in vitro

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Summary. Kernels of wild type maize (*Zea mays* L.) and the mutants *shrunken-1* and *shrunken-2* developed as much as in vivo when excised at five days post-pollination and grown in culture using existing methods. Mature kernels from culture exhibited their expected phenotypes. Starch, sugar and enzyme levels of kernels grown in culture were similar to those known to occur in kernels of the same genotypes grown in vivo. Differences in percentage germination of kernels grown in vitro were similar to those of kernels grown in vivo.

Key words: Tissue culture – Seed development – Starch synthesis – Starch mutants – *Zea mays* L.

Introduction

A potential technique for examining starch metabolism in the maize endosperm is the use of various tissue culture systems. Such systems would be open to manipulation and would offer a unique opportunity to study starch synthesis under controlled conditions. To this end, Shannon and Batey (1973) reported establishing callus cultures of maize endosperm for analysis of starch synthesis. Chu and Shannon (1975) showed that supplementing the growth medium with sucrose led to starch accumulation in the endosperm cells, indicating that enzymes necessary for starch synthesis were present in the cultured cells. Total starch content of the cells, however, was low in comparison to intact kernels. From these data the authors suggested that the cultured cells were not capable of carrying out normal starch synthesis.

A culture method that may be useful for examining starch synthesis is the in vitro kernel culture method developed by Gengenbach (1977). He found that blocks of tissue consisting of kernels and supportive tissue would develop normally in culture. Mature kernels derived from this method would germinate. This technique has been used to investigate the effects of temperature on kernel growth (Jones et al. 1981) as well as to examine the uptake of metabolites into excised maize kernels (Shimamoto and Nelson 1981).

For kernel culture or any other in vitro method to be useful in studies of starch biosynthesis it must allow for normal starch synthesis to proceed. The primary objective of this investigation was to examine development of wild type, *shrunken-1* (*sh1*) and *shrunken-2* (*sh2*) kernels grown with the method of Gengenbach (1977) in order to determine if starch synthesis in vitro is comparable to starch synthesis in vivo. Here we report that wild type maize kernels as well as kernels of the mutants *shrunken-1* and *shrunken-2* will develop to maturity in vitro and express their expected phenotype. Furthermore, we show that starch synthesis in vitro, as judged by the presence of the starch synthetic enzymes and by starch levels, is comparable to that which occurs in vivo.

Materials and methods

Biological material

Wild type maize and the starch deficient mutants *shrunken-1* (*sh1*) and *shrunken-2* (*sh2*) were analyzed. Normal maize was the F_2 progeny of W64A × 182E. The *sh1* allele, 7205, which has approximately 5% of the wild type level of sucrose synthetase in the cleavage and the synthetic reactions was generously provided by Dr. Prem Chourey, University of Florida. The *sh2* material, having approximately 5% of the wild type level of ADP-glucose pyrophosphorylase, was the commercial cultivar 'Florida Stay Sweet'. Plants were grown in the field or greenhouse in Gainesville, Florida.

Kernel culture procedure

Ears were harvested at 2 to 5 dpp (days post-pollination) with harvest at 5 dpp being used for most of the studies reported here. After harvest, the ears were transported to the laboratory and were held at 4 °C. All ears were placed in culture within 4 h of harvest, although ears could be stored for 24 h without apparent damage. Kernel blocks were dissected using the method of Gengenbach (1977). Fertilization percentage was based upon ovule enlargement at 10 dpp.

Culture medium

Kernel blocks were placed in a Murashige and Skoog medium as modified by Gengenbach (1977) and as described here. The concentration of sucrose was 150 g/l. The medium was filter sterilized or sterile sugars were added after the medium, minus sugars, was autoclaved.

The medium was supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 g/l of casein hydrolysate. Streptomycin sulfate (10 mg/l) was added to control bacterial contamination. Two to four kernel blocks were placed in each 100×25 mm petri dish containing 50 ml of culture medium. All cultures were grown at 26 °C in darkness.

Germination measurements of kernels grown in culture

After 30 days in culture, kernels judged to be mature were removed, air-dried for at least 2 weeks, and germinated. Field grown seeds of the three genotypes were also used in order to compare germination of in vivo and in vitro grown seeds. Only undamaged kernels that had embryos and endosperms based on visual observations were used.

Seeds were placed between sheets of moist blotting paper and germinated in the dark at 25 °C for 1 week. Three replicates of at least 60 seeds were used for germination tests. Total germination was the percentage of seeds that had germinated after 1 week. Germination was defined as radical emergence.

Extraction procedures

Frozen kernels were removed from the cob and the endosperms were separated from the embryo and pericarp. The endosperms were ground 1:1 (W:V) in chilled 0.01 M TRIS-maleate buffer (ph 7.0). The homogenate was centrifuged at 34,000 g for 25 min. The supernatant and the pellet were separated. An aliquot of the supernatant was removed, diluted 5 times with water and immediately used for ADP-glucose pyrophosphorylase assay.

An equal volume of saturated ammonium sulfate was added to the remainder of the supernatant to separate the soluble enzymes from the soluble sugars. After incubation in ice for 1 h, the preparation was centrifuged at 27,000 g for 15 min. The pellet containing the enzymes was resuspended in one-half the original volume of the extraction buffer and dialyzed overnight against buffer. The dialyzed solution was then used for sucrose synthetase and starch synthetase assays.

The supernatant containing the soluble sugars was treated with 10 volumes of 95% ethyl alcohol to precipitate the ammonium sulfate which was removed by filtration through Whatman no. 4 filter paper.

The starch-containing pellet from the initial centrifugation was boiled in 10 volumes of 95% ethyl alcohol for 30 min. After filtration through Whatman no. 1 paper, the filtrate was pooled with the other soluble sugar fraction and used in the sugar analysis.

At least 3 replicates consisting of 12 to 20 kernels were extracted for use in enzyme assays as well as for determination of starch and sugar levels.

Enzyme assays

Sucrose synthetase (UDP-glucose:D-fructose-2-glucosyltransferase, E.C.2.4.1.13) was assayed by the method of Chourey and Nelson (1979).

ADP-glucose pyrophosphorylase (ATP: α-D-glucose-1-phosphate adenyltransferase, E.C.2.7.7.27) was assayed by the formation of ADP-[^{14}C] glucose from [^{14}C] glucose-1-phosphate using a modification of the method of Dickinson and Preiss (1969) as described by Hannah et al. (1980).

Soluble starch synthetase (soluble ADP-glucose-starch glucosyltransferase, E.C.24.1.21) was assayed by incorporation of [^{14}C] glucose from ADP-[^{14}C] glucose into amylopectin by a modification of the methods of Ozbun et al. (1971), Tsai et al. (1970) and Hawker and Downton (1974). ADP[^{14}C] glucose was prepared by a modification of the methods of Preiss and Greenberg (1972).

The reaction mixture for soluble starch synthetase consisted of 12 µmoles HEPES buffer (pH 7.5), 0.5 µmoles ADP-[^{14}C] glucose (46,000 cpm), 0.5 µmoles EDTA, 5 mg of amylopectin, and 10 µl of the partially purified enzyme preparation in a final volume of 100 µl. Controls lacked amylopectin. The reaction was carried out for 30 min at 37 °C.

The reaction was terminated and the amylopectin was solubilized by the addition of 1.0 ml of 0.1 N NaOH. Methanol was added to a final concentration of 75%. The starch was pelleted by centrifugation at 34,000 g and washed 3 times by NaOH solubilization followed by methanol precipitation and centrifugation. After the third wash, the starch pellet was solubilized in 0.2 ml of 0.1 N NaOH followed by adding 1.0 ml of methanol to suspend the starch.

The starch suspension was placed on glassine filter disk and washed with 10 ml of methanol. After drying, the disk was monitored for radioactivity by scintillation spectrometry.

Starch determinations

For measurement of starch in dried seeds, the seeds were extracted twice in 5 volumes of 95% ethyl alcohol. After filtering through Whatman no. 4 paper, the residue was dried. One hundred milligrams was solubilized in 10 ml of 0.5 N NaOH, followed by addition of 70 ml of distilled water. The pH was adjusted to 4.5 with acetic acid and the volume adjusted to 100 ml. Ten milligrams of amyloglucosidase were added to 1.0 ml of the solution followed by incubation at 55 °C for 1 h. Starch was then measured as free glucose using a Yellow Springs Instrument Model 27 Glucose Oxidase Analyzer.

Gas-liquid chromatography

Two milliliters of the soluble sugar extract were placed in a vial and dried in an oven at 20 °C. Lipids were removed from the samples by addition of 1.0 ml of hexane and 1.0 ml of water for lipid : sugar partitioning. The samples were mixed and allowed to stand until the layers separated. The hexane phase containing the lipids was removed and the aqueous phase dried in an oven at 60 °C. By use of radiolabelled sugars it was revealed that no sugars were lost into the hexane phase during partitioning.

The sugars were derivatized using the methods of Ferguson, Dickinson and Rhodes (1979). One microliter of the silylat-

ed sugars was injected into a Hewlett Packard 5710A gas chromatography programmed at 150 °C for 2 min followed by a linear rise in temperature to 250 °C at a rate of 8 °C/min. Injection port temperature was 300 °C and the detector temperature was 350 °C. The column was stainless steel (1.83 m × 3.17 mm) packed with 3% OV-17 on Chromosorb W(HP) 80/100 mesh (Altech). The carrier gas was helium at 40 ml/min. The retention times of the derivatives of sucrose, glucose and fructose under these conditions were approximately 11.3 min, 4.8 min, and 4.0 min, respectively.

Results

Characteristics of kernels grown to maturity in culture

The percentage of wild type kernels that developed normally was dependent upon the age of the kernels at harvest (Table 1). Kernels were classified as normal if at maturity they had an embryo and endosperm and did not exhibit any external damage to the seed coat. Twenty-three percent of the kernels harvested 2 dpp showed normal development whereas 78% of the kernels harvested at 5 dpp developed normally (Table 1). Kernels harvested at 5 dpp were therefore used for all subsequent experiments.

Mature kernels of all three genotypes exhibited phenotypes identical to those of mature kernels produced in vivo. Wild type kernels were plump whereas the mutant kernels exhibited their characteristic mutant phenotype. Kernels of *sh1* had concave crowns and *sh2* kernels were collapsed as is characteristic of this severe deficiency in starch content.

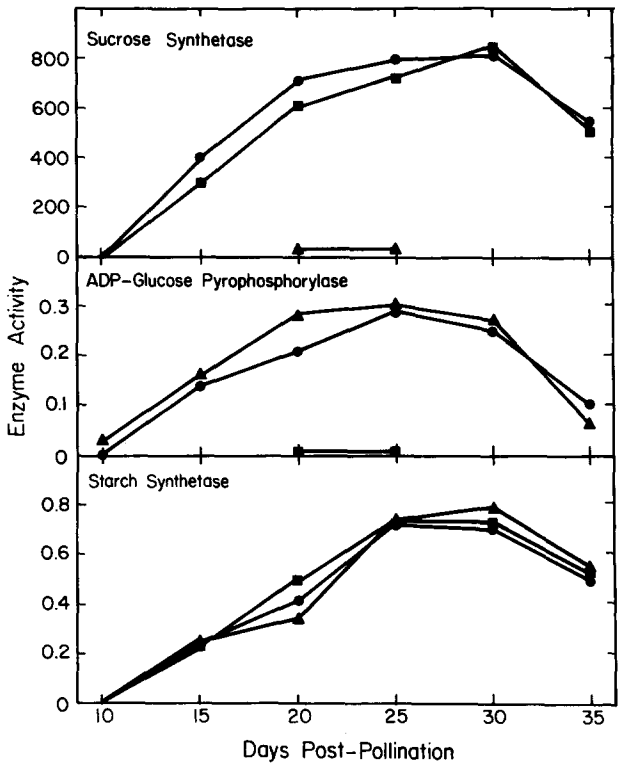

Fig. 1. Activity of sucrose synthetase, ADP-glucose pyrophosphorylase and starch synthetase throughout development in endosperms of wild type (●), *sh1* (▲), and *sh2* (■) kernels grown in vitro

Table 1. Percentage of wild type kernels that showed normal development as a function of kernel age at time of placement in culture. Approximately 200 kernels were used for each treatment

Age (days) at harvest	Percentage of fertilized kernels that developed to maturity[a]
2	23
3	44
4	55
5	78

[a] Fertilization rate based on kernel enlargement at 10 dpp

Table 2. Germination and starch content of kernels grown in culture

Genotype	Total germination		Starch content[a]	
	in vivo	in vitro	in vivo	in vitro
Wild Type	92±6.2	96±3.2	77±4.2	81±3.3
Shrunken-1	68±5.6	52±3.8	46±6.9	40±3.9
Shrunken-2	53±4.1	43±3.8	36+5.2	32±1.6

[a] Percent of dry weight

Mature kernels of all three genotypes grown in vitro germinated and produced normal seedlings (Table 2). Differences in percent germination and in starch content among the three genotypes grown in vitro and in vivo were detected (Table 2). Wild type kernels had the greatest starch content and highest percentage of germination (Table 2). The *sh1* kernels followed wild type in percentage germination and starch content while *sh2* kernels had the lowest percentage germination and starch content. The differences in starch content and germination among the kernels grown in vitro parallel the relative differences found in starch content and germination of kernels grown in vivo (Table 2).

Examination of kernels throughout development

Activities of sucrose synthetase, ADP-glucose pyrophosphorylase and soluble starch synthetase were measured throughout development in kernels of the three genotypes except for sucrose synthetase in *sh1* and ADPglucose pyrophosphorylase in *sh2* which were examined only at 20 dpp and 25 dpp (Fig. 1). Sucrose synthetase activity in *sh1* was approximately 5% of that found in wild type or *sh2* endosperms produced in vitro (Fig. 1). This is similar to the results obtained by Chourey and Nelson (1976) for sucrose synthetase activity of *sh1*

grown in vivo. ADP-glucose pyrophosphorylase activity in *sh2* was approximately 3% of the amount in wild type and *sh1* endosperms (Fig. 1) in agreement with results obtained (Dickinson and Preiss 1969; Hannah and Nelson 1975, 1976) for *sh2* grown in vivo.

Specific activities of sucrose synthetase in wild type and *sh2* kernels were similar throughout development (Fig. 1). No activity was found at 10 dpp and maximal activity occurred at 25 to 30 dpp, the period of most active starch synthesis. Activity declined considerably by 35 dpp.

ADP-glucose pyrophosphorylase activity in wild type and *sh1* kernels was similar throughout development (Fig. 1). Maximal levels were reached at 25 dpp and activity declined by 35 dpp.

Starch synthetase levels in wild type, *sh1* and *sh2* kernels were similar (Fig. 1). As observed with the other enzymes, maximum activity corresponded to the period of greatest starch synthesis as reported by Tsai et al. (1970).

Sucrose, glucose and fructose levels were measured throughout development in kernels of the three genotypes. Sugar levels in kernels of the three genotypes grown in vitro were markedly different (Fig. 2). The mutants showed an increase in sugars that was not found in wild type kernels. Mutant phenotypes and the build up of sugars in the mutants grown in culture would be expected if growth in vitro mimicked in vivo growth.

Sucrose in wild type remained low throughout development and peaked at 20–25 dpp (Fig. 2). Fructose and glucose levels in wild type increased gradually through 25 dpp and declined thereafter (Fig. 2). From 20 dpp onward, sucrose content was greater than the combined amounts of fructose and glucose.

The sucrose levels in *sh1* increased approximately three-fold between 15 dpp and 20 dpp (Fig. 2) and declined thereafter. The amount of sucrose in *sh1* from 15 dpp to 35 dpp was approximately 3 times greater than that found in wild type. As in wild type, there was little change in the amount of fructose or glucose during kernel development (Fig. 2). At the later times examined, the sucrose level in *sh1* was greater than the level of fructose plus glucose. In general, the amounts of glucose and fructose were greater in *sh1* than in normal.

Sucrose levels in *sh2* were greater than those in the other genotypes (Fig. 2). Sucrose increased three-fold from 15 dpp to 20 dpp and continued to increase to 30 dpp. There was a slight decline in sucrose from 30 to 35 dpp. Fructose and glucose increased from 15 to 20 dpp and declined through 35 dpp (Fig. 2). Sucrose levels were greater than the amount of glucose plus fructose at all time intervals examined.

Sugar levels in wild type and *sh2* kernels grown in vitro mimic those obtained by Creech (1968) with kernels grown in vivo in that sugars increase in *sh2* in comparison to wild type. The amount of sucrose in *sh1* also increased in comparison to wild type levels in agreement with results obtained by Chourey and Nelson (1976).

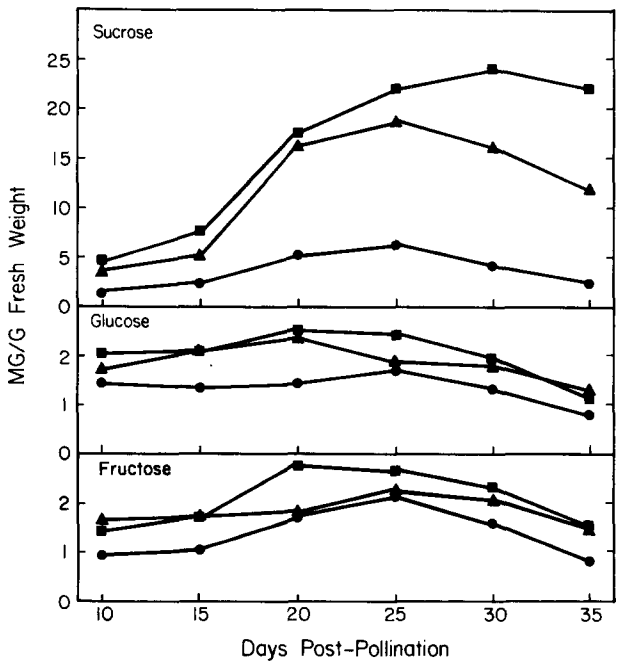

Fig. 2. Levels of sucrose, glucose and fructose throughout development in endosperms of wild type (●), *sh1* (▲) and *sh2* (■) kernels grown in vitro

Discussion

Our results indicate that growth of kernels in vitro, using a slightly modified version of the method devised by Gengenbach (1977), is similar to growth in vivo. Firstly, the expression of sucrose synthetase, ADP-glucose pyrophosphorylase and starch synthetase as a function of development was characteristic of kernels grown in vivo. The three enzymes were present in wild type kernels and sucrose synthetase and ADP-glucose pyrophosphorylase were deficient in *sh1* and *sh2* kernels respectively. That the enzymes are deficient in the mutants indicates that kernels grown in culture are expressing the genes normally expressed in the kernel. The enzymes also show distinct developmental profiles similar to results obtained by Tsai et al. (1971) and Ozbun et al. (1973) for field grown maize suggesting that activity changes in the enzymes associated with starch biosynthesis are expressed in culture as they are in vivo. Maximal activity of the starch synthetic enzymes examined here occurred at 25 dpp or 30 dpp which is in agreement with results obtained by Tsai et al. (1971) and Ozbun et al. (1973) who found maximal activity at 22–28 dpp.

Secondly, elevated sucrose levels were found in the endosperm tissue of the mutants grown in vitro as is the case for those mutants grown in vivo. Creech (1965, 1968) found that the level of sucrose in *sh2* kernels was 8–10 times the level found in wild type kernels at

22–28 dpp. Chourey and Nelson (1976) reported that the level of sucrose in *sh1* kernels at 22 dpp was approximately 4 times the level of sucrose found in wild type kernels. We found that the level of sucrose in *sh2* kernels grown in vitro was 5–8 times that found in wild type kernels whereas the level of sucrose found in *sh1* kernels grown in vitro was 3–5 times the level found in wild type kernels. Thus, elevated levels of sucrose are found in kernels grown in vitro as in kernels grown in vivo. Also, in vitro grown *sh2* kernels exhibit a greater accumulation of sucrose than do *sh1* kernels grown in vitro.

Thirdly, each genotype exhibited its distinctive, mature phenotype. Wild type seeds were plump whereas the mutants were shrunken. The *sh1* kernels had a characteristic concave crown whereas *sh2* endosperms were quite collapsed.

Fourthly, the starch contents of the three genotypes were similar for both in vivo and in vitro grown seeds. The starch content in mature *sh2* seeds grown in vitro was 32% which is similar to that found in seeds grown in vivo and is in agreement with Laughnan (1953) who found that mature *sh2* seeds contained 24.8% starch. The starch content in mature *sh1* seed was similar in both in vivo and in vitro grown seeds.

Finally, the total germination was similar for both in vivo and in vitro grown seeds. We found that both *sh1* and *sh2* seeds had less germination than did wild type seeds which is in agreement with results reported by Hannah and Cantliffe (1976) for *sh2* and Nass and Crane (1970) for *sh1*.

These results indicate that kernels actively grow and synthesize starch in culture in a manner similar to that of field-grown maize. Growth in culture of wild type, *sh1* and *sh2* kernels is representative of growth in the field.

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References

Chourey PS, Nelson OE (1976) The enzymatic deficiency conditioned by the *shrunken-1* mutations in maize. Biochem Genet 14:1041–1055

Chourey PS, Nelson OE (1979) Interallelic complementation at the *sh* locus in maize at the enzyme level. Genetics 91:317–325

Chu LC, Shannon JC (1975) In vitro cultures of maize endosperm – A model system for studying in vivo starch biosynthesis. Crop Sci 15:814–819

Creech RG (1965) Genetic control of carbohydrate synthesis in maize endosperm. Genetics 52:1175–1186

Creech RG (1968) Carbohydrate synthesis in maize. Adv Agron 20:275–322

Dickinson DB, Preiss J (1969) ADP-glucose pyrophosphorylase from maize endosperm. Arch Biochem Biophys 130:119–128

Ferguson JE, Dickinson DB, Rhodes, AM (1979) Analysis of endosperm sugars in a sweet corn inbred which contains the sugary enhancer gene and comparison of *se* with other corn genotypes. Plant Physiol 63:416–420

Gengenbach BG (1977) Development of maize caryopses resulting from in vitro pollination. Planta 134:91–93

Hannah LC, Cantliffe DJ (1976) Levels of various carbohydrate constituents and percentage germination of four everlasting heritage sweet corns. Proc Fla State Hortic Soc 89:80–82

Hannah LC, Nelson OE (1975) Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol 55:297–302

Hannah LC, Nelson OE (1976) Characterization of ADP-glucose pyrophosphorylase from *Shrunken-2* and *Brittle-2* mutants of maize. Biochem Genet 14:547–560

Hannah LC, Tuschall DM, Mans RJ (1980) Multiple forms of maize endosperm ADP-glucose pyrophosphorylase and their control by *Shrunken-2* and *Brittle-2*. Genetics 95:961–970

Hawker JS, Downton J (1974) Starch synthetases from *Vitis vinifera* and *Zea mays*. Phytochemistry 13:893–900

Jones RJ, Gengenbach BG, Cardwell VB (1981) Temperature effects on in vitro kernel development in maize. Crop Sci 21:761–766

Laughnan JR (1953) The effects of the *Sh2* factor on carbohydrate reserves in the mature endosperm of maize. Genetics 38:485–499

Nass HG, Crane PL (1970) Effect of endosperm mutants on germination and early seedling growth rate in maize (*Zea mays* L.). Crop Sci 10:139–140

Ozbun JL, Hawker JS, Greenberg E, Lammel C, Preiss J, Lee EYC (1973) Starch synthetase, phosphorylase, ADP-glucose pyrophosphorylase, and UDP-glucose pyrophosphorylase in developing maize kernels. Plant Physiol 51:1–5

Ozbun JL, Hawker JS, Preiss J (1971) Adenosine diphosphoglucose starch glucosyltransferases from developing kernels of waxy maize. Plant Physiol 48:765–769

Preiss J, Greenberg E (1972) ADP-[^{14}C] glucose. Methods Enzymol 28:279–281

Shannon J, Batey J (1973) Inbred and hybrid effects on establishment of in vitro cultures of *Zea mays* L. endosperm. Crop Sci 13:491–493

Shimamoto K, Nelson OE (1981) Movement of ^{14}C compounds from maternal tissue into maize seeds grown in vitro. Plant Physiol 67:429–432

Tsai C, Salamini F, Nelson OE (1970) Enzymes of carbohydrate metabolism in the developing endosperm of maize. Plant Physiol 46:299–306