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[^{14}C]Sucrose Uptake and Labeling of Starch in Developing Grains of Normal and *segl* Barley[1]

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ABSTRACT

Previous work showed that the *segl* mutant of barley (*Hordeum vulgare* cv Betzes) did not differ from normal Betzes in plant growth, photosynthesis, or fertility, but it produced only shrunken seeds regardless of pollen source. To determine whether defects in sucrose uptake or starch synthesis resulted in the shrunken condition, developing grains of Betzes and *segl* were cultured in [^{14}C]sucrose solutions after slicing transversely to expose the endosperm cavity and free space. In both young grains (before genotypes differed in dry weight) and older grains (17 days after anthesis, when *segl* grains were smaller than Betzes), sucrose uptake and starch synthesis were similar in both genotypes on a dry weight basis. To determine if sucrose was hydrolyzed during uptake, spikes of Betzes and *segl* were allowed to take up [fructose-U-^{14}C]sucrose 14 days after anthesis and the radioactivity of endosperm sugars was examined during 3 hours of incubation. Whereas less total radioactivity entered the endosperm and the endosperm cavity (free space) of *segl*, in both genotypes over 96% of the label of endosperm sugars was in sucrose, and there was no apparent initial or progressive randomization of label among hexose moieties of sucrose as compared to the free space sampled after 1 hour of incubation. We conclude that *segl* endosperms are capable of normal sucrose uptake and starch synthesis and that hydrolysis of sucrose is not required for uptake in either genotype. Evidence suggests abnormal development of grain tissue of maternal origin during growth of *segl* grains.

Mechanisms for the control of assimilate movement into developing grains are not known. One approach to the problem has been to examine a series of barley (*Hordeum vulgare* L.) shrunken endosperm mutants which are inherited as maternal plant monofactorial recessives and do not express xenia (5). We investigated *segl*, a mutant of the cultivar Betzes, which produces seed with 35 to 55% of normal dry weight (2). Mutant plants did not differ from normal plants in mean relative growth rate, net assimilation rate, or carbon exchange rate. Results of spike culture experiments and distribution of ^{14}C-labeled assimilates suggested that maternal spike or grain tissue is affected by the mutation, which causes premature cessation of grain filling.

Hydrolysis of sucrose appears to be involved in the transport of assimilates into the endosperm of corn (*Zea mays* L.) (10, 11), but not of wheat (*Triticum aestivum* L.) (6–8). Sucrose hydrolysis during transport into barley endosperm has not been studied using ^{14}C-labeled sugars, but in a recent study of wheat and barley involving enzyme assays and sugar analysis it was suggested that sucrose is not cleaved as it enters the endosperm of either species (1).

The purpose of this study was to characterize further the mechanism of the *segl* mutation by incubating endosperms of normal Betzes and *segl* in solutions of [^{14}C]sucrose to compare the sucrose uptake and starch synthesis capacities. We also compared their ability to randomize the label of [fructose-U-^{14}C] sucrose when supplied to cut spikes to determine whether sucrose hydrolysis occurred during sucrose uptake by either genotype.

MATERIALS AND METHODS

Endosperm Incubation. Plants were grown in an environmental chamber as described previously (2). Under these conditions, neither anther nor spike emergence correspond exactly to date of pollen shedding. Therefore, for accurate determination of developmental stage, spikes were selected on the basis of kernel fresh weight for each experiment and these weights were later compared with typical grain growth curves to estimate days after anthesis. The experiment was conducted at 5, 9, and 17 d after anthesis. The palea and lemma were removed from each grain, and the grains from several spikes of each genotype were pooled for each experiment. Five grains were incubated in 10-ml beakers containing 1 ml of 2% (w/v) sucrose in 50 mM phosphate buffer (pH 5.0). Each beaker contained 1 μCi [U-^{14}C]sucrose. Immediately before grains were placed in the incubating solution, they were cut in half transversely under water with a razor blade to expose the endosperm cavity and apoplast to the incubation solution. After 1, 2, 4, or 6 h incubations at room temperature, the sucrose solution was removed and replaced with water at 1°C. The half-grains were rinsed with additional changes of cold water for 2 min to remove the incubation solution from the surface. Then the half-grains were blotted dry, frozen, and lyophilized.

Sugar and Starch Analysis. After being weighed, grains were ground with a mortar and pestle with a small amount of 80% (v/v) ethanol. The extracts were rinsed into test tubes and heated to 80°C for 2 h, cooled, filtered through Whatman No. 1 filter paper in a Büchner funnel with rinsing, and the filtrates were brought to an equal volume. Aliquots of the ethanol extracts and the incubation solution were counted by liquid scintillation spectrometry, and sucrose uptake was determined by relating radioactivity of the ethanol extracts to the specific activity of sucrose in the medium.

The dried filter papers bearing the residues after ethanol extraction were placed in 50-ml Erlenmeyer flasks containing 10

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ml of 0.1 M phosphate buffer (pH 6.9) with 10 mM NaCl and boiled for 5 min. After cooling to room temperature, 1 ml of 1% (w/v) α-amylase (Sigma)[2] was added and the flasks were incubated at 40°C overnight to hydrolyze the starch. The contents of each flask were then filtered through Whatman No. 1 filter paper in a Büchner funnel, rinsed thoroughly with 80% ethanol, and the filtrates were brought to equal volume. Aliquots were taken for scintillation counting, and starch synthesized during the incubation period was expressed as cpm. Preliminary experiments established that no additional ^{14}C was liberated by subsequent treatment of the residue with α-amylase.

Sucrose Hydrolysis Experiment. Spikes of Betzes and *segl* were selected 14 d after anthesis and the culms were cut under water 5 mm below the rachis. Each spike was transferred to 0.1 ml water containing 1.5 μCi [fructose-U-^{14}C]sucrose (New England Nuclear). The spikes were placed under cool white fluorescent lights providing a photosynthetic photon flux density of 100 μE m^{-2} s^{-1} at 25°C. When nearly all the sucrose was taken up by the spike, water was added as needed. Each spike bore 20 grains which were numbered from bottom to top. Preliminary trials with [U-^{14}C]sucrose provided a basis for choosing incubation periods and a sampling technique. At each sampling time, 5-grain samples were taken from the spikes as follows: 0.5 h, grains 1, 5, 9, 13, 17; 1 h, grains 2, 6, 10, 14, 18; 2 h, grains 3, 7, 11, 15, 19; and 3 h, grains 4, 8, 12, 16, 20. Paleas and lemmas were removed and the pericarps were carefully peeled off. The endosperms and pericarps from each 5-grain sample were combined and rinsed in 5 ml of water at 1°C for 10 s to remove most of the endosperm cavity fluid which adhered to both tissues after dissection. Tissues and rinse water were immediately frozen. Later, endosperm samples were ground with a mortar and pestle in 80% ethanol, held for 1 h with occasional stirring, and then centrifuged at 300*g* for 10 min. The supernatants were evaporated to dryness, dissolved in 0.05 ml of 80% ethanol, and spotted equally onto two lanes of 9-cm strips of Whatman 3MM chromatography paper with a 2-cm reference lane for standards in the center. Chromatograms were developed in the descending mode for 48 h in butanol:acetic acid:water (3:1:1, v/v/v) (9). Reference lanes with sucrose, glucose, and fructose standards were stained using a procedure adapted from that of Trevelyan *et al.* (14) as follows: dried strips were dipped in acetone:40% (w/v) $AgNO_3$ (40:1), dried, dipped in ethanol:10 N NaOH (20:1), held 3 min at 25°C, dipped in 0.5 M $Na_2S_2O_3$, and dried. Those portions of one outer lane corresponding to the identified sugar zones were cut into 1-cm segments which were placed in scintillation vials. To elute the sugars, 4.5 ml of 80% ethanol was added to each vial. After at least 2 h, the vials were shaken, the filter paper was removed, and 10 ml Aquasol (New England Nuclear) was added for liquid scintillation counting. After background subtraction, counts of all vials comprising a sugar peak were summed and the relative amount of radioactivity determined for glucose, fructose, and sucrose. In preliminary experiments, no difference in recovery of different sugars from chromatograms could be detected. The sucrose zone from the other outer lane of each chromatogram was cut out and eluted with 80% ethanol which was then evaporated to dryness. The sucrose was dissolved into 1 ml water, 0.1 ml of 2 N H_2SO_4 was added, and the solution was heated in a boiling water bath for 15 min. After cooling and neutralizing with NaOH, the solutions were passed through an Amberlite MB-3 (Sigma) mixed bed deionizing column, eluted with water, and the eluate evaporated to dryness. The hydrolysate was dissolved in 80% ethanol and chromatographed as described above. A preliminary experiment showed that sucrose was completely hydrolyzed by this method and that the asymmetrically labeled sucrose preparation contained 98.5% of the radioactivity in fructose and 1.5% in glucose.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

RESULTS

Sucrose uptake by cultured endosperm halves was approximately linear between 1 and 6 h (Fig. 1). If the lines were extrapolated to 0 h, the level of sucrose would represent free space sugars which rose rapidly to a maximum level during incubation and were not rinsed out after sampling. No consistent difference was observed between Betzes and *segl*, and the rates of uptake obtained with linear regression equations (Table I) were not significantly different between genotypes based on *t* tests of the slopes of each pair of regression lines (12). Although data were expressed on a dry weight basis to eliminate grain size differences, the total amount of sugar taken up per grain increased greatly with age; at 17 d, when Betzes and *segl* grains differed significantly in dry weight, much more sugar entered Betzes than *segl* endosperms. Up to 9 d, grains of Betzes and *segl* were not significantly different in dry weight (data not shown). By 5 d the grains had achieved nearly their maximum length, but had not yet expanded laterally, under the growth conditions used in this study.

Incorporation of ^{14}C-sugars into starch (Fig. 2) exhibited a pattern similar to that of sucrose uptake, with no consistent genotype difference in the amount or rate of starch accumulation. Again, on a per grain basis, considerably more starch was labeled with increasing age, and more starch accumulated in Betzes than *segl* grains at 17 d. Rates of incorporation of ^{14}C-sugars into starch are shown in Table I. Although the difference in the slopes of the regression lines of Betzes and *segl* was barely significant at the 5% level based on a *t* test for 5-d-old grains, rates were not significantly different for 9- or 17-d-old grains.

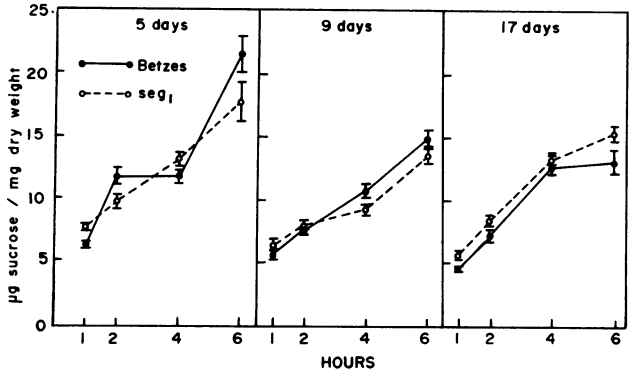

FIG. 1. Sucrose accumulation by Betzes and *segl* grains incubated in [U-^{14}C]sucrose 5, 9, or 17 d after anthesis. Points are the average of three replicate samples each consisting of five grains cut in half transversely. Bars indicate ± SE.

Table I. *Rates of Sucrose Uptake and Incorporation of ^{14}C into Starch of Barley Half-Grains Incubated in [^{14}C]Sucrose*

Age	Sucrose Uptake		^{14}C Labeling of Starch	
	Betzes	*segl*	Betzes	*segl*
	μg (mg dry weight)$^{-1}$ h^{-1}		cpm (mg dry weight)$^{-1}$ h^{-1}	
5	2.65	1.92[a]	6.34	7.58[b]
9	1.78	1.34[a]	8.18	8.83[a]
17	1.72	1.95[a]	4.74	5.37[a]

[a] Rates not significantly different between genotypes based on *t* test ($P > 0.05$).

[b] Rates significantly different ($0.05 > P > 0.02$).

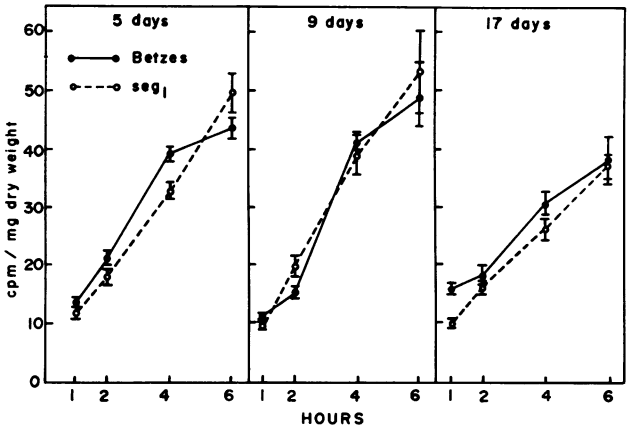

FIG. 2. Incorporation of ^{14}C into starch by grains represented in Figure 1. Bars indicate ± SE.

Table II. *Distribution of Radioactivity in Free Space and Endosperm Sugars after Incubation of Spikes in [Fructose-U-^{14}C]Sucrose*

Free space fractions consisted of rinse water from all five endosperms and pericarps of that sampling time. Values represent the percent contribution of each sugar to the total radioactivity in each sample.

Fraction	Incubation Time	Sucrose	Glucose	Fructose	Total
	h		%		*cpm*
Betzes					
Free space	1	86.6	5.2	8.2	4,208
Endosperm	0.5	96.7	0.8	2.5	4,490
	1	96.7	1.1	2.2	19,113
	2	96.5	1.1	2.4	36,917
	3	96.4	1.1	2.5	41,318
*seg*l					
Free space	1	86.5	5.8	7.7	986
Endosperm	0.5	96.6	0.6	2.8	1,042
	1	97.3	0.5	2.2	2,392
	2	97.2	1.0	1.8	9,851
	3	96.7	1.2	2.1	24,466

Whereas in the endosperm incubation experiments the grains were cut to expose the endosperm cavity and free space, thus eliminating maternal tissue as a required uptake pathway, in the sucrose hydrolysis experiment the labeled sugar was supplied to the cut spike, whereby the endosperm sugars would have traversed grain tissue of maternal plant origin. Radioactivity recovered from the endosperm cavity would represent mostly sugars which passed through the chalaza and nucellar projection and were not yet absorbed by the endosperm cells. Rinse water from endosperms sampled at 0.5 h contained levels of radioactivity only slightly above background. After 1 h of incubation, the free space of 14-d-old *seg*l grains contained only 986 cpm as compared to 4,208 cpm in the free space of Betzes grains (Table II). The pattern of radioactivity accumulation in the endosperm over time also differed between genotypes. Total cpm increased rapidly from 0.5 to 2 h in Betzes, but more slowly in *seg*l, in which grains had only one-fourth as much radioactivity as Betzes grains at 2 h (Table II). This difference probably reflects a slower movement of sugars into the free space and not a lack of ability of the endosperm to absorb sugars, because at 3 h the radioactivity in *seg*l endosperms was more than double that at 2 h.

Most of the radioactivity recovered from the endosperms at all sampling times was in the sucrose fraction, with no apparent increase in the percentage of labeled hexoses (Table II). Although

Table III. *Distribution of Radioactivity among Hexose Moieties of Sucrose in Free Space and Endosperm Sugars after Incubation of Spikes in [Fructose-U-^{14}C]Sucrose*

Fractions are the same as Table I and represent the hydrolysate of the sucrose portion of those extracts.

Fraction	Incubation Time	Glucose	Fructose
	h	%	
Betzes			
Free space	1	30.1	69.9
Endosperm	0.5	17.8	82.2
	1	32.6	67.4
	2	36.0	64.0
	3	25.2	74.8
*seg*l			
Free space	1	25.6	74.4
Endosperm	0.5	23.6	76.4
	1	29.3	70.7
	2	25.2	74.8
	3	28.1	71.9

the solution supplied to the spike contained 100% of the radioactivity in sucrose (data not shown), evidently some hydrolysis had taken place by the time labeled sugars reached the endosperm cavity. Because 13.5% of the free space radioactivity was in hexose, but only 3 to 4% of the endosperm radioactivity was in hexose, it is likely that sucrose was the primary sugar absorbed by the endosperms. There was clearly no difference between genotypes in the distribution of radioactivity among the sugars.

Distribution of radioactivity among glucose and fructose moieties of sucrose extracted from endosperms and contained in endosperm cavity (free space) fluid is shown in Table III. No trend towards randomization of label was seen from 1 to 3 h in the endosperm fractions, and the label of endosperm sucrose was not more randomized than that of free space sucrose. Because the 0.5-h Betzes endosperm sucrose exceeded the 1-h free space sucrose in asymmetry, it is possible that some randomization of label may have occurred in the endosperm cavity during 1 h of incubation. Although 4,490 and 1,042 cpm were recovered from 0.5 h extracts of Betzes and *seg*l endosperms, respectively, only 295 and 71 cpm (insufficient for meaningful analysis) were contained in 0.5-h Betzes and *seg*l rinse water, suggesting that in both genotypes sugars were rapidly absorbed by the endosperm without accumulating first in the endosperm cavity free space.

DISCUSSION

Our endosperm culture experiments suggest that the *seg*l endosperm is capable of normal sucrose uptake and starch synthesis when the barrier of maternal tissue is removed by slicing the grain in half. Gifford and Bremner (4) used this technique for endosperm culture of wheat and found that starch synthesis was more rapid for the first 3 d of culture than when the pericarp tissue was peeled off. We chose to culture sliced rather than peeled grains to reduce the wounded surface area, and also to incubate endosperms at stages when pericarp removal would be very difficult (17 d) or would result in severe endosperm damage (5 d).

Incorporation of ^{14}C into starch was essentially linear over the 6-h incubation period (Fig. 2), although the radioactivity of the ethanol-soluble fraction increased progressively (Fig. 1). If starch precursors were being progressively labeled, an exponential increase in ^{14}C incorporation into starch would be expected. We suggest that the starch precursor pool (cytoplasmic sugars) was rapidly equilibrated (within 1 h) with [^{14}C]sucrose, and that the increase in 80% ethanol-soluble radioactivity over the 6-h period

was accounted for by vacuolar sugars, fructosans, and other metabolites which were not precursors of starch. Rates of starch synthesis based on the specific activity of sucrose in the medium were much lower than normal starch synthesis rates of intact grains. This could be a result of incomplete adaptation of the sliced grains to the incubation conditions, which required at least 6 h for similarly cultured wheat grains (4). Nevertheless, 17-d-old Betzes grains, whose average dry weight was 16.6 mg, accumulated exogenous sucrose at a rate of 0.685 mg/d, which is of the same order as normal grain growth rates although somewhat low. Why most of the absorbed sugars entered soluble pools is not clear.

The *segl* character is inherited as a maternal plant monofactorial recessive gene not expressing xenia (5), and therefore *segl* plants produce shrunken seeds regardless of pollen source, and no segregation of kernels occurs within plants. This inheritance pattern is not typical of endosperm mutations in which sugar metabolism or starch synthesis enzymes are affected. Our previous experiments showed that the effects of the *segl* gene are not expressed before about 10 d after anthesis (2). The difference between Betzes and *segl* in total radioactivity entering 14-d-old grains (Table II) when spikes were supplied with equal amounts of [fructose-U-^{14}C]sucrose confirms that grains of *segl* do not exhibit normal sugar transport into the endosperm when maternal tissue must be traversed by incoming sugars. This along with the endosperm culture data indicates that maternal grain tissue is affected by the *segl* gene.

If sucrose hydrolysis were a prerequisite to uptake by the endosperm, maternal tissue might be involved. Therefore, a comparison of the results of providing [fructose-U-^{14}C]sucrose to Betzes and *segl* spikes was considered the most direct approach to testing whether the sucrose hydrolysis mechanism, if it existed in barley, was affected by the *segl* gene. In testing this hypothesis, we have obtained evidence for the intact transport of sucrose without hydrolysis in both normal and *segl* barley. This finding supports a similar suggestion by Chevalier and Lingle (1) based on sugar analysis of barley pericarp and endosperm tissues at different ages. The insoluble invertase activity in pericarp tissue which they found may have accounted for the hydrolysis and randomization of label of sucrose seen in our experiments, but the activity was not high enough to suggest an obligatory role in transport.

Sakri and Shannon (8) injected [fructose-U-^{14}C]sucrose into the peduncles of 14-d-old wheat spikes and analyzed the distribution of radioactivity among the hexose moieties of sucrose extracted from the rachis, pericarp, and endosperm after various times. At 1 h after injection, rachis sucrose was labeled 8% in glucose, and pericarp and endosperm sucrose was labeled approximately 15% in glucose. Thus, some hydrolysis and randomization occurred during transport from the stem to the pericarp, which was also found in our study. Because the progressive randomization of label in wheat endosperm sucrose closely paralleled that of pericarp and rachis sucrose, the authors suggested that sucrose was not cleaved during movement into the endosperm. In all wheat tissues examined, randomization had progressed within 4 h to about 30% in glucose, a percentage close to that at which randomization stabilized in our barley experiment. Our results are also similar to a study by Jenner (6) in which spikes of wheat were cultured on [fructose-U-^{14}C]sucrose. Endosperm sucrose extracted after 4 h retained 71% of its radioactivity in the fructose moiety, leading him to conclude that sucrose was not hydrolyzed during uptake into wheat. In addition to the above studies of wheat and barley, nonhydrolysis of sucrose upon import into developing sinks was also suggested for sugar beets (3) and soybeans (13).

In conclusion, we have shown that the *segl* gene does not impair the ability of the endosperm to absorb sucrose or synthesize starch, which is consistent with its inheritance pattern. This, in conjunction with previous data (2), indicates that maternal grain tissue such as the nucellar projection, chalaza, or phloem must be affected. In determining that Betzes and *segl* barley did not differ with respect to sucrose hydrolysis associated with transport into the endosperm, we have obtained supporting evidence that such hydrolysis is not required for phloem unloading or uptake in developing barley endosperm. Histological evidence for abnormal crease tissue development in *segl* will be described in a subsequent report.

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