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Single-kernel Analysis for the Presence of the *sugary enhancer (se)* Gene in Sweet Corn

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Abstract. **This paper presents the methodology for the extraction and quantitative analysis of the sugars from single kernels of maize (*Zea mays* L. var. *saccharata* Bailey). Concentrations of sorbitol, fructose, glucose, sucrose, and maltose were determined for individual kernels of sweet corn inbreds homozygous for the endosperm carbohydrate mutants *sugary (su)* and *sugary enhancer (se)* by gas chromatographic analysis. The extraction procedure was efficient and precise. Single-seed sugar analysis of kernels from the inbreds IL451b *(su)* and IL677a *(su se)* revealed that genetic differences between the inbreds was the primary source of phenotypic variation in kernel sugar content. Differences between ears of the same inbred was also a significant source of variation, whereas, in most instances, kernel-to-kernel variation on ears was not. Fifty-eight percent of the variation in the predominant corn sugar, sucrose, was attributed to genetic differences between the two inbreds. Analysis of the observed and predicted distribution in a mature-dry F_2 kernel population for sucrose content indicated that single-kernel analysis can isolate the action of the *se* gene in segregating populations. This procedure can be used to simplify the incorporation of *se* into elite inbreds, map its chromosomal location, and uncover other potentially useful alleles that modify corn endosperm carbohydrate metabolism.**

In recent years growers have been encouraged by consumer demand and high market value to increase production of sweet corn cultivars containing endosperm mutations that enhance kernel sugar content. High sugar content is important in sweet corn, since sugars are converted rapidly to starch during the time that ears are harvested, processed, and marketed. The consumer associates high sugar content of sweet corn with superior quality and freshness.

In the past 20 years, endosperm gene mutations other than the traditional *su* gene have been indentified and incorporated into sweet corn hybrids (1, 7). The endosperm mutations *brittle–2 (bt_2)* and *shrunken–2 (sh_2)* have been introduced into sweet corn to increase the level of sucrose, the most important corn sugar by percentage weight. One endosperm gene currently under study is the *se* gene, a recessive modifier of the standard *su* allele (5). When homozygous, the *se* allele nearly doubles endosperm sucrose content in a standard *su* background while maintaining high levels of phytoglycogen, the water soluble starch that imparts a creamy texture to the kernel (6).

Unlike other endosperm carbohydrate mutations, such as sh_2 and bt_2, which can be identified in the homozygous state by their effects on the morphology of mature seeds, kernels homozygous for *se* are not always visually distinct from *su* kernels. Transfer of the *se* gene into *su* genotypes and studies of its genetic nature have been aided by the association of several visual characteristics of the original inbred source of the *se* gene, 'IL677a' (3). Relative to *su* kernals, these characters include a lighter kernel color, a thinner and more finely wrinkled pericarp in the mature seed, and a tendency for kernels homozygous for *se* to dry more slowly during seed maturation (6). To date, breeders have had only limited success at incorporating *se* into sweet corn inbreds using these phenotypic parameters for selection. The only completely reliable method for identifying homozygous *se* individuals in segregating populations is laboratory analysis of kernel sugar content. This procedure is impractical for most breeders due to the expense of instrumentation and labor needed to analyze the large number of required samples.

A technique for individual kernel analysis for the fructose, glucose, sucrose, maltose, and sorbitol content should reduce sources of environmental variation and improve the accuracy for genetic analysis, since segregating populations of kernels will originate from the same ear and be of similar maturity. Such a method would be valuable in clarifying the genetic nature of *sugary enhancer* and other genes modifying carbohydrate metabolism in sweet corn.

The following procedure was used for single kernel sugar analysis. Individual kernels were removed from the frozen immature ears, weighed, freeze-dried, reweighed, placed in 1.5-ml plastic microfuge tubes, and ground into powder with a Phillip's screwdriver. Mature-dry kernels were ground in a rotary mill with a 20-mesh screen after weighing. Ground powder (100 to 200 mg) from a mature dry kernel or the entire freeze-dried immature kernel was placed in a microfuge tube and extracted four times with 1.0-ml portions of 95% ethanol on a rotary-evaporator at 70°C for 15 min per extraction. After each extraction, the vials were centrifuged in a tabletop centrifuge for 10 min at 8000× *g*. The supernatant fluids from the repeated extractions of each sample were combined and brought up to a 5.0-ml volume with ethanol. Previous experiments had revealed that these

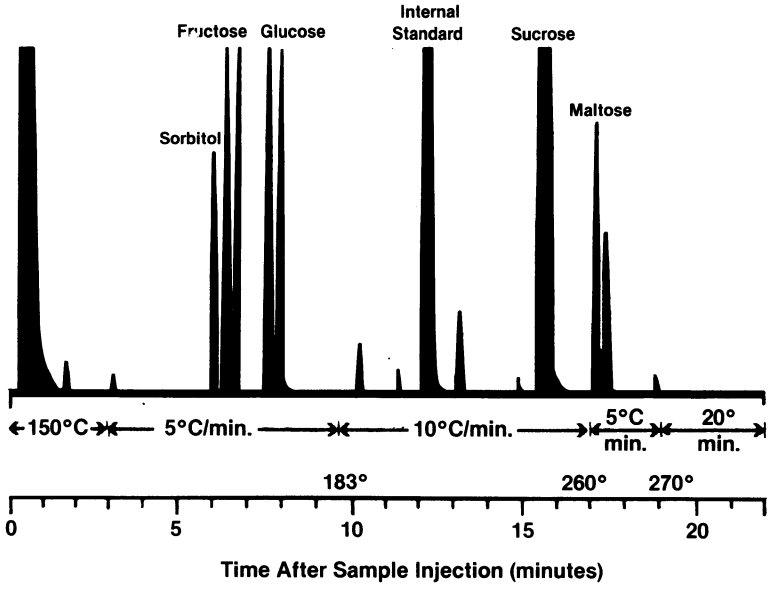

Fig. 1. Capillary gas chromatogram of the trimethylsilylated sugars from a single kernel of the sweet corn inbred, IL677a.

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Table 1. Mean sugar concentrations of kernels from the sweet corn inbreds IL451b (*su*) and IL677a (*su se*) with a listing of the relative contribution of each source of variation to the total variation in sugar content.

Kernel maturity	Sugar	Mean sugar concentrations ($mg \cdot g^{-1}$ dry wt) and SDs		Sources of variation (% of total) and their level of significance[z]			
		IL677a	IL451b	Inbreds	Ears (inbreds)	Kernels (ears)	Remainder[y]
21 DAP	Sorbitol	5.41 ± 1.9	3.2 ± 1.5	44^{***}	8^{NS}	17^{NS}	31
	Fructose	40.1 ± 15.2	17.8 ± 8.2	62^{***}	6^{*}	11^{NS}	21
	Glucose	36.7 ± 12.1	14.7 ± 7.2	64^{***}	7^{*}	12^{NS}	17
	Sucrose	172 ± 75.9	38.6 ± 9.6	65^{***}	15^{**}	2^{*}	18
	Maltose	3.8 ± 8.3	0.6 ± 2.3	57^{***}	6^{NS}	6^{NS}	31
	Total Sugar	257 ± 99.8	75.0 ± 20.2	72^{**}	12^{***}	2^{*}	14
Mature Dry	Sorbitol	1.02 ± 0.71	0.51 ± 0.19	18^{***}	17^{**}	18^{NS}	47
	Fructose	2.31 ± 1.82	1.60 ± 0.90	7^{*}	18^{*}	19^{NS}	56
	Glucose	3.12 ± 1.92	4.88 ± 1.62	20^{**}	6^{*}	34^{NS}	40
	Sucrose	49.7 ± 8.8	32.4 ± 5.5	52^{***}	7^{*}	13^{NS}	28
	Maltose	4.12 ± 2.69	0.29 ± 0.28	51^{***}	8^{*}	18^{NS}	24
	Total Sugar	60.4 ± 14.3	39.7 ± 5.2	48^{***}	10^{**}	13^{NS}	29

[z] Degrees of significance for inbreds, ears within inbreds, and kernels within ears are presented as NS when $P > 0.05$, as * when $0.01 < P < 0.05$, as *** when $0.001 < P < 0.01$, and as ** when $P < 0.001$.

[y] The remainder of the variation in kernel sugar concentrations was from the interaction between inbreds and kernels plus the three-way interaction (inbreds × ears × kernels).

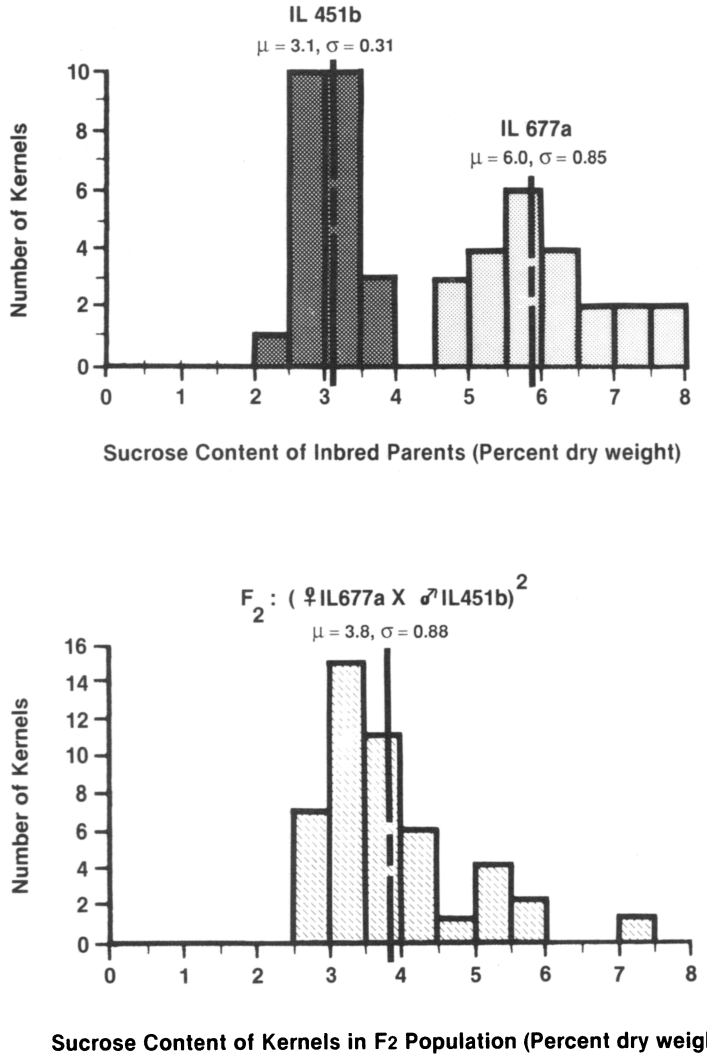

Fig. 2. Frequency distribution for sucrose content of mature dry kernels of the inbred parents and an F_2 population generated from their hybridization.

procedures were effective in extracting >98% of the sugars in the ground kernel sample. A 150-μl portion of the extract then was placed in a 500-μl reactivile, evaporated to dryness, and capped. Fifty microliters of stox-oxime reagent (Pierce) with phenyl-B-D-glucopyranoside mixed in as an internal standard was injected into the vial, vortexed, heated for 15 min at 70°C, and again vortexed. Fifty microliters of *N*-trimethylsilyl imidazole (TMSI) (Pierce) then was injected into the vial prior to gas chromatographic analysis (5). These reagents result in the formation of trimethylsilyl (TMS) derivatives of the sugars in the sample after their stabilization as oximes.

The reactiviles then were loaded onto a gas chromatograph (Hewlett Packard model 5760) with an autosampler (Hewlett Packard model 7671A), a 12.5-m crosslinked methyl silicone capillary column (0.1 mm i.d.), a flame ionization detector, and an integrator (Hewlett Packard 3390A). About 1μl of each sample was injected by the autosampler into the injection port where the derivatized constituents were loaded onto the column with helium as a carrier gas at a 100:1 split ratio. Figure 1 presents a typical chromatogram of the derivatized ethanol extract from a kernel of 'IL677a' with information on the temperature program used for these analyses. This program provides good peak separation with a relatively short sample recycling time. The reducing sugars—fructose, glucose, and maltose—form two isomeric oxime derivatives with two slightly different chromatographic retention times. The peak areas for the two isomeric forms are summed for quantitative analysis. The relative retention times (retention time of specific sugar/retention time of internal standard) under these conditions were 0.69 for sorbitol, 0.70 and 0.71 for fructose, 0.77 and 0.78 for glucose, 1.22 for sucrose, and 1.36 and 1.37 for maltose. The respective peak areas for each TMS corn sugar is converted into a fraction of the peak area of the internal standard. These values for each sample then can be converted into milligrams of specific sugar (sorbitol, fructose, glucose, sucrose, and maltose) per gram of kernal wet or dry weight by comparison with chromatograms from a set of premixed standards. Previous studies have shown close agreement in total sugar content of similar subsamples as determined by this procedure and the one described by Dickinson et al. (3).

To test the reproducibility of this extraction and analytical procedure, 100 mature dry kernels from a single ear of 'IL779a' (a sweet corn inbred homozygous for both *su* and *se*) were bulked and ground into a composite sample. Six separate subsamples were

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then extracted and analyzed as described above. The mean and standard deviation (milligrams per gram of dry kernel weight) of each sugar over the six samples were as follows: Fructose (2.5 ± 0.084), glucose (6.6 ± 0.14), sucrose (56.4 ± 0.64), and maltose (31.4 ± 0.58). The low standard deviation for sugar content is indicative of the precision of this method.

An experiment then was conducted using single-kernel analysis to measure the phenotypic variation in sugar content between a *su* and a *su se* inbred, between ears of the same inbred, and between kernels from the same ear. Four selfed ears of the inbreds IL451b (homozygous for *su*) and IL677a (homozygous for *su* and *se*) were harvested at 21 days after pollination (DAP) and at kernel maturity. The 21 DAP ears were promptly husked, frozen in liquid nitrogen, and stored in a freezer at -80°C for analysis.The mature ears were harvested at 55 DAP and air-dried in a forced-air oven at 33°. Six kernels from each corn were selected for analysis, the results of which are presented in Table 1, along with an analysis of variance.

At both harvest dates, kernels of the *su se* inbred, IL677a, possessed significantly greater amounts of all sugars (except glucose in mature dry kernels) than IL451b. The primary source of the total variability in kernel sucrose content, which accounts for 68% of the total sugars at 21 DAP in 'IL677a' and 53% in 'IL451b', comes from genetic differences between the two inbreds. Sources of variation due to ears within inbreds and kernels within ears accounted for much less variability for all the sugars. With the exceptions of sorbitol and maltose at the 21 DAP harvest, ear-to-ear differences within inbreds was also a significant source of kernel sugar variation. Kernel within ear variation was significant only for sucrose at 21 DAP, although it represented only 2% of the total variation in sucrose compared to 65% due to genetic differences between the inbreds. The significant difference for sucrose was due primarily to kernel-to-kernel differences in ears of IL677a, which, on average, contained 4.4 times as much sucrose as kernels of IL451b. The kernel-to-kernel significant difference ($P = 0.049$) at 21 DAP for total sugar resulted from kernel sucrose variation.

These results indicate that kernels homozygous for endosperm carbohydrate mutations, such as *se*, might be identified by single-kernel analysis in segregating plant populations, particularly when segregation is occurring on an individual ear. Since individual ears of corn can possess up to 500 kernels, and kernel-to-kernel variation for sugar on the same ear is small compared to genetic differences, kernel populations on the same ear segregating for endosperm carbohydrate mutations could be subjected effectively to genetic analysis. To test this hypothesis, a cross was made between 'IL677a' and 'IL451b' to generate F_1 seed. A plot of this F_1 seed was planted adjacent to plots of the inbred parents, and several plants in each of these populations were selfed to generate F_2 kernels. Several ears of the selfed inbreds and hybrids were harvested at 55 DAP and dried in a forced-air over at 33°C. Fifty kernels from a single selfed F_1 ear and 25 kernels from one ear of each of the parent inbreds were harvested and analyzed individually for sugar content. According to Ferguson et al. (4), the *se* gene is a recessive modifier of the *su* locus in sweet corn and should segregate phenotypically in an F_2 population from a cross between a *su* and *su se* inbred in a bimodal proportion of three *su* kernels (lower sucrose content) to one *su se* kernel (higher sucrose content). Figure 2 presents histograms of the kernel sucrose content of the 25 mature-dry kernels of each parental genotype and that of 47 mature dry F_2 kernels generated from the cross between these inbreds. The kernels at the upper end of the F_2 sucrose distribution should be homozygous for *se*. Since statistical analysis of the parental kernel populations indicated that they were distributed normally, it is possible to generate a theoretical distribution for the F_2 where, according to a single recessive gene model, 75% of the population would be distributed about the mean of IL451b parent and 25% distributed about the mean of the *se* inbred. Thirty-seven of the F_2 kernels fell within the mean and standard deviation of IL451b and the remaining 10 within the mean and standard deviation of IL677a. The χ^2 test for the goodness of fit between the expected and the observed F_2 distribution generated a value of 0.348. With 1 df, this value indicates that the probability of a significant difference existing between the theoretical and observed frequency distributions is 0.58. The model adequately describes the F_2 kernel sucrose distribution.

These findings suggest that single-kernel analysis can isolate the action of the *se* gene in segregating populations.

The single-kernel analytical procedure also can be used to map the chromosomal location of the *se* gene. Linked morphological or molecular genetic markers potentially could be used to select indirectly for *se* in segregating populations even in the heterozygous state. This analytical technique also could uncover other potentially beneficial endosperm carbohydrate-modifying genes that could be of use in sweet corn cultivar improvement programs.

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