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Exotic Corn Lines with Increased Resistant Starch and Impact on Starch Thermal Characteristics

Kim A. Rohlfing
Iowa State University

Linda M. Pollak
United States Department of Agriculture

Pamela J. White
Iowa State University, pjwhite@iastate.edu

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Abstract
Ten parent corn lines, including four mutants (dull sugary2, amyloseextender sugary2, amylose-extender dull, and an amylose-extender with introgressed Guatemalen germplasm [GUAT ae]) and six lines with introgressed exotic germplasm backgrounds, were crossed with each other to create 20 progeny crosses to increase resistant starch (RS) as a dietary fiber in corn starch and to provide materials for thermal evaluation. The resistant starch 2 (RS2) values from the 10 parent lines were 18.3–52.2% and the values from the 20 progeny crosses were 16.6–34.0%. The %RS2 of parents was not additive in the offspring but greater RS2 in parents was correlated to greater RS2 in the progeny crosses (r = 0.63). Differential scanning calorimetry (DSC) measured starch thermal characteristics, revealing positive correlations of peak gelatinization temperature and change in enthalpy with %RS2 (r = 0.65 and r = 0.67, P ≤ 0.05); however, % retrogradation (a measure of RS3) and retrogradation parameters did not correlate with %RS2. The %RS2 and onset temperature increased with the addition of the ae gene, likely because RS delays gelatinization.

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Kim A. Rohlfing,[1] Linda M. Pollak,[2] and Pamela J. White[1,3]

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ABSTRACT

Ten parent corn lines, including four mutants (*dull sugary2, amylose-extender sugary2, amylose-extender dull*, and an *amylose-extender* with introgressed Guatemalen germplasm [GUAT *ae*]) and six lines with introgressed exotic germplasm backgrounds, were crossed with each other to create 20 progeny crosses to increase resistant starch (RS) as a dietary fiber in corn starch and to provide materials for thermal evaluation. The resistant starch 2 (RS2) values from the 10 parent lines were 18.3–52.2% and the values from the 20 progeny crosses were 16.6–34.0%. The %RS2 of parents was not additive in the offspring but greater RS2 in parents was correlated to greater RS2 in the progeny crosses (r = 0.63). Differential scanning calorimetry (DSC) measured starch thermal characteristics, revealing positive correlations of peak gelatinization temperature and change in enthalpy with %RS2 (r = 0.65 and r = 0.67, $P \leq 0.05$); however, % retrogradation (a measure of RS3) and retrogradation parameters did not correlate with %RS2. The %RS2 and onset temperature increased with the addition of the *ae* gene, likely because RS delays gelatinization.

Four types of resistant starch (RS) have been defined. RS1 is resistant because of the surrounding food matrix; RS2 is present in ungelatinized, raw starches; RS3 is created by retrogradation; and RS4 is produced through chemical alteration (Englyst et al 1996). Incorporation of RS into the diet provides many health benefits: it serves as a prebiotic, or fermentable substrate, for the growth of probiotics; lowers the pH of the colon (Cherrington et al 1991); increases mineral absorption (Courdray et al 1997); and increases cell turnover (Young et al 2005). Cholesterol metabolism may be down-regulated by RS, by production of short-chain fatty acids that may either suppress cholesterol synthesis in the liver (Hara et al 1999) or decrease cholesterol absorption (Vahouny et al 1988).

Corn endosperm mutants can affect appearance of the kernel or underlying component quality, while double mutants may synergistically affect endosperm appearance and quality. Corn starch properties in the germplasm can be modified by traditional plant breeding methods using major (e.g., naturally occurring mutant genes), or minor (modifying genes) genetic factors (Ji et al 2004) where the effect is enhanced by crossing the lines. Exotic corn lines may provide unusual traits of interest including increased %RS through the presence of modifying genes. High-amylose (*amylose-extender*, *ae*) corn lines provide greater amounts of RS2 than *normal* corn through a major (mutant) gene. Thus, crossing *ae* and exotic corn types could increase the RS, provide unique materials for food use, and possibly provide cooking properties better than *ae* corn lines used alone.

Whereas four types of RS have been defined, only RS2, RS3, and RS4 are routinely measured. The process of extraction may alter RS1 because it destroys the surrounding food matrix. There are several options available for measuring RS2, RS3, and RS4. The Megazyme RS kit measures RS2 effectively using Approved Method 32-40 (AACC International 2010) and is designed to screen large numbers of samples (McCleary and Monaghan 2002). However, most starch is not eaten in an ungelatinized form; the starch is generally cooked. Thus, the Megazyme kit, which includes no gelatinization step, may not be an accurate measure of RS as eaten.

[1] Graduate student and professor, respectively, E262 Lagomarcino, College of Human Sciences and Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011.
[2] Research geneticist, USDA-ARS, Corn Insects and Crop Genetics Research Unit, Department of Agronomy, Iowa State University, Ames, IA 50011.
[3] Corresponding author. Phone: 515-294-5380. Fax: 515-294-7802. E-mail: pjwhite@iastate.edu

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Differential scanning calorimetry (DSC) measures starch gelatinization characteristics, including retrogradation, the cause of RS3. Thus, starches with a high percentage of retrogradation should have high RS3 percentages (Haralampu 2000). The starch properties from many corn mutants, including *sugary2 (su2)*, *amylose extender (ae)*, and *amylose dull* (*ae du)*, were examined by DSC (Tziotis et al 2005). Less work has been done on double mutants, and especially on other mutants that vary in RS. An *ae* starch might provide a large amount of both RS2 and RS3. The *ae* starches do not completely gelatinize under boiling temperatures (Champ 1992), leaving some of the RS2 intact; the starch that does gelatinize would be available for retrogradation and, thus, a source of RS3.

Thermal characterization of starches from corn lines with elevated levels of RS has not been done, especially in relation to RS2 measured with the Megazyme kit and RS3 measured with DSC. These evaluations would be useful in predicting behavior of the corn starches in food products.

The objectives of this study were to identify new corn breeding crosses containing high %RS2 by crossing four mutants and six lines with introgressed exotic backgrounds with each other and to relate the percentage of RS2 in the starches measured by using the Megazyme Resistant Starch kit (RS2), with the %RS3, and other thermal characteristics measured on DSC.

MATERIALS AND METHODS

Corn Materials

Ten corn lines (*Zea mays* L.), four mutants and six lines with introgressed exotic backgrounds (Table I), termed 'parents', were crossed with each other to create 20 progeny crosses (Table II). The GUAT *ae* parent is the first public 70% amylose line (Campbell et al 2007). For easier discussion and comparison, the progeny crosses are separated into four groups and identified by the first parent, creating the mutant groupings of *dull sugary2* (*du su2), amylose-extender sugary2(ae su2), amylose-extender dull (ae du)*, and an *amylose-extender* with introgressed Guatemalan germplasm (GUAT *ae)*.

All progeny crosses were grown at Juana Diaz, Puerto Rico, in 2006 and 2007 and were the second-selfed generation in the process of developing inbred lines. Ears were harvested at full maturity, and dried at 37.5°C to ≈12% moisture. Seeds were stored at 4°C and 10% rh until needed for starch extraction. Seeds from individual ears were pooled and 15 kernels were randomly selected for each starch extraction. Commercial cornstarch (Sigma, St. Louis, MO) was used as a typical corn starch control. A high-amylose control (High Am-C, amylogel 03001, 50% apparent amylose, Cargill, Cedar Rapids, IA) was used as an *ae* control in the analyses.

Starch Extraction

From each parent and progeny cross, starch was extracted twice from two sets of 15 randomly selected kernels from an individual ear. The two extractions were treated as replicates. Starch was extracted from the 15 randomly selected kernels based on the procedures of Krieger et al (1997) with modifications. A 100-µm filter (N100C Cellmicrosieves, Biodesign, New York, NY) was used as suggested in the Megazyme RS kit (McCleary and Monaghan 2002). The filtrate was allowed to settle at 4°C for 24 hr, after which the supernatant was discarded. The remaining slurry was centrifuged at 1,000 × *g* for 10 min and supernatant was again discarded. The pellet was dried at 45°C for 24–48 hr. After extraction, starches were stored in a desiccator until needed for analyses.

RS Determination

The Megazyme RS kit (K-RSTAR, Megazyme International, Bray, Ireland) was used to determine the RS content of ungelatinized starch according to Approved Method 32-40 (AACC International 2010). Modifications included 50 µL of amyloglucosidase was added to 50 µL of diluted RS solution to ensure all RS was converted to glucose before the addition of glucose oxidase-peroxidase reagent (GOPOD). All analyses for each replicate were conducted twice and the averages were computed.

DSC

The method of White et al (1990) with modifications by Krieger et al (1997) was followed. Briefly, starch (4 mg, db) was weighed in stainless steel pans, 8 µL of distilled water was added, and the pan was sealed. Pans were added to the DSC Diamond using an autosampler and Intercooler 2P (Perkin-Elmer, Norwalk, CT). The intercooler kept the pans in the autosampler at –100°C. Once the pans entered the DSC oven they were equilibrated at 25°C for 5 min and then scanned from 25 to 180°C at 10°C/min. Data was then analyzed with Pyris Step Scan software (v.3.7, Perkin-Elmer). All analyses for each replicate were conducted twice and the averages were computed. Onset temperature (T_{oG}), peak temperature (T_{pG}), and change in enthalpy (ΔH_G) were computed for initial gelatinization. The gelatinized pans were stored for seven days at 4°C and rescanned to determine retrogradation using the same program as for gelatinization and measuring retrogradation onset (T_{or}), retrogradation peak temperature (T_{pr}), and change in enthalpy of retrogradation (ΔH_r) (White et al 1989). Endpoint was recorded but not presented because differences were not significant. Retrogradation (*%r*) is ΔH_r divided by ΔH_G.

Statistical Analyses

The proc ANOVA program (SAS Institute, Cary, NC) was used to determine significant differences ($\alpha = 0.05$) in %RS2 between lines. Correlations were evaluated among RS2 and all DSC parameters, including *%r* as RS3, using Excel software (Microsoft Office 2007, Seattle, WA). Regression analyses using SAS were done between the %RS2 averages of progeny crosses and the parents of those progeny crosses to determine whether %RS2 was an additive trait.

TABLE I
Pedigree Information and ID Labels for 10 Parent Lines

Pedigree	ID Label	When/Where Grown	Generation
HSY99 *du su2*	*du su2*	Juana Diaz Puerto Rico, 2005-2006	Inbred line
HSY99 *ae su2*	*ae su2*	Juana Diaz Puerto Rico, 2005-2006	Inbred line
HSY99 *ae du*	*ae du*	Juana Diaz Puerto Rico, 2005-2006	Inbred line
GUAT209:S13//Oh43*ae*/H99*ae*-1-2-1	GUAT *ae*	Iowa Agronomy Farm, 2006	S_3
AR01105:S01-1082	AR	Iowa Agronomy Farm, 2005	S_1
UR13061:S22-1092	URS	Iowa Agronomy Farm, 2006	S_1
CU110:N17-1172	CUBA	Iowa Agronomy Farm, 2005	S_1
B/3/DK212T:S610-8-1-3-4-8-2	DK	Iowa Agronomy Farm, 2003	S_6
BR52051:S17-1112	BR	Iowa Agronomy Farm, 2005	S_1
UR13085:N214-14-1	URN	Iowa Agronomy Farm, 2006	S_2

TABLE II
Pedigree Information and ID Labels for 20 Progeny Crosses of the S_2 Generation[a]

Cross Pedigree	ID Label
(HSY99 *du su2*/AR011050:S01-1082)-2-2	(*du su2*/AR)-2-2
(HSY99 *du su2*/CU110:N17-1122)-3-1	(*du su2*/CU)-3-1
(HSY99 *du su2*/CUBA110:N17-1122)-3-2	(*du su2*/CU)-3-2
(HSY99 *du su2*/AR011050:S01-1082)-2-1	(*du su2*/AR)-2-1
(HSY99 *du su2*/AR011050:S01-1082)-2-3	(*du su2*/AR)-2-3
(HSY99 *du su2*/CUBA110:N17-1122)-3-1	(*du su2*/AR)-3-1
(HSY99 *du su2*/CUBA110:N17-1122)-3-2	(*du su2*/AR)-3-2
(HSY99 *ae su2*/AR011050:S01-1082)-3-1	(*ae su2*/AR)-3-1
(HSY99 *ae su2*/UR13061:S22-1092)-2-1	(*ae su2*/URS)-2-1
(HSY99 *ae su2*/AR011050:S01-1082)-03)-02	(*ae su2*/AR-)3-2
(HSY99 *ae du*/AR011050:S01-1082)-1-1	(*ae du*/AR)-1-1
(HSY99 *ae du*/UR13085:N215-14-1)-2-2	(*ae du*/URN)-2-2
(HSY99 *ae du*/AR011050:S01-1082)-1-2	(*ae du*/AR)-1-2
(HSY99 *ae du*/UR13061:S22-1092)-3-2	(*ae du*/URS)-3-2
(GUAT209:S13/Oh43*ae*/H99*ae*/AR011050:S01-1082)-1-1	(GUAT *ae*/AR)-1-1
(GUAT209:S13/Oh43*ae*/H99*ae*/UR13061:S22-1092)-2-1	(GUAT *ae*/URS)-2-1
(GUAT209:S13//Oh43*ae*/H99*ae*/UR13061:S22-1092)-2-2	(GUAT *ae*/URS)-2-2
(GUAT209:S13//Oh43*ae*/H99*ae*/3/BR52051:S17-1112)-1-2	(GUAT *ae*/BR)-1-2
(GUAT209:S13//Oh43*ae*/H99*ae*/3/DK212T:S610-8-1-3-4-8-2)-1-1	(GUAT *ae*/DK)-1-1
(GUAT209:S13//Oh4 *ae*/H99*ae*/3/DK212T:S610-8-1-3-4-8-2)-1-2	(GUAT *ae*/DK)-1-2

[a] Grown in Juana Diaz Puerto Rico in 2006-2007. Numbers outside of pedigree parenthesis refer to ear number.

RESULTS AND DISCUSSION

Resistant Starch 2

Of the parents studied, those with the *ae* gene in their backgrounds had the greatest %RS2 (Table III). The GUAT *ae* parent had greatest overall in RS2 at 52.2%, which was different from all other lines. The *ae du* parent also had a high %RS2 at 30.6 and was intermediate compared with the GUAT *ae* parent and the remaining parent with values of 18.3–23.9%. The progeny crosses had more variability than the parents. Progeny crosses with GUAT *ae* in their background had the greatest %RS2, 23.7–34.0%, but none retained the %RS of the GUAT *ae* parent (52.2%). In general, the %RS2 increased in the order *du su2* < *ae su2* < *ae du*. The exotic parent did not affect %RS2 enough to produce differences between the lines of the first three mutant parent groups (*du su2*, *ae su2*, *ae du*). For example, %RS2 of the progeny crosses, (*du su2*/AR)-3-1 through (*du su2*/CU)-3-2, were not different (Table III). The AR and CU parents provided similar amounts of RS2 to their progeny. Mutant progeny crosses with *ae su*2 and *ae du* followed similar trends. The progeny crosses with the mutant parent, GUAT *ae,* differed between exotic crosses. URS and BR, the exotic sources, contributed less RS2 than any of the AR crosses and the DK parents. All of the progeny crosses are of the S_2 generation when homozygosity of a trait throughout an entire ear of corn is not ensured. Successive generations of the GUAT *ae*/AR cross should be grown and analyzed.

All parents, with the exception of GUAT *ae,* and progeny crosses had RS2 values higher than the commercial cornstarch (8.9%) but lower than the High Am-C sample (40.2%). Regression analysis showed that higher RS2 in the parents lead to higher RS2 in the progeny crosses ($r = 0.59$, $P \leq 0.05$) but expression of RS2 was not additive. Parents with higher %RS2 did not combine to create progeny crosses with RS2 higher than the parent with greater %RS2. The recessive genes, *ae*, *su2*, and *du,* previously increased amylose expression (Shannon and Garwood 1984). Presence of the *ae* gene increased the amounts of amylose, which lead to increased amounts of RS2, as previously noted by Shu et al (2007). The *ae du* gene also increased the amylose content of corn starch by 10–15% (Shannon and Garwood 1984).

Thermal Analyses by DSC

The parents had less differentiation in both gelatinization and retrogradation profiles than did the progeny crosses (Table III). The GUAT *ae* parent showed a large ΔH_G of 34.8 J/g but a very small ΔH_r of 3.2 J/g. The progeny crosses showed a slightly greater T_{pG} range (68.3–76.4°C) than the parents (61.8–74.7°C). There was a strong correlation between %RS2 and T_{pG} ($r = 0.65$; individual correlations not shown). The presence of RS2 delays gelatinization, logically relating to a higher T_{pG}. Another strong correlation occurred between %RS2 and ΔH_G ($r = 0.67$); however, understanding the second of these correlations is more complex. The presence in the corn lines of known mutants (*ae, du, su*$_2$) and modifying genes from the introgressed exotic backgrounds, coupled with their interactions, brought several factors into play. In particular, the *ae* and *su*$_2$ genes, both with elevated RS2 levels, behave quite differently during gelatinization. The greatest ΔH_G was for the (GUAT ae/DK)-1-1 cross with ΔH_G of 17.5 J/g. Starch from all progeny crosses had ΔH_G near that of the commercial corn starch (13.1 J/g).

The starch from the GUAT *ae* group had among the highest onset and peak temperatures, with the rest of the progeny crosses following the trend *du su2* < *ae su2* < *ae du* (Table III). Previ-

TABLE III
%RS2 of Parents and 20 Progeny Crosses

		Gelatinization[c]			Retrogradation[c]			
Parent ID[a]	RS (%)[b]	T_{oG} (°C)	T_{pG} (°C)	ΔH_G	T_{or} (°C)	T_{pr} (°C)	ΔH_r	% *r*
BR	18.3c	70.6a	74.6a	15.3ab	44.9b	59.9ab	9.0a	0.62a
DK	18.8c	67.6ab	72.6a	13.5ab	47.7ab	52.4b	1.0bc	0.07bc
CUBA	22.0c	70.4a	74.7a	13.5ab	49.7ab	58.6b	2.4bc	0.17abc
URS	21.9c	69.0a	73.7a	11.8ab	53.2ab	57.8b	1.0bc	0.10bc
du su2	23.9c	63.8bc	66.9b	3.3b	53.0ab	73.5a	1.9bc	0.56a
ae su2	18.5c	60.95c	61.8b	8.5ab	54.2ab	57.9b	0.5c	0.05c
ae du	30.6b	69.0a	73.3a	6.4ab	51.3ab	57.2b	5.9ab	0.53ab
AR	18.7c	69.6a	72.7a	10.1ab	54.8ab	60.0ab	1.7bc	0.18a–c
GUAT *ae*	52.2a	62.3c	66.7b	34.8a	60.8a	62.7ab	3.2bc	0.30a–c
URN	19.1c	71.3a	73.7a	11.1ab	59.8ab	63.2ab	1.0bc	0.09bc
Cross ID Label								
(*du su2*/AR)-3-1	19.5d–g	65.7f–h	71.5f–h	11.3d–f	52.7b–d	60.5b–h	2.1b–d	0.19a–d
(*du su2*/AR)-3-2	20.6c–g	64.0gh	68.3ij	10.7d–g	53.0b–d	58.9d–h	1.7cd	0.15b–d
(*du su2*/AR)-2-1	16.9g	62.5h	67.5j	7.9h–j	54.2b–d	59.4c–h	3.2b–d	0.43a–d
(*du su2*/AR)-2-2	16.6g	63.9gh	71.1g–i	8.2h–j	55.9a–d	57.7e–h	1.7cd	0.21a–d
(*du su2*/AR)-2-3	17.1g	66.7e–g	71.8e–h	7.2ij	50.4cd	56.4gh	2.7b–d	0.28a–d
(*du su2*/CU)-3-1	17.3g	67.6d–g	72.6d–h	9.4e–i	48.8d	57.5f–h	3.0b–d	0.32a–d
(*du su2*/CU)-3-2	16.9g	64.6gh	70.2h–j	12.3cd	53.1b–d	59.8c–h	2.2b–d	0.19a–d
(*ae su2*/AR)-3-1	17.9fg	62.3h	68.5ij	8.8g–j	51.3b–d	58.3e–h	1.4d	0.17a–c
(*ae su2*/AR)-3-2	18.2fg	65.6f–h	72.9c–h	8.4g–j	51.3b–d	57.6e–h	1.2d	0.14cd
(*ae du*/AR)-1-1	20.9c–g	68.6b–f	73.2b–g	9.7efgh	59.3a–c	64.1a–d	1.2d	0.13cd
(*ae du*/AR)-1-2	20.6c–g	69.4a–f	73.8a–g	6.5j	56.9a–d	61.0b–g	2.8b–d	0.44a–d
(*ae du*/URN)-2-2	22.6b–e	71.9ab	75.8ab	8.0h–j	50.8b–d	54.8h	4.0b–d	0.50a–c
(*ae du*/URS)-3-2	22.4b–f	67.7c–g	71.9e–h	9.3f–i	50.8b–d	56.5gh	1.3d	0.06d
(GUAT *ae*/AR)-1-1	34.0a	66.8e–g	74.2a–f	12.4cd	64.6a	67.3a	5.7a–c	0.47a–c
(GUAT *ae*/AR)-1-2	25.6b	71.1a–d	74.3a–e	8.3h–j	50.7b–d	58.5d–h	4.4a–d	0.47a–c
(GUAT *ae*/BR)-1-2	24.4bc	70.5a–e	75.0a–d	13.7bc	48.1d	56.8f–h	2.7b–d	0.20a–d
(GUAT *ae*/URS)-2-1	23.7b–d	73.2a	76.4a	12.7cd	60.4ab	63.3a–e	4.3a–d	0.31a–d
(GUAT *ae*/URS)-2-2	24.6bc	71.6a–c	75.7a–c	16.0ab	55.1a–d	66.2ab	8.4a	0.52ab
(GUAT *ae*/DK)-1-1	33.2a	70.2a–e	73.0c–h	17.5a	59.5a–c	64.9a–c	6.1ab	0.34a–d
(GUAT *ae*/DK)-1-2	31.4a	69.0b–f	74.2a–f	11.7c–e	55.2a–d	62.4a–f	5.9ab	0.40a–c

[a] ID labels as listed in Table I.
[b] Measured by Megazyme Resistant Starch kit.
[c] Gelatinization and retrogradation characteristics measured by differential scanning calorimetry (DSC), scanning at 25–180° C. Values are average of three replicates. Values followed by the same letter in the same column are not significantly different ($\alpha = 0.05$).

ously, the presence of the *su2* gene increased the starch component amylose by ≈10%, (Campbell et al 1994). In other work, the *su2* gene decreased the T_{oG} and the ΔH_G values, especially compared with *normal* corn starch (Inouchi et al 1984). Although increased amylose leads to increased RS2, the *su2* gene causes increased digestibility, possibly because of the long B-chains and few branch points of the amylose in *su2* mutants (Takeda and Preiss 1993). Branching slows digestion, thus fewer branch points could increase digestibility and reduce the %RS. These observations were reflected in the data with progeny crosses containing the *su2* gene, which had among the lowest onset temperatures and among the lowest RS2. This observation and explanation does not take into account the possibility of RS creation through tangled amylose chains forming enzyme-inaccessible areas. Starches with higher gelatinization temperatures might retain more RS2 during heating. Temperatures at ≤180°C may be needed to fully gelatinize starches containing amylose ≥50% (Inouchi et al 1984; Sanders et al 1990).

The *%r* was highly variable among the mutants (6–52%) with no correlations between RS2, the presence of the *ae* gene, or any retrogradation parameter (Table III). Differences did not depend on mutant groups. However, the parent with the highest *%r* was the BR parent, which also had the lowest %RS2 as measured by the Megazyme kit. Retrogradation of starch from individual mutants should vary according to the amylose-to-amylopectin ratios (Zhang et al 2008). Amylose recrystallizes quickly upon cooling, whereas amylopectin recrystallizes more slowly; thus more amylose (%) should provide greater retrogradation (*%r*), which did not occur in the current study. The amount of RS3 formed, however, could vary, based on the factors just mentioned, ease of granule rupture which would reduce starch realignment, and annealing during the gelatinization process (Yao et al 2009). Both parents and progeny crosses in this study showed unusual DSC peak shapes with a large amount of tailing, thus reducing the accuracy of the *%r* measurement and conclusions regarding RS3.

CONCLUSIONS

Twenty exotic crosses of maize from 10 parents with mutant or exotic backgrounds were evaluated for RS2 concentrations. The progeny crosses did not have greater %RS2 than their parents, showing no transgressive segregation. There was a moderate correlation between %RS2 and T_{pG}, likely because the presence of RS2 delays gelatinization. The GUAT *ae* mutants had the greatest %RS2 and T_{pG}, and the starches from the parents and progeny crosses had more RS2 than did commercial corn starch. Only small amounts of RS3 (or *%r*) in the corn types were revealed by the retrogradation evaluation on the DSC. That is, the ΔH_r values were smaller than values for normal corn starch, likely because of the greater amounts of RS2. Understanding the effect of RS2 on the gelatinization characteristics of starches will help the food industry understand its effect on food processing, especially processing involving heating. Using traditional plant breeding to develop corn lines for increased RS2 seems promising, particularly with the identification of parent lines high in amylose (>50%). The development of high-amylose, high-RS2 corn types could provide new sources of high-fiber products useful to the food industry in its quest for healthful foods and food ingredients.

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