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Variations in Starch Physicochemical Properties from a Generation-Means Analysis Study Using Amylomaize V and VII Parents

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GEMS-0067 (PI 643420) maize line is a homozygous mutant of the recessive *amylose-extender* (*ae*) allele and an unknown number of high-amylose modifier (HAM) gene(s). GEMS-0067 produces starch with a ~25% higher resistant-starch (RS) content than maize *ae* single-mutant starches. The objective of this study was to understand how the HAM gene(s) affected the RS content and other properties of *ae*-background starches. Nine maize samples, including G/G, G/F1, G/H, F1/G, F1/F1, F1/H, H/G, H/F1, and H/H with HAM gene-dosages of 100, 83.3, 66.7, 66.7, 50, 33.3, 33.3, 16.7, and 0%, respectively, were produced from self- and intercrosses of GEMS-0067 (G), H99ae (H), and GEMS-0067×H99ae (F1) in a generation-means analysis (GMA) study. RS contents of examined starches were 35.0, 29.5, 28.1, 32.0, 28.2, 29.4, 12.9, 18.4, and 15.7%, respectively, which were significantly correlated with HAM gene-dosage ($r = 0.81$, $p < 0.01$). Amylose content, number of elongated starch granules, and conclusion gelatinization temperature increased with the increase in HAM gene-dosage. X-ray diffraction study showed that the relative crystallinity (%) of starch granules decreased with the increase in HAM gene-dosage. The results suggested that the HAM gene-dosage was responsible for changes in starch molecular structure and organization of starch granules and, in turn, the RS formation in the maize *ae* mutant starch.

KEYWORDS: High-amylose maize; *ae* mutant; resistant starch; amylose; starch physicochemical properties; high-amylose modifier gene

INTRODUCTION

Normal (nonmutant) maize starch consists of two polysaccharides: amylose and amylopectin. Amylose molecules are essentially linear chains of (1→4)-linked α-D-glucopyranose; some amylose molecules possess a few branches. Amylopectin has highly branched structures consisting of (α1→6)-linked chains of (1→4)-linked α-D-glucopyranose (*1*, *2*). In addition to amylose and amylopectin, high-amylose maize starch also contains intermediate components (IC), which are branched molecules having molecular weights similar to those of amylose molecules (*3*, *4*).

Starch is widely used for food and nonfood applications, such as an energy source, thickeners, binders, films, and foams (*5*). In the human digestive system, starch is digested predominantly in the small intestine by enzymes. Resistant starch (RS), however, resists enzymatic hydrolysis in the small intestine and passes to the large intestine, where it is subjected to bacterial fermentation (*6*). Studies have shown that RS provides many benefits to human health, including prevention of colon cancer, lowering blood glucose, and reduction of LDL-cholesterol (*7*). RS is classified into five types (*8*−*10*). Type I RS is the physically inaccessible starch, resulting from entrapment in nondigestible plant tissue. Type II RS is raw starch granules from potato, pea, or high-amylose maize starch, which has the B- or C-type polymorphism. Type III RS is retrograded amylose. Type IV RS is chemically modified starch. Type V RS is amylose−lipid complexed starch.

Normal-maize starch consists of ~30% amylose (*11*). Commercial maize varieties possessing high-amylose starch have been classified into amylomaizes V, VI, and VII, reflecting amylose contents of 50, 60, and 70%, respectively (*12*). A high-amylose maize line, GEMS-0067 (PI 643420), has been developed by the USDA-ARS Germplasm Enhancement of Maize (GEM) Project (*13*). Starches of GEMS-0067 lines consist of significantly larger amylose/IC contents (86.1−89.3%) and RS contents (39.4−43.2%) than maize *amylose-extender* (*ae*) single-mutant starches of H99*ae*, OH43*ae*, B89*ae*, and B84*ae* (66.5−74.6 and 11.5−19.1%, respectively) (*14*). The GEMS-0067 line is a homozygous mutant of the recessive *ae* gene and an unknown number of high-amylose modifier (HAM) gene(s). Wu et al. (*15*) conducted a generation-means analysis (GMA) study using the GEMS-0067 line and H99*ae* line to understand the effect of the HAM gene(s)

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on the amylose content of the maize *ae* mutant starch. It has been observed that the HAM gene mainly has an additive effect rather than a dominance effect on the amylose content of starch when it is present in maize of a homozygous *ae* mutant background (*15*). There is no information, however, regarding the impact of the HAM gene(s) on either RS content or other starch characteristics. Such information could provide guidelines for maize breeders in the development of new germplasms with increased grain yields and RS contents. This study aimed to understand how HAM gene-dosage affected the RS content and other physicochemical properties of maize *ae* mutant starches obtained from the GMA study conducted by Wu et al. (*15*).

MATERIALS AND METHODS

Preparation of Maize Samples. This study used a GMA model for traits under triploid inheritance as described by Bogyo et al. (*16*). This model enables estimates of the genetic parameters of a greater contribution of the female parent. The generations, including G/G, G/F1, G/H, F1/G, F1/F1, F1/H, H/G, H/F1, and H/H, were obtained from self- and intercrosses of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1). A diagram of the production of nine maize sample lines through crossing is shown in **Figure 1**. Pedigrees and descriptions of maize samples are given in **Table 1**. Plants were grown at the Truman State University research farm near Kirksville, MO, in the summer of 2006 and established in a randomized complete block design.

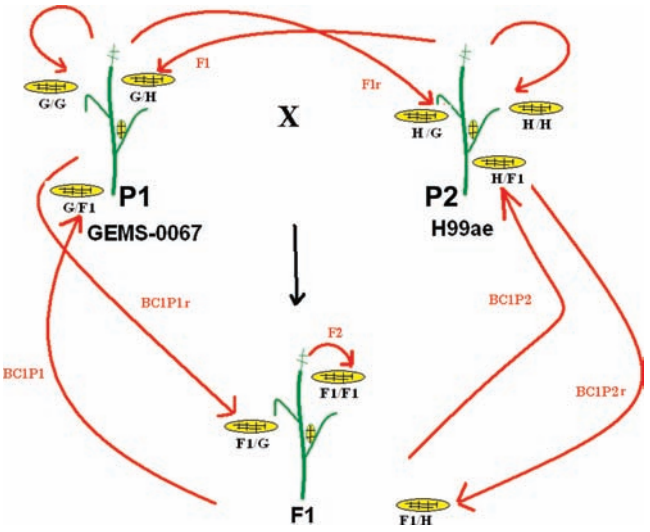

Figure 1. Nine maize samples derived from self- and intercrosses of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1). G/G, GEMS-0067×GEMS-0067; G/F1, GEMS-0067×(GEMS-0067 × H99*ae*); G/H, GEMS-0067×H99*ae*; F1/G, (GEMS-0067×H99*ae*)×GEMS-0067; F1/F1, (GEMS-0067×H99*ae*)×(GEMS-0067 × H99*ae*); F1/H, (GEMS-0067×H99*ae*)×H99*ae*; H/G, H99*ae*×GEMS-0067; H/F1, H99*ae*×(GEMS-0067×H99*ae*); H/H, H99*ae*×H99*ae*; P1, parent 1; P2, parent 2; F1, first generation; BC1, first cycle of backcross.

Total Starch Determination. A method using enzymatic hydrolysis, described by McCleary et al. (*17*) and Hall and Mertens (*18*) with modifications, was used to determine the total starch content of the grain sample. The samples were ground using a Tecator sample mill (Foss North America, Eden Prarie, MN) to pass a screen with an opening of 1 mm. The ground sample (0.1 g) was mixed with 1 mL of dimethyl sulfoxide (DMSO) and then incubated in a water bath at 58 °C for 24 h. The starch dispersion was mixed with acetate buffer (30 mL, 0.1 M, pH 4.5) and then hydrolyzed using a thermally stable α-amylase (0.1 mL, Spezyme Xtra, Genencor, Rochester, NY) at boiling-water temperature for 1 h. The hydrolysate was further digested with glucoamylase (0.3 mL, G-Zyme 480, Genencor) at 58 °C for 2 h. The glucose concentration (g/L) was determined using a YSI 2700 Select Biochemistry monitoring system (Yellow Springs Instruments Inc., Yellow Springs, OH). The total starch content of the sample was calculated as follows: % total starch = 100 × 0.9 × (total glucose released from starch)/total dried weight of sample.

Starch Isolation. Starch was isolated from maize kernels using a wet-milling method in the presence of neutral proteases as described by Wittrock et al. (*19*).

Determination of Amylose Content. The amylose content of starch was determined using an iodine−colorimetric method described by Wittrock et al. (*19*) and using Sepharose CL-2B gel permeation chromatography (GPC) (*20*) followed by a total carbohydrate (phenol−sulfuric acid) assay (*21*).

RS Content of Starch. The RS content of each starch sample was determined using AOAC method 991.43 for total dietary fiber (*22*) as described by Li et al. (*14*).

Scanning Electron Microscopy (SEM). Starch was coated with gold−palladium (60:40). Starch granules were viewed under a scanning electron microscope (JEOL JSM-6100) at 5.0 kV accelerating voltage.

Light Microscopy. Polarized and phase-contrast light micrographs of starch granules were obtained using a light microscope (Zeiss Axioplan 2, Thornwood, NY) equipped with a digital microscopy imaging system including an AxioCam MRC color camera and Axiovison rel. 4.5 software. Images were analyzed for the number of starch granules displaying weak or no birefringence and for the area, perimeter, and circularity of starch granules using the online program ImageJ from the National Institutes of Health (NIH), which is available by ftp at zippy.nimh.nih.gov/ or http://rsb.info.nih.gov/nih-imageJ.

X-ray Diffraction and Starch Crystallinity. Starch samples were equilibrated in a chamber at 100% relative humidity at 25 °C for 24 h. X-ray diffraction pattern and relative crystallinity of starch were determined using the method described by Ao and Jane (*23*).

Thermal Properties of Starch. Thermal properties of the starch were analyzed using a differential scanning calorimeter (DSC) (DSC-7, Perkin-Elmer, Norwalk, CT) (*24*). Starch (~6.0 mg, dry starch basis) was mixed with water (~18 μL) in a stainless steel pan, equilibrated at room temperature for 1 h, and then heated from 10 to 150 °C at a rate of 10 °C/min. A sealed empty stainless steel pan was used as reference. Onset (T_o), peak (T_p), and conclusion (T_c) gelatinization temperatures and enthalpy change (ΔH) of starch were analyzed by using Pyris software (Perkin-Elmer, Norwalk, CT).

Table 1. Pedigrees and Descriptions of Nine Maize Samples Derived from Self- and Intercrosses of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1)

sample	breeding pathway	pedigree	endosperm genotype	HAM gene-dosage in endosperm[b] (%)
G/G	P1	GEMS-0067×GEMS-0067	bbb[a]	100
G/F1	BC1P1	GEMS-0067×(GEMS-0067×H99*ae*)	Bbb, bbb	83.3
G/H	F1	GEMS-0067×H99*ae*	Bbb	66.7
F1/G	BC1P1r	(GEMS-0067×H99*ae*)×GEMS-0067	BBb, bbb	66.7
F1/F1	F1/F1	(GEMS-0067×H99*ae*)×(GEMS-0067 × H99*ae*)	BBB, BBb, Bbb, bbb	50
F1/H	BC1P2r	(GEMS-0067×H99*ae*)×H99*ae*	BBB, Bbb	33.3
H/G	F1r	H99*ae*×GEMS-0067	BBb	33.3
H/F1	BC1P2	H99*ae*×(GEMS-0067 × H99*ae*)	BBB, BBb	16.7
H/H	P2	H99*ae*×H99*ae*	BBB	0

[a] b represents HAM gene(s) assuming a single locus,; B represents wild type allele. [b] % HAM gene-dosage = 100 × the total number of b/the sum of total numbers of B and b.

Statistical Analysis. Data were analyzed using the SAS program (SAS 9.1).

RESULTS AND DISCUSSION

HAM Gene-Dosage of Maize Samples. Maize endosperm is produced by fusion of two maternal nuclei and one paternal nucleus during fertilization, resulting in a triploid tissue (*25*). Therefore, ears of G/G, G/H, H/G, and H/H had kernels with endosperms of a homogeneous genotype bbb, Bbb, BBb, and BBB, respectively, in which b represents the HAM gene(s) (assuming a single locus) and B represents the wild type allele (**Table 1**). Each of the other samples (G/F1, F1/G, F1/F1, F1/H, and H/F1) had endosperm genotypes with mixtures of bbb, Bbb, BBb, or BBB because of segregation of alleles from the F1 gametes (**Table 1**). The HAM gene-dosage of endosperm was calculated as the total number of b divided by the sum of total numbers of B and b (**Table 1**). Consequently, the nine maize samples, G/G, G/F1, G/H, F1/G, F1/F1, F1/H, H/G, H/F1, and H/H, had HAM gene-dosage levels of 100, 83.3, 66.7, 66.7, 50, 33.3, 33.3, 16.7, and 0%, respectively (**Table 1**).

The GEMS-0067 line has been demonstrated to be homozygous of *ae* and HAM gene(s), whereas the H99*ae* line is an *ae* single mutant (*13*). The HAM gene mainly has an additive effect rather than a dominance effect on the amylose content of maize *ae* mutant starch (*15*). Preliminary results showed that the *sbeI* gene could be partially or entirely HAM gene(s) (*26*). It is known that biosynthesis of the starch granule typically involves several enzymes including ADP-glucose pyrophosphorylase, starch synthase, starch branching enzyme (SBE), and starch debranching enzyme. The branched structure of amylopectin results from the activity of SBEs. The SBEs catalyze the formation of (α1→6) glycosidic bonds by means of cleavage within a linear chain and transfer of the free reducing end to the C6 of an adjacent glucose unit. There are two major SBE isoforms in maize endosperm, SBEI and SBEII. SBEII is known to transfer shorter chains in vitro, whereas SBEI transfers longer chains. In maize endosperm, two closely related forms of SBEII (SBEIIa and SBEIIb) have been reported. Mutation at the *sbeI* locus reduces enzyme activity of SBEI, whereas mutation at the *ae* (*sbeIIb*) locus results in a decrease in the SBEIIb enzyme activity. SBEIIb plays a major role in the biosynthesis of branch structures of amylopectin in normal-maize endosperm (*27*, *28*).

Amylose, RS, and Total Starch Contents. Amylose contents of starches determined using GPC followed by total carbohydrate (phenol−sulfuric) detection and using an iodine−colorimetric method are summarized in **Table 2**. The amylose content determined using GPC followed by total carbohydrate determination, which includes both amylose and IC molecules, increased from 68.9 to 88.2% as HAM gene-dosage increased ($r = 0.98$, $p < 0.001$). A similar trend was also observed for the amylose content determined using an iodine−colorimetric method (**Table 2**). These results were in agreement with the result previously reported (*15*). Amylose contents of maize *ae* mutant starches are typically around 55% but can vary significantly depending on the parental background genes (*12*). The effect of SBEIIb activity on starch characteristics has been reported. Increasing doses of the recessive *ae* allele decrease the enzyme activity of SBEIIb proportionally (*29*). Although an increase in amylose content has been reported between *AeAeAe* and *Aeaeae* endosperm genotypes (*30*), there is no significant difference between *AeAeAe* and *AeAeae*. Zuber et al. (*31*) reported amylose contents up to 70.3%, which could be a result of modifying factors that were not allelic to *ae*. Sidebottom et al. (*32*) reported that an amylomaize VII line contained an elevated amylose content, which could result from losses of SBEIIb and SBEI enzyme activities. A low-amylopectin starch has been reported to have an even higher amylose level than the amylomazie VII starch because of an additional loss of SBEIIa enzyme activity in the low-amylopectin maize line (*32*).

Table 2. Amylose and RS Contents of Native Maize Starches and Total Starch Contents of Grain Samples Derived from Self- and Intercrosses of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1)

sample	amylose-GPC[a] (%)	amylose-C[b] (%)	RS[c] (%)	total starch (%)
G/G	88.2 ± 1.5	69.9 ± 0.0	35.0 ± 0.5	59.3 ± 1.5
G/F1	86.7 ± 0.1	68.3 ± 0.6	29.5 ± 0.8	63.4 ± 0.6
G/H	81.7 ± 1.6	64.9 ± 0.4	28.1 ± 0.8	65.1 ± 0.7
F1/G	84.0 ± 0.1	61.9 ± 0.2	32.0 ± 1.4	61.4 ± 1.3
F1/F1	77.2 ± 1.3	63.1 ± 0.5	28.2 ± 0.7	62.5 ± 1.0
F1/H	74.7 ± 0.4	58.5 ± 0.2	29.4 ± 0.7	68.8 ± 2.2
H/G	77.9 ± 0.8	61.1 ± 0.1	12.9 ± 0.2	62.8 ± 1.0
H/F1	72.8 ± 0.2	58.2 ± 0.6	18.4 ± 1.3	64.1 ± 1.3
H/H	68.9 ± 1.6	56.3 ± 0.4	15.7 ± 0.2	64.7 ± 0.1
linear[d]	***[e]	***	**	ns

[a] Amylose content of starch was determined using Sepharose CL-2B gel permeation chromatography followed by total carbohydrate determination. [b] Amylose content of starch was determined using an iodine−colorimetric method. [c] Resistant starch (RS) content of starch was determined using AOAC method 991.43 for total dietary fiber. [d] Linear relationship between HAM gene-dosage and contents of amylose, RS, and total starch. [e] ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

As shown in **Table 2**, the RS contents could be divided into two groups according to the HAM gene-dosage. The first group had RS contents of 28.1−35.0%, which had G or F1 as the female parent. Another group (RS, 12.9−18.4%) had H as the female parent (**Table 2**). These results along with endosperm genotypes of the samples (**Table 1**) suggested that two doses or more of HAM gene(s) in the maize *ae* mutant substantially increased RS content of the starch. However, it was noted that one dose of HAM gene(s) had little effect on the RS content.

Although the total starch content of maize *ae* mutant sample did not show a clear trend with the dosage of HAM gene(s), the lowest starch content (59.3%) was found in G/G with homozygous HAM gene(s) (**Table 2**). The total starch contents (59.3−68.8%) of the maize *ae* mutants were less than that of normal maize (~72%) (*11*). It is known that the grain yields and total starch contents of maize *ae* mutants were lower than that of the normal maize (*33*).

Morphology of Starch Granules. SEM images of starch granules of selected genotypes, G/G, G/H, H/G, and H/H, are shown in **Figure 2**. Two types of starch granules, spherical and elongated granules, were observed for all starch samples, which were similar to the results previously reported (*34*). G/G starch contained a larger number of elongated granules than other starches (**Figure 2**). When the GEMS-0067 line was pollinated by the H99*ae* line, some starch granules showed protrusions and began to have a less extreme rod shape (G/H, **Figure 2B**). When the H99*ae* line was pollinated by the GEMS-0067 line, some starch granules showed minor protrusions (H/G, **Figure 2C**) that were less pronounced than those found in G/H starch granules (**Figure 2B**). These results suggested that the HAM gene(s) affected the formation of elongated granules, which could result from the increase in amylose content of the starch granules (**Table 2**). It has been reported that the proportion of elongated starch granules in maize *ae* mutant starch increases with the increase in amylose content of the starch (*34*). The elongated starch granules in maize *ae* mutant are developed by fusion of multiple granules through amylose interaction in the amyloplast at the early stage of the granule development (*35*).

The average circularity, area, and perimeter of starch granules are summarized in **Table 3**. The average area ($r = -0.90$, $p < 0.01$) and perimeter ($r = -0.92$, $p < 0.001$) of starch granules

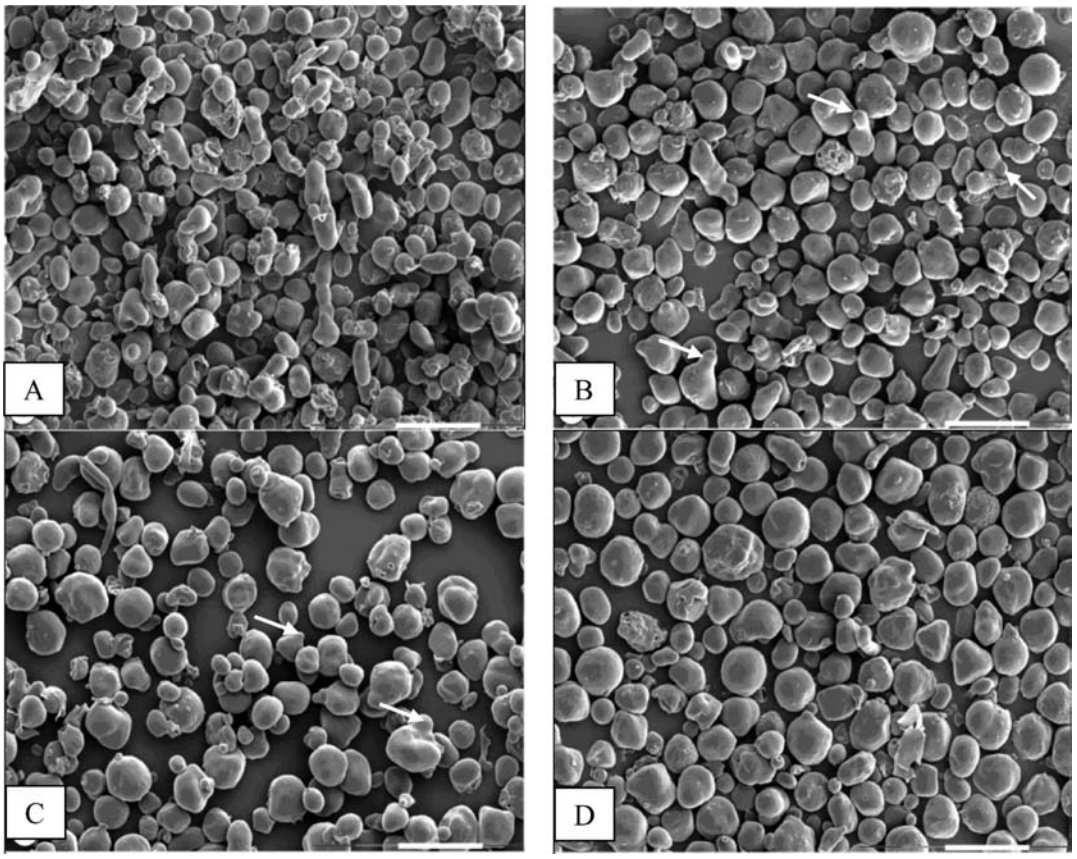

Figure 2. SEM images of starches isolated from maize endosperm homozygous for *ae* gene with decreasing doses of the HAM gene(s) from G/G (GEMS-0067): (**A**) G/G; (**B**) G/H, (**C**) H/G; (**D**) H/H. Arrow indicates protrusion. Scale bar = 20 μm.

Table 3. Morphological Properties of Starch Granules Isolated from Kernels of Self- and Intercrossed Lines of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1)

sample	SG[a] (%)	circularity[b] (4π(area/perimeter^2))	area (μm^2)	perimeter (μm)
G/G	27.2 ± 1.8	0.76 ± 0.005	35.2 ± 8.2	24.1 ± 0.3
G/F1	26.9 ± 2.1	0.81 ± 0.003	46.9 ± 1.0	26.4 ± 0.3
G/H	8.0 ± 2.5	0.80 ± 0.003	57.2 ± 1.5	29.5 ± 0.4
F1/G	13.5 ± 3.5	0.80 ± 0.003	55.7 ± 1.5	28.3 ± 0.4
F1/F1	10.7 ± 1.5	0.81 ± 0.003	60.1 ± 1.9	29.1 ± 0.5
F1/H	6.1 ± 1.8	0.78 ± 0.003	64.5 ± 1.6	31.4 ± 0.4
H/G	9.7 ± 1.0	0.79 ± 0.003	60.1 ± 1.6	30.2 ± 0.4
H/F1	8.0 ± 2.0	0.78 ± 0.003	68.9 ± 1.9	32.2 ± 0.5
H/H	6.0 ± 1.5	0.78 ± 0.003	64.5 ± 1.8	31.4 ± 0.4
linear[c]	**[d]	ns	**	***

[a] Number of starch granules that displayed weak or no birefringence under a polarized-light microscope. [b] A circularity value of 1.0 indicates a perfect circle. Values approaching 0.0 indicate increasingly elongated polygons. [c] Linear relationship between HAM gene-dosage and starch properties. [d] ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

increased as the HAM gene-dosage decreased. Circularity of starch granules varied slightly between starches examined. However, the lowest value (0.76) was observed for the G/G starch in which the HAM gene was fixed (**Table 3**). These findings confirmed that morphological properties of starch granules were influenced by the HAM gene-dosage.

Polarized and phase-contrast light micrographs of G/G starch granules are shown in **Figure 3**. The starch granules showed various birefringence patterns as previously reported (*35*), which included one Maltese cross on a whole single granule, several Maltese crosses overlapping in one granule, a single granule containing one or more Maltese crosses and weak to no birefringence on the remainder of the granule, and granules showing weak or no birefringence. The starch granules that displayed weak or no birefringence were mostly elongated granules and were found in greater proportions for G/G and G/F1 starches (~27.1%) than other starches (6.0−13.5%) (**Table 3**). These results suggested that the increase in HAM gene-dosage resulted in changes in the birefringence patterns of the starch granules, indicating changes in the molecular organization of the granules. It was noted that three doses of HAM gene(s) in the endosperm of the maize *ae* mutant played a major role in the proportion of starch granules that displayed weak or no birefringence pattern when viewed under a polarized light microscope. Because the increase of HAM gene-dosage in maize *ae* mutant increased the amylose content of the starch (**Table 2**), the change in birefringence patterns of starch granules was a result of an increase in amylose content of starch granules.

Crystallinity of Starches. X-ray diffraction patterns of native starches of selected genotypes, G/G, G/H, H/G, and H/H, are shown in **Figure 4**. Although the doses of HAM gene(s) decreased from 3, 2, 1, to 0 for the samples of G/G, G/H, H/G, and H/H, respectively, all of the starches displayed a B-type polymorph, indicating no effect of HAM gene-dosage on the starch polymorph of maize *ae* mutant. The percentages of starch crystallinity increased from 16.3 to 23.7% with the decrease in the HAM gene-dosage (**Figure 4**) as well as the decrease in the amylose content (**Table 2**). This result was in agreement with a previous paper (*34*). The V-type X-ray diffraction pattern of the amylose−lipid complex with 2θ peaks at 8°, 13°, and 20° (*36*) was not observed for the starches, indicating no crystalline amylose−lipid complex present in the starch granules as previous reported (*14*, *34*).

Thermal Properties of Starches. DSC thermograms of the nine maize starches are shown in **Figure 5**, and their thermal properties

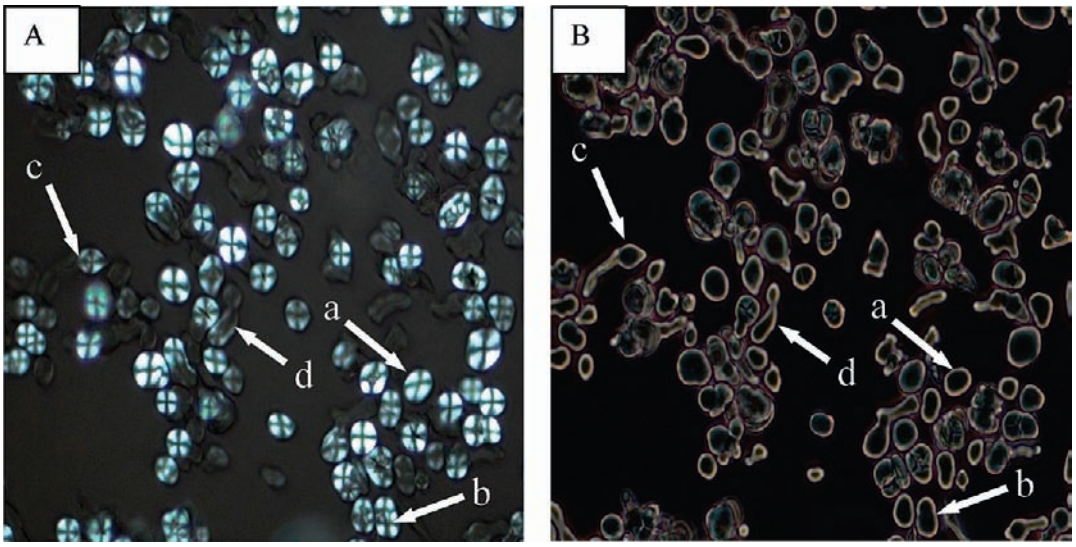

Figure 3. Polarized (**A**) and phase contrast (**B**) light micrographs of G/G (GEMS-0067) maize starch. Arrows indicate birefringence patterns of (a) one Maltese cross on the whole granule, (b) Maltese crosses overlapping in one granule, (c) a single granule containing one Maltese cross and no birefringence on the remainder of the granule, and (d) starch granule showing weak or no birefringence.

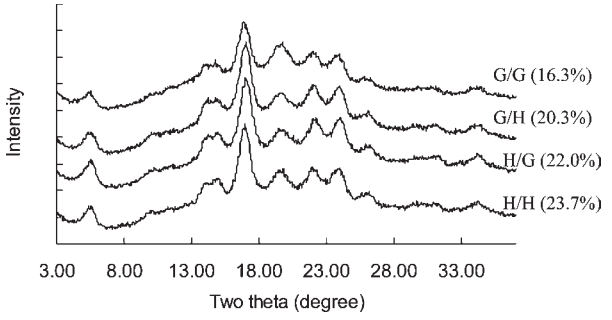

Figure 4. X-ray diffraction patterns of native maize starches isolated from kernels of self- and intercrossed lines of GEMS-0067 (G) and H99*ae* (H). Percentage crystallinity is given in parentheses.

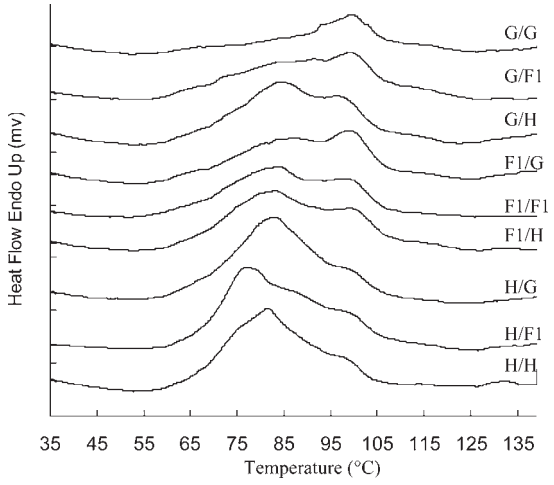

Figure 5. DSC gelatinization thermograms of native maize starches isolated from kernels of self- and intercrossed lines of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1).

are summarized in **Table 4**. Broad thermal transition peaks were observed for all of the starch samples (**Figure 5**), which was in agreement with other observations of high-amylose starches (*14*, *37*). The range of the thermal transition peak decreased when the HAM gene-dosage decreased (**Figure 5**).

Table 4. Thermal Properties of Nine Maize Starches Isolated from Kernels of Self- and Intercrossed Lines of GEMS-0067 (G), H99*ae* (H), and GEMS-0067×H99*ae* (F1)[a]

	gelatinization				
sample	T_o (°C)	T_{p1} (°C)	T_{p2} (°C)	T_c (°C)	ΔH (J/g)
G/G	60.3 ± 0.4	nd[b]	99.7 ± 0.2	122.2 ± 0.2	7.8 ± 0.2
G/F1	60.1 ± 0.4	91.9 ± 0.1	99.5 ± 0.4	123.0 ± 0.6	10.8 ± 0.9
G/H	60.3 ± 0.4	84.2 ± 0.2	99.1 ± 0.8	119.0 ± 0.3	13.7 ± 0.8
F1/G	60.3 ± 0.4	84.9 ± 0.2	99.0 ± 0.0	121.1 ± 2.1	9.3 ± 0.6
F1/F1	59.6 ± 0.8	83.5 ± 0.7	99.7 ± 0.1	120.3 ± 0.4	14.4 ± 0.9
F1/H	59.9 ± 0.1	83.0 ± 0.0	99.8 ± 0.1	119.1 ± 0.1	13.3 ± 0.1
H/G	61.3 ± 0.4	82.5 ± 0.7	98.6 ± 0.1	108.9 ± 0.1	16.2 ± 0.8
H/F1	60.8 ± 0.4	78.6 ± 1.7	99.1 ± 0.6	108.5 ± 0.7	15.5 ± 0.4
H/H	60.1 ± 0.1	81.1 ± 0.8	99.0 ± 0.3	105.5 ± 0.2	15.1 ± 0.8
linear[c]	ns[d]	**	ns	**	**

[a] T_o, T_p, T_c, and ΔH are onset temperature, peak temperature, conclusion temperature, and enthalpy change, respectively. [b] nd, not detected. [c] Linear relationship between HAM gene-dosage and starch thermal properties. [d] ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

Two endothermic peaks were observed for most starches except G/G (**Figure 5**). The first peak (T_{p1}, 78.6−91.9 °C; **Table 4**), corresponding to the melting of short-chain double-helical crystallites of mainly amylopectin molecules (*14*, *34*, *38*), decreased with an increase in HAM gene-dosage (**Figure 5**). The second peak (T_{p2}, ∼99.4 °C; **Table 4**), corresponding to the melting of long-chain double-helical crystallites of amylose/IC (*14*, *38*) and the dissociation of amylose−lipid complexes (*14*, *39*), increased with an increase in HAM gene-dosage (**Figure 5**). The conclusion gelatinization temperatures were ∼120.8 °C for G/G, G/F1, G/H, F1/G, F1/F1, and F1/H starches, ∼108.7 °C for H/G and H/F1 starches, and 105.5 °C for H/H starch (**Table 4**), which decreased with the decrease in HAM gene-dosage ($r = 0.87$, $p < 0.01$). These results supported the view that the HAM gene-dosage affected the molecular structures of maize *ae* mutant starches, resulting in different gelatinization temperatures. It has been reported that the long-chain double-helical crystallites in maize *ae* mutant starches have gelatinization temperatures above 100 °C, retain semicrystalline structures at 95−100 °C, and are resistant to enzymatic hydrolysis at 95−100 °C (*14*, *34*). The lipids present in maize *ae* mutant starches also reduce the enzyme digestibility of the starch

Table 5. Pearson Correlation Coefficients (r) between HAM Gene-Dosage and Starch Physicochemical Properties

	amylose-GPC (%)	amylose-C (%)	RS (%)	total starch (%)	SG (%)	circularity (4π(area/perimeter^2))	area (μm^2)	perimeter (μm)	T_o[e] (°C)	T_{p1} (°C)	T_{p2} (°C)	T_c (°C)	ΔH (J/g)
HAM gene-dosage	0.98***[f]	0.96***	0.81**	−0.55	0.83**	0.12	−0.90**	−0.92***	−0.18	0.84**	0.41	0.87**	−0.83**
amylose-GPC[a] (%)	1	0.93***	0.72*	−0.59	0.84**	0.14	−0.88**	−0.91**	−0.03	0.83*	0.25	0.80**	−0.82*
amylose-C[b] (%)		1	0.70*	−0.59	0.81**	0.11	−0.92***	−0.93***	−0.12	0.86**	0.38	0.76*	−0.70*
RS[c] (%)			1	−0.24	0.59	0.07	−0.65	−0.69*	−0.60	0.58	0.72*	0.94**	−0.84**
total starch (%)				1	−0.64	−0.08	0.67*	0.72*	−0.19	−0.19	0.10	−0.23	0.53
SG[d] (%)					1	−0.05	−0.92***	−0.93***	−0.08	0.90**	0.38	0.61	−0.78*
circularity (4π(area/perimeter^2))						1	0.17	0.04	−0.26	0.74	−0.09	0.30	0.16
area (μm^2)							1	0.99***	0.11	−0.96***	−0.36	−0.65*	0.84**
perimeter (μm)								1	0.17	−0.93***	−0.39	−0.72*	0.85**

[a] Amylose content of starch was determined using Sepharose CL-2B gel permeation chromatography followed by total carbohydrate determination. [b] Amylose content of starch was determined using an iodine-colorimetric method. [c] Resistant starch (RS) of starch was determined using AOAC method 991.43 for total dietary fiber. [d] Number of starch granules displayed weak or no birefringence under a polarized-light microscope. [e] T_o, T_p, T_c, and ΔH are onset temperature, peak temperature, conclusion temperature, and enthalpy change, respectively. [f] *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

granules (*34*). A decrease in enthalpy change with the increase in HAM gene-dosage was also observed ($r = -0.83$, $p < 0.01$), which could result from the reduction in the starch crystallites (**Figure 4**) or the total double helices in starch granules.

Relationship between HAM Gene-Dosage and Starch Physicochemical Properties. The Pearson correlation coefficients between HAM gene-dosage and starch physicochemical properties are given in **Table 5**. The HAM gene-dosage positively correlated with amylose contents determined using both GPC and iodine−colorimetric methods ($r = 0.98$ and 0.96, $p < 0.001$, respectively) (**Table 5**). A positive effect of HAM gene-dosage on RS content ($r = 0.81$, $p < 0.01$) and number of starch granules that displayed weak or no birefringence ($r = 0.83$, $p < 0.01$) was also observed. The HAM gene-dosage, however, showed negative correlation with some morphological properties of starch granules, that is, average area ($r = -0.90$, $p < 0.01$) and perimeter ($r = -0.92$, $p < 0.001$).

The increase in HAM gene-dosage positively correlated with the peak gelatinization temperature of the short-chain double-helical crystallites ($r = 0.84$, $p < 0.01$) (first peak, **Table 5**) and conclusion gelatinization temperature ($r = 0.87$, $p < 0.01$), but negatively correlated with enthalpy change ($r = -0.83$, $p < 0.01$) (**Table 5**). It was of particular interest in the good correlation between conclusion gelatinization temperature and RS content of the examined starches ($r = 0.94$, $p < 0.01$; **Table 5**). Because much of the recent interest in high-amylose maize has been focused on its RS content, the conclusion gelatinization temperature could be potentially used as an indicator for estimation of RS content in high-amylose maize starch.

As listed in **Table 5**, the amylose content, like the HAM gene-dosage, also significantly correlated with RS content, number of starch granules that displayed weak or no birefringence, average area and perimeter of starch granules, and thermal properties including first peak and conclusion gelatinization temperatures and enthalpy change. The results confirmed that the increase of HAM gene-dosage in the maize *ae* mutant resulted in an increase in amylose content of the starch, leading to changes in molecular organization of the granule, granule morphology, starch thermal properties, and RS content of the starch.

In conclusion, an increase of HAM gene-dosage in maize *ae* mutant resulted in higher amylose content of the starch, which, in turn, significantly affected the molecular organization of the granule, granule morphology, starch crystallinity, and starch thermal properties. The increase of amylose content in maize *ae* mutant starch contributed to the formation of long-chain double-helical crystallites and amylose−lipid complex resulting in RS formation in the starch. Two or more doses of HAM gene(s) in maize *ae* mutant substantially increased the RS content of the starch, whereas one dose of HAM gene(s) had little effect on the RS content.

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