Project Title: Development of GEM Line Starch to Improve Nutritional Value and Bio-Fuel Production

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Project Overview

This research project is aiming to characterize starches produced by the Germplasm Enhancement of Maize (GEM) projects and to develop value-added utilization of these starch lines. Two types of starch, high-amylose maize starch developed from GEM amylomaize project of Dr. Mark Campbell at Truman State University (GEMS-0067 *ae*-lines), and normal maize starch extracted from GEM lines supplied by the GEM Coordinator, Dr. Mike Blanco, were the main genotypes used in the study. Three GEMS-0067 *ae*-lines: GUAT209:S13 × (OH43*ae*×H99*ae*) B-B-4-1-2-1-1, GUAT209:S13 × (OH43*ae*×H99*ae*) B-B-4-4-2-1-1, and GUAT209:S13 × (OH43*ae*×H99*ae*) B-B-4-4-2-1-2 and four existing *ae*-lines: H99*ae*, OH43*ae*, B89*ae*, and B84*ae* have been used in this study. The GEMS-0067 *ae*-line starches displayed significantly larger resistant-starch contents (39.4-43.2%) than that of the existing *ae*-lines (11.5-19.1%) determined using AOAC method 991.43 for total dietary fiber. The GEMS-0067 *ae*-line starches had much larger apparent-amylose contents (83.1-85.6%) than did the existing *ae*-line starches (61.7-67.7%). The resistant-starch content was positively correlated with the apparent-amylose content (r = 0.99). The conclusion gelatinization-temperatures of the GEMS-0067 *ae*-line starches (122.0-130.0ºC) were substantially higher than that of the existing *ae*-line starches (100.5-105.3º). The high gelatinization temperature of the GEMS-0067 *ae*-line starch was likely to cause the large resistant-starch content in the cooked starch.

Maize *ae*-mutant starches are known for consisting of two types of starch granules: spherical granules and elongated granules. The GEMS-0067 *ae*-line starches consisted of 22.6%-32.0% elongated starch granules larger than did the existing *ae*-line starches (5.2%-7.7%). After digesting the *ae*-line starches with thermally stable α-amylase at 95-100°C, mainly elongated starch granules and the periphery of spherical starch granules remained in resistant-starch residues. All the resistant-starch residues displayed the B-type polymorph and showed onset gelatinization-temperatures above 100°C. All the resistant-starch residues consisted of mostly partially-preserved amylose and IC molecules. The long-chain double-helical crystallites of amylose/IC molecules in the native *ae*-line starches having gelatinization-temperature above 100°C retained the semi-crystalline structures and were resistant to the enzymatic hydrolysis at 95-100°C.

To understand the internal structures of the elongated starch granules in the GEMS-0067 *ae*-line and the development of elongated starch granule, we harvested the GEMS-0067 *ae*-line at early stage of maize development. GEMS-0067 *ae*-line is homozygous mutant of *ae* gene and high-amylose modifier (HAM) gene. To understand how the HAM gene dosage affected the resistant-starch content of the maize *ae*-mutant starch, we analyzed the physicochemical properties of the starches isolated from endosperms with 0, 1, 2, and 3 doses of HAM gene.

In 2009, two manuscripts were published; one was submitted; and two abstracts were presented. The poster "Effect of high-amylose modifier (HAM) gene dosage on resistant-starch content of maize amylose-extender (*ae*) mutant starch" authored by H. Jiang et al., received a second place award at the 8th Annual Borlaug Poster Competitions for world food issues from the Nutritional Sciences Council at Iowa State University, Oct 2009.

Publications:

- Jiang, H., Campbell, M., Blanco, M., and Jane, J. 2008. Characterization of maize amylose-extender (*ae*) mutant starches. Part II. Structures and properties of starch residues remaining after enzymatic hydrolysis at boiling-water temperature. Carbohydrate Polymers, In press.
- Srichuwong, S., Gutesa, J., Blanco, M., Duvick, S. A., Gardner, C., and Jane, J. 2009. Characterization of corn grains for dry-grind ethanol production. Journal of ASTM International, Accepted.
- Jiang, H., Horner, H., Pepper, T., Blanco, M., Campbell, M., and Jane, J. 2009. Formation of elongated starch granules in high-amylose maize. Carbohydrate Polymers, Submitted.

Presentations:

- Jiang, H., Campbell, M, Jane, J. 2009. Sep 13-16, 2009. Dosage effect of high-amylose modifier (HAM) gene on physicochemical properties of maize amylose-extender (*ae*) starch. Annual Meeting of American Association of Cereal Chemists in Baltimore, MD, U.S.A.
- Jiang, H., Campbell, M, Jane, J. 2009. June 6-9, 2009. Effect of high-amylose modifier (HAM) gene dosage on resistant-starch content of maize amylose-extender (*ae*) mutant starch. IFT Annual Meeting & Food Expo in Anaheim, CA, USA.

Objectives

The objectives of this research project were to characterize starches produced by the GEM projects and to develop value-added utilization of these starch lines.
Specific aims of the study in 2009 were:

1. To understand the formation mechanism of the elongated starch granules in the

GEMS-0067 *ae*-line.

2. To understand how the HAM gene dosage affected the resistant-starch content of maize *ae*-mutant starch.

Progress Made in 2008

Objective 1. To understand the formation mechanism of the elongated starch granules in the GEMS-0067 *ae*-line.

GEMS-0067 *ae*-line and normal maize (B73) were planted in the field of the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA in 2007. Plants were self-pollinated by hand pollination.

GEMS-0067 *ae*-line starch consists of up to 32% elongated starch granules, which differs from normal maize starch containing no elongated starch granules. To understand how the elongated starch granules formed in the amyloplast, we used transmission electron microscopy (TEM) to study the sub-aleurone layers of the endosperms harvested on 20 DAP. The TEM images of the endosperm of B73 maize are shown in Fig. 1. The protrusion of amyloplast was observed for normal maize B73 (Fig. 1A & 1B). In general, one amyloplast contained one starch granule in normal maize (Fig. 1A). Occasionally, some amyloplasts contained two starch granules (Fig. 1B). It was likely that the amyloplast started dividing after two granules were developed (Fig. 1B).

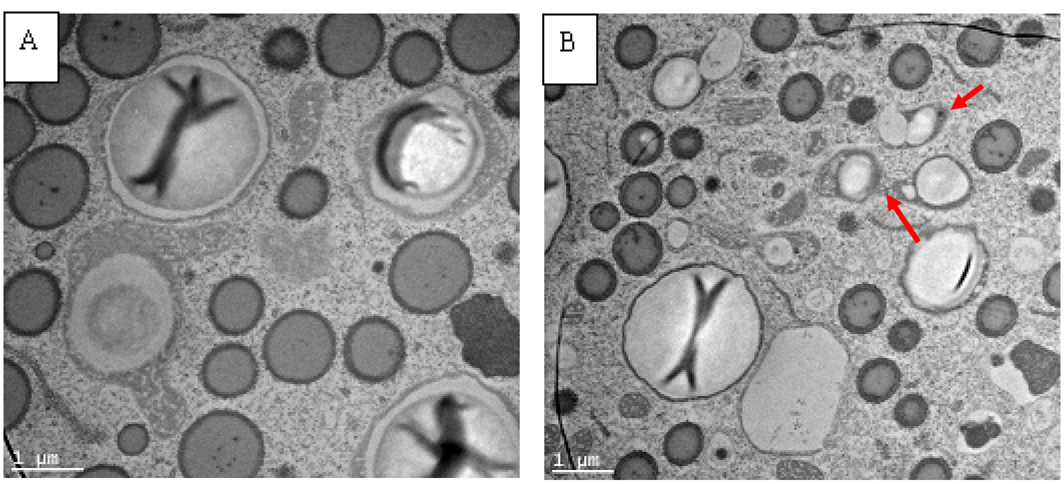


Fig. 1. TEM images of B73 endosperm tissue harvested on 20 DAP. A: amyloplast containing one starch granule; B: amyloplast containing two starch granules indicated by arrows.

TEM images of sub-aleurone layers of GEMS-0067 endosperm harvested on 20 DAP are shown in Fig. 2. Many amyloplasts contained two or more starch granules (Fig. 2A-B), and some granules consisted of multiple small granules fused together as evidenced by multiple hila in one granule (Fig. 2C-D). These results strongly supported that the elongated granules in GEMS-0067 starch were formed by fusion of several small granules, likely through amylose interaction during granule development because of the high concentration of amylose in GEMS-0067 starch. Amylose synthesized on the later developmental stage surrounded the fused granules and resulted in elongated granules.

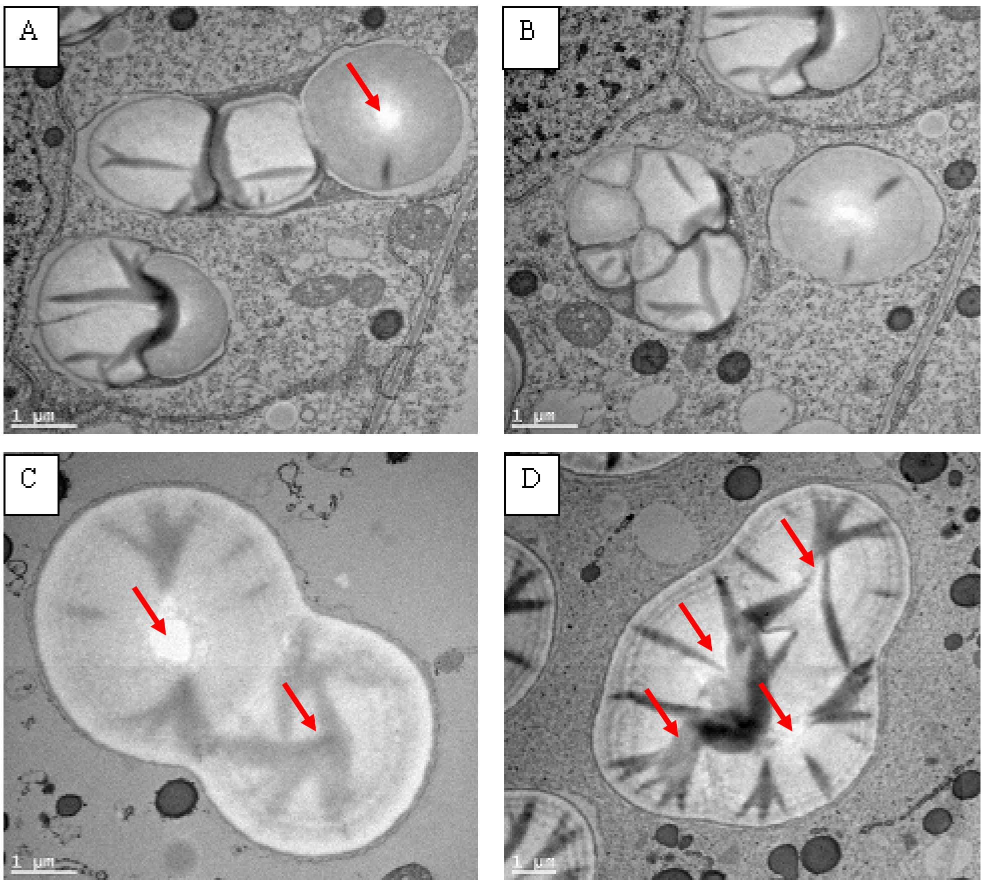


Fig. 2. Transmission electron micrographs of GEMS-0067 endosperm tissue harvested on 20 DAP. Arrow indicates hilum.

Objective 2. To understand how the HAM gene dosage affected the resistant-starch content of maize *ae*-mutant starch.

The maize endosperm is a triploid tissue containing 2 doses of gene from female and one dose of gene from male. Four samples, including G/G, G/H, H/G, and H/H with 3, 2, 1, and 0 doses of HAM gene, respectively, were obtained from self-and inter-crosses of GEMS-0067 (G) and H99*ae* (H). Plants were grown in Kirksville, MO in 2006.

The resistant-starch contents of starches differing in HAM gene dosage are given in Table 1. The resistant-starch contents were 35.0, 28.1, 12.9, and 15.7% for G/G, G/H, H/G, and H/H starches, respectively. This result suggested that two doses of HAM gene substantially increased the resistant-starch content of maize *ae*-mutant starch, while one dose of HAM gene had little effect on the resistant-starch content.

The Sepharose CL-2B gel-permeation chromatograms (GPC) of four native starches are shown in Fig. 3. The dispersed whole-starch was separated into two peaks. The first peak (fractions 11-18) was amylopectin molecules, whereas the second peak (fractions 19-45) was mixture of amylose/intermediate component (IC) (Fig. 3). The amylopectin contents of starches, calculated on the basis of the total carbohydrate shown in the peaks of GPC (Fig. 3), are summarized in Table 1. The amylopectin contents of starches increased from 11.8 to 31.1% as the decrease in HAM gene dosage (Table 1), which negatively correlated with RS content ($r = -0.83$, $p < 0.05$). These results suggested that amylose/IC contributed to the RS formation in maize *ae*-mutant starch.

Table 1 Resistant-starch (RS), amylopectin, amylose, and IC contents[a] and starch crystallinity

Sample	RS[b] (%)	Amylopectin (%)	Amylose (%)	LIC[c] (%)	SIC[c] (%)	Starch crystallinity (%)
G/G	35.0 ± 0.5	11.8 ± 1.5	63.7	7.7	16.7	16.3
G/H	28.1 ± 0.8	18.3 ± 1.6	53.3	8.1	20.3	20.3
H/G	12.9 ± 0.2	22.1 ± 0.8	55.8	7.5	14.6	22.0
H/H	15.7 ± 0.2	31.1 ± 1.6	42.1	15.0	11.8	23.7

[a] Amylopectin, amylose, and IC contents were determined using GPC and normal butanol precipitation method.

[b] Determined using AOAC Method 991.43 for total dietary fiber.

[c] LIC: large molecular-weight IC (Fractions 19-30, see Fig. 2); SIC: small molecular-weight IC (Fractions 31-45, see Fig. 2).

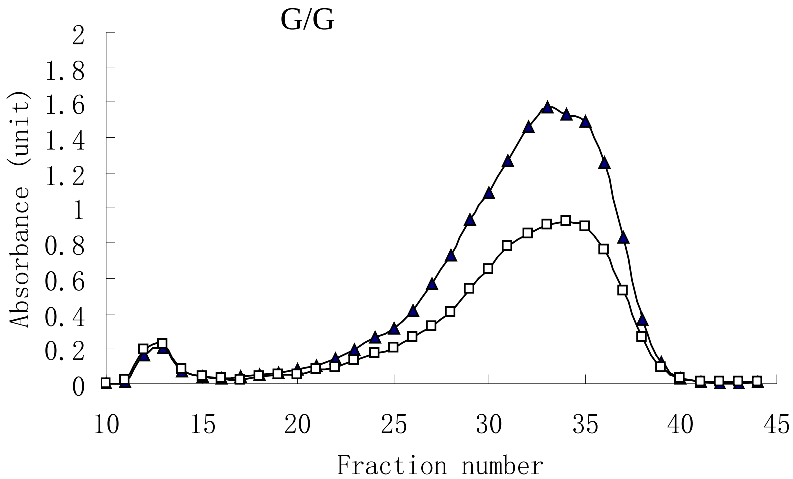


Fig. 3. Sepharose CL-2B gel-permeation profile of native starch. —▲—, blue value; —□—, total carbohydrate.

After the amylose molecules were precipitated using 1-butanol, the mixture of amylopectin and IC was further separated using GPC (Fig. 4). The amylose molecules were removed as indicated by the ratio of blue value to total carbohydrate (Fractions 19-45, Fig. 4). The amylopectin molecules were eluted out between fractions 11 and 18. The large molecular-weight IC molecules were collected from fractions between 19 and 30, and the small molecular-weight IC were collected from fractions between 31 and 45. The contents of amylose, large molecular-weight IC, and small molecular-weight IC, calculated on the basis of the total

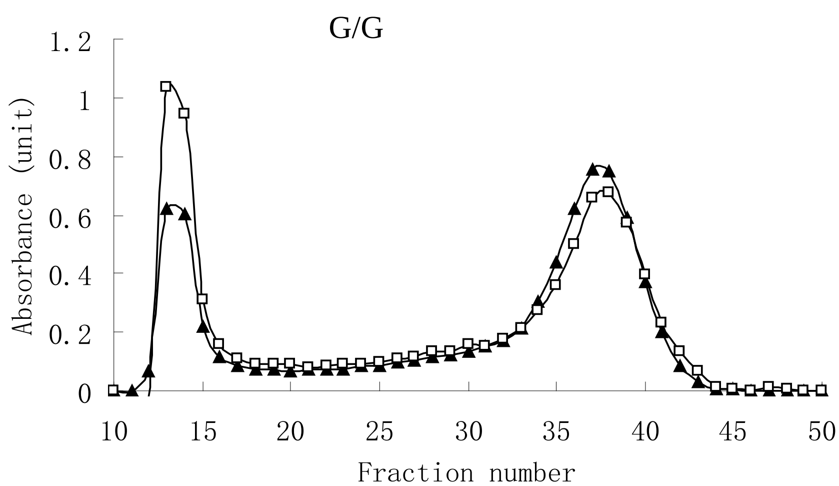
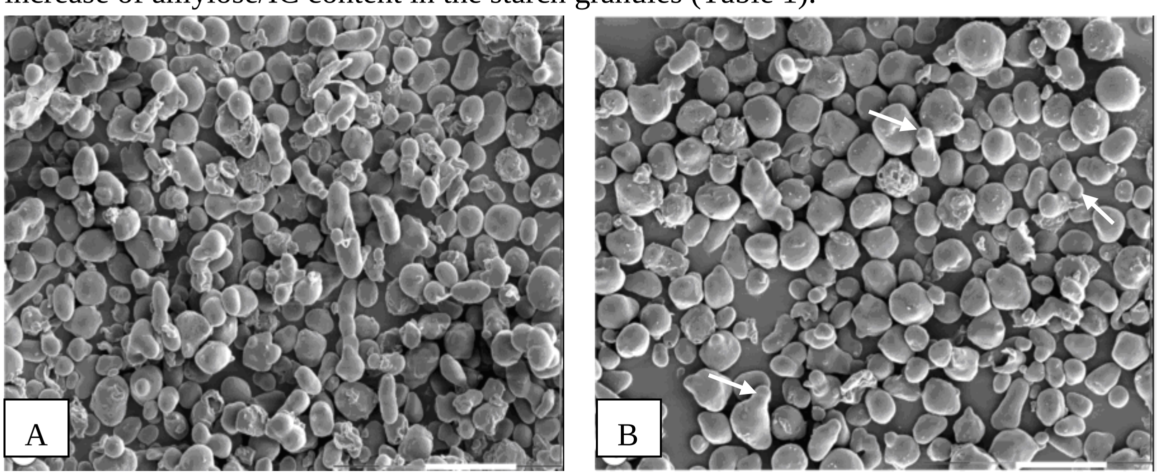


Fig. 4. Sepharose CL-2B gel-permeation profile of amylopectin/IC mixture. —▲—, blue value; —□—, total carbohydrate.

carbohydrate shown in the peaks of GPC (Figs. 3 & 4), are summarized in Table 1. G/G starch had more amylose (63.7%) and less small molecular-weight IC (16.7%) than G/H starch (53.3% and 20.3%, respectively) (Table 1). Similar content of large molecular-weight IC was observed in G/G (7.7%) and G/H (8.1%) starches (Table 1). These results suggested that 3 doses of HAM gene in G/G maize increased amylose content of starch and, thus, the greater resistant-starch content of G/G starch (35.0%) than G/H starch (28.1%) was attributed to the increased amylose content in the G/G starch.

The contents of amylose and large molecular-weight IC of G/H and H/G starches (2 and 1 doses of HAM gene, respectively) were similar (53.3 and 55.8 %, and 8.1 and 7.5%, respectively). Thus, the larger content of small molecular-weight of G/H (20.3%) than H/G (14.6%) indicated that 2 doses of HAM gene in G/H maize increased the content of small molecular-weight IC contributing to the RS formation. This result suggested that the small molecular-weight IC formed long-chain double-helical crystallites, which had gelatinization temperature above 100°C and were resistant to enzymatic hydrolysis at 95-100°C.

Scanning electron microscopy (SEM) images of G/G, G/H, H/G, and H/H starches are shown in Fig. 5. All starch contained spherical and elongated granules. G/G starch contained a large number of elongated granules while other starches contained a smaller proportion of elongated granules (Fig. 5A-D). Fig. 5B shows that some G/H starch granules began to take on a less extreme protrusion. Fig. 5C shows that some H/G starch granules had minor protrusion that was less pronounced than those found in G/H starch granules (Fig. 5B). The results suggested that the HAM gene affected the formation of elongated granules, which resulted from the increase of amylose/IC content in the starch granules (Table 1).



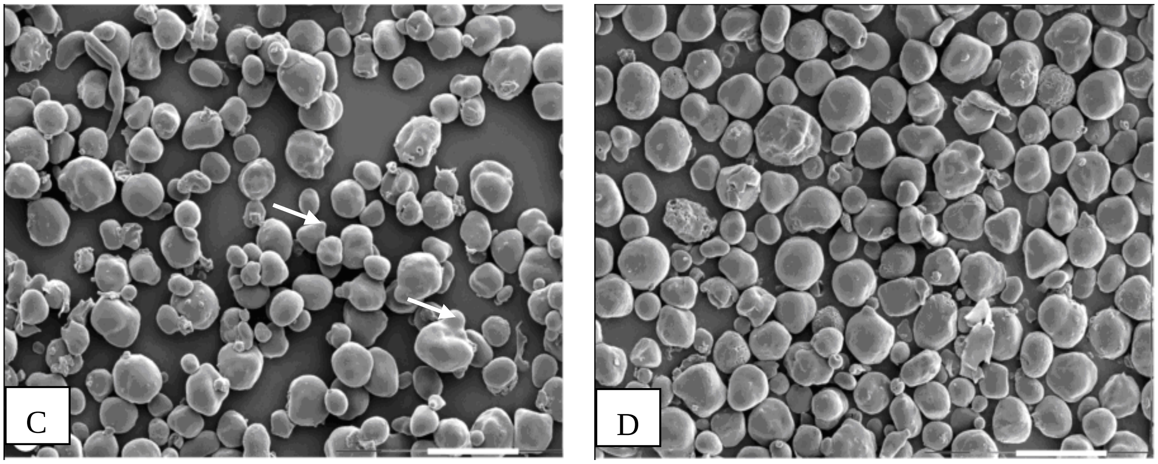


Fig. 5. SEM images of starches. A, G/G; B, G/H, C, H/G; D, H/H. Scale bar = 20μm. Arrow indicates protrusion.

G/G, G/H, H/G, and H/H starches displayed B-type polymorph. The percentages of starch crystallinity increased from 16.3 to 23.7% as the decrease in HAM gene dosage (Table 1). Although both amylose and amylopectin molecules contribute to the crystallinity of the *ae*-mutant starch, the decrease in starch crystallinity with the increase of amylose content suggested that the amylose molecules had less efficiency to form crystallites in the *ae*-line starch granules. V-type X-ray diffraction pattern of amylose-lipid complex with 2θ peaks at 8°, 13°, and 20° was not observed in the starches, indicating that no crystalline amylose-lipid complex was present in the starch granules.

A broad thermal-transition was observed for all starch samples (Fig. 6). The transition range became narrower as the HAM gene dosage decreased (Fig. 6). Onset gelatinization-temperatures ranged from 61.3 to 65.9°C (Table 2), which was similar to that of normal maize starch. Two endothermic peaks were observed for most starches except for G/G. The first peak (81.1-84.2°C), corresponding to the melting of crystallites resulting from mainly amylopectin molecules, decreased with the increase in HAM gene dosage (Table 2; Fig. 6). The increase in HAM gene dosage correlated with an increase in second peak (~99.1°C) (Table 2; Fig. 6), which was attributed to melting of amylose-lipid complex and long-chain double-helical crystallites of amylose/IC. The conclusion gelatinization-temperatures were 122.2°C for G/G, 119.0°C for G/H, 108.9°C for H/G, and 103.2°C for H/H starch (Table 2), which decreased with the decrease in HAM gene dosage. These findings supported that HAM gene dosage significantly affected molecular structure of starch, resulting in changes of gelatinization temperature. A decrease in enthalpy change with the increase of HAM gene dosage was also observed (Table 2), which could be resulted from the reduction of starch crystallites and total double helices in starch granules.

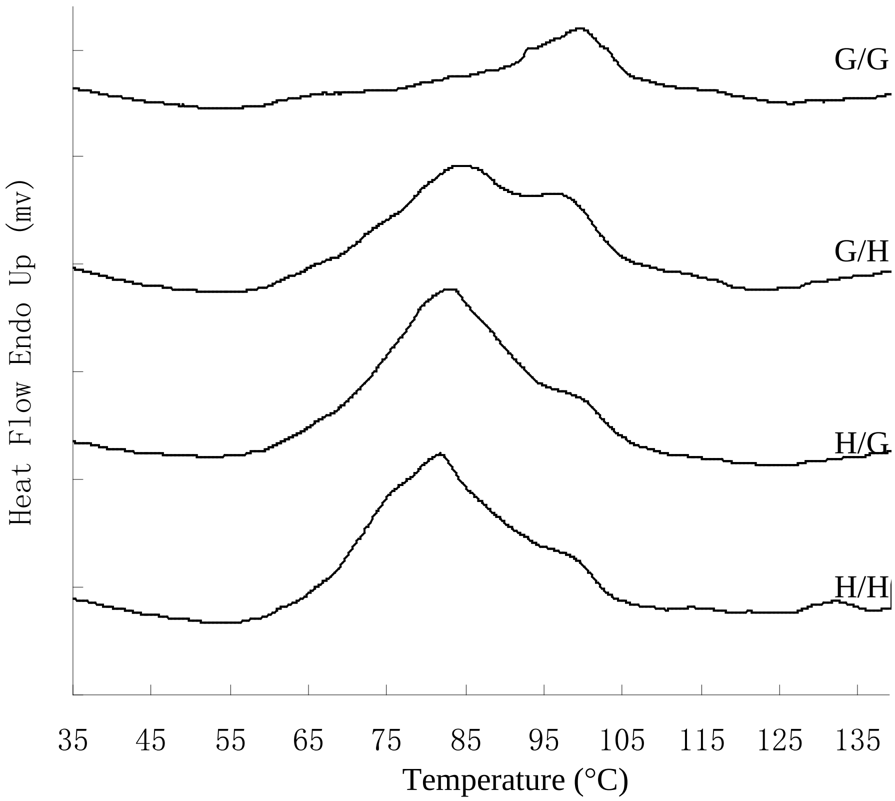


Fig. 6. Differential scanning calorimetry (DSC) gelatinization-thermograms of starches.

Table. 2. Thermal properties of starches[a]

Sample	Gelatinization				
	T_o (°C)	T_{p1} (°C)	T_{p2} (°C)	T_c (°C)	ΔH (J/g)
G/G	61.8 ± 0.4	nd [b]	99.7 ± 0.2	122.2 ± 0.2	7.8 ± 0.2
G/H	61.3 ± 1.1	84.2 ± 0.2	99.1 ± 0.8	119.0 ± 0.3	13.7 ± 0.8
H/G	64.1 ± 2.3	82.5 ± 0.7	98.6 ± 0.1	108.9 ± 0.1	16.2 ± 0.8
H/H	65.9 ± 1.0	81.1 ± 0.8	99.0 ± 0.3	103.2 ± 0.5	15.1 ± 0.8

[a] T_o, T_p, T_c, and ΔH are onset temperature, peak temperature, conclusion temperature, and enthalpy change, respectively.

[b] nd = not detected.

The increased HAM-gene dosage increased the amylose/IC content in maize *ae*-mutant starches, which resulted in formation of long-chain double-helical crystallites and amyloose-lipid complex and changes in granule morphology, starch crystallinity, and starch thermal properties. Two doses of HAM gene substantially increased the RS content of maize *ae*-mutant starch, while one dose of HAM gene had little effect on the RS content.

In summary, during this report period, we have revealed the mechanism of elongated granule formation in the amyloplast of GEM 0067 line. The HAM gene dosage had significant effect on the resistant-starch content and physicochemical properties of the maize *ae*-mutant starch. The dosage effect of HAM gene on the resistant-starch content provided information for corn breeders to develop corn with high yield and larger resistant starch contents.