DIFFERENCE ANALYSIS OF RESISTANT STARCH ACCUMULATION IN BANANA FRUITS

Gao Huijun[1,*], Huang Shaobo[1], Lu Wei[1], Ling Xuexi[1], & Yi Ganjun[2]

[1]Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, China
[2]Guang Dong Academy of Agricultural Sciences, China

*Corresponding author: huijun_gao@aliyun.com

ABSTRACT

Bananas are well known as good sources of starch. Resistant starch (RS) is a functional component having health care function. 'Cavendish' and plantain bananas were used to investigate the influences of hydrolases and granule structure on starch degradation. The levels of RS, non-resistant starch (non-RS), total starch, and amylose content during the fruit-ripening process were determined. For starch-synthesis, yeast one-hybrid assay, Real Time q-PCR, double luciferase experiment, banana genetic transformation were used to screen and verify the transcription factors regulating the synthesis of RS in developing banana fruit. For banana starch processing, the physicochemical properties of starch were analyzed from 3 banana cultivars and potato as comparison. Compared to 'Cavendish', plantain had a higher content of total starch and RS, a faster starch-degradation rate, and a lower decrease in the ratio of RS/total starch. Two a-amylases, one starch phosphorylase, and one starch debranching enzyme were specifically upregulated in plantain, which might hydrolyze more non-RS compared with Cavendish; *MaGBE4* was specifically upregulated during banana fruit developing, 5 transcription factors were selected, which might regulate starch synthesis by binding *MaGBE4*; Banana starch had higher gelatinization temperature than potato starch, which corresponded to the rich resistant starch in banana and in conclusion there are differences in the synthesis and degradation of resistant starch among different banana varieties. Transcription factors can regulate the synthesis of resistant starch by regulating the activity of *MaGBE4*. The resistant starch in banana has great potential for processing.

Keywords: banana; resistant starch; transcription factors; processing

1. INTRODUCTION

Starch is a product of photosynthesis and is present as a semicrystalline form in plant cell plastids. In animals, starch is digested at different rates according to the structure of the starch in the starch granules. There are three categories: 1) Rapidly digestible starch, which can be digested and absorbed orally and in the small intestine rapidly (digestion time < 20 min); 2) Slowly digestible starch, which can be digested in the small intestine (digestion time between 20 and 120 min); 3) Resistant starch (RS), which refers to the starch that cannot be digested or absorbed in the small intestine (digestion time > 120 min). The existence of resistant starch was first proposed by Englyst *et al.* (1982). The physiological function of RS is similar to dietary fiber, as in that it can delay blood glucose and insulin response, and reduce the concentrations of cholesterol and triglycerides. Resistant starch is fermented and degraded to short-chain fatty acids that acidify the intestine contents and may play a role in the prevention of colon cancer (Fuentes-Zaragoza *et al.*, 2010). As more attention is being paid to healthy diets, RS has become a focus for research activity.

2. MATERIALS AND METHODS

Fruit fingers were selected for uniformity of shape, size, and color. After ethylene treatment (500mL L^{-1}) for 16 hours, fruit was stored at 24°C and 90% moisture. The samples were collected at 0, 2, 3, 4, and 5 days in storage time, and subsequently frozen in liquid nitrogen and stored at -80°C prior to further analysis. Five banana fingers were selected for each replicate. All samples were prepared with at least three biological replicates.

Fruit flesh (0.5 g fresh weight) frozen with liquid nitrogen was ground into powder using mortar and pestle, then treated with successive washes of 80% alcohol, 50% alcohol, and water to remove soluble sugar and other soluble substrates. Resistant starch and non-RS contents were analyzed using a Resistant Starch Assay Kit (Megazyme, Bray Business Park, Bray, Wicklow, Ireland) according to the manufacturer, Park, Bray, Wicklow, Ireland). Assayrc is the sum of RS and non-RS.

Starch granules were isolated from the fruit pulp at five ripening stages using a reported protocol (Soares *et al.*, 2011) with the following modifications. Frozen flesh pulp was ground into powder then a 5 g sample was directly suspended in 0.005 L of pectinase solution (15 g L^{-1}, pH 4.0, Sigma Chemical Co.) for enzymic removal of cell walls. The homogenate was laid in a shaking water bath at 45°C for 2 h, then filtered using Miracloth membrane (Calbiochem). After being centrifuged at 3000g for 10 min, the pellet was washed with distilled water three times. The pellet was dried in a drying oven, and stored at room temperature for SEM and amylose content analysis.

Amylose content was determined using a two-wavelength method with potassium iodide (Zhu *et al.*, 2008). Dry starch granules (0.1 g) were completely dissolved in 0.01 L potassium hydroxide solution (0.5 mol L^{-1}), then diluted to 0.6 L with distilled water; 0.03 L were titrated to pH 3.5 with hydrochloric acid (0.1 mol L^{-1}), then 0.002 L iodine solution was added. After color development, samples were scanned at 624 and 472 nm using a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Results were evaluated using the method described by Zeng *et al.* (2012).

The dried starch granules were fixed onto stubs using doublesided tape and coated with a 10 nm thick platinum layer using the JEOL JFC-1600 (JEOL, Tokyo, Japan) coating system. The samples were examined on a JEOL JSM-6360LV (JEOL, Tokyo, Japan) scanning electron microscope. Scanning electron microscopy was performed in secondary electron mode at 15 kV. After SEM analysis, the images were loaded in SmileView (JEOL Ltd, Tokyo, Japan) software. The length of starch granules was measured using "apan" software. The length of starch granules was measured for each sample and the average particle size of the starch nanoparticles was determined.

3. RESULTS

3.1. Changes in starch content during fruit ripening

The total starch content decreased gradually in both Cavendish and Plantain during the ripening process (Table 1). At 0 day, 91% of total starch was RS in Plantain, which dropped to 83% at 5 days. Meanwhile, the proportion of RS in total starch decreased from 87% to 69% in Cavendish. That means the ratio of RS in total starch declined at 8% and 18% in Plantain and Cavendish, respectively. This suggested that the ratio of RS in total starch decreased more slowly in Plantain

than in Cavendish, and there was obviously a different preference in starch degradation between Cavendish and Plantain.

Table 1. Resistant (RS) and non resistant starch (non-RS) content in Cavendish and Plantain at five ripening stages

Starch	Species	0 d	2 d	3 d	4 d	5 d
RS	Cavendish	0.81±0.007 b	0.114±0.008 c	0.075±0.004 d	0.057±0.003 e	0.027±0.002 f
	Plantain	0.253±0.011 a	0.201±0.026 b	0.140±0.015 c	0.080±0.008 d	0.053±0.012 e
Non-RS	Cavendish	0.028±0.001 a	0.028±0.003 a	0.017±0.001 c	0.020±0.003 c	0.012±0.002 d
	Plantain	0.024±0.004 b	0.017±0.002 c	0.017±0.002 c	0.012±0.001 d	0.011±0.001 d
Total	Cavendish	0.209	0.142	0.092	0.077	0.039
	Plantain	0.277	0.218	0.157	0.092	0.064

3.2. Starch-degradation-related gene expression analysis

Compared with b-amylases, a-amylase had different expression patterns (Figure 1). Two genes (GSMUA_Achr3T07130_001 and GSMUA_Achr3T07190_001) encoding a-amylases were downregulated during the whole ripening process in both Cavendish and Plantain, while two (GSMUA_Achr5T10560_001 and GSMUA_Achr8T04140_001) were up-regulated. Specifically, a-amylase (GSMUA_Achr7T18920_001) was down-regulated in the Cavendish fruit-ripening process but up-regulated in Plantain, especially at later stages. This suggested that GSMUA_Achr7T18920_001 might play a vital role in the differential starch degradation between Cavendish and Plantain. Isoamylase is one type of debranching enzyme, which preferentially removes short branches and participates in amylopectin synthesis. Interestingly, one gene encoding isoamylase (GSMUA_Achr7P12210_001) was strongly induced gradually during the Plantain ripening process, reaching a 49-fold increase at 5 days compared with 0 day.

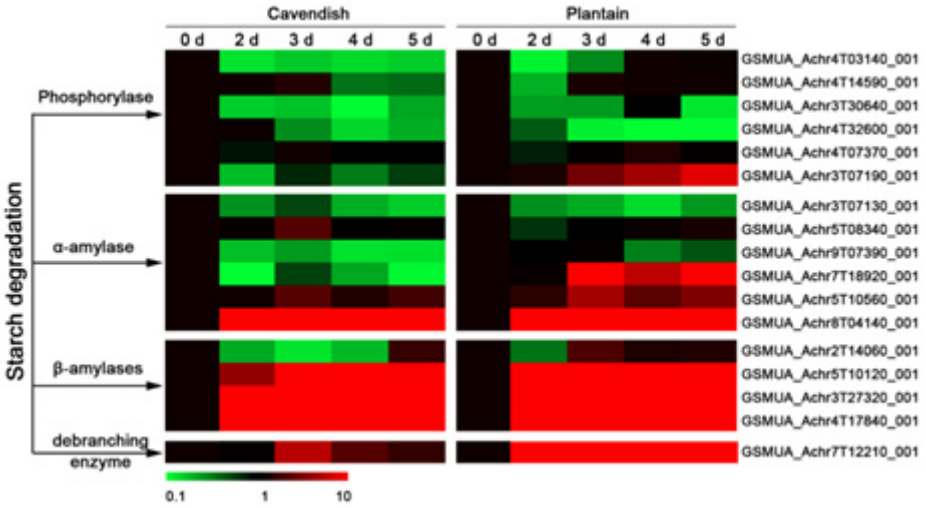

Figure 1. Starch degradation-related genes in fruit ripening

3.3. MaGBE4 expression analysis and transcription factors selected

Five MaGBEs genes were cloned from banana, which belong to three different subtypes. Among them, subtype 2 is reported to be the most functional family. The results of Real Time q-PCR

showed that the expression of *MaGBE4* was significantly higher than other homologous genes during banana fruit development. Therefore, we indicate that *MaGBE4* is the key gene regulating the synthesis of resistant starch in banana fruit. The promoter region of *MaGBE4* was then cloned and constructed as bait vectors. Five candidate transcription factors were screened by the yeast one-hybrid system. Then we verified that these 5 transcription factors could interact with *MaGBE4* by double luciferase assay, subcellular localization and Point-to-Point Y1H.

3.4. Physicochemical Properties of Different Starches

Starch physicochemical properties were analyzed in 3 banana cultivars and potato (Table 2). Pasting temperature in 3 banana cultivars was higher than in potato, while peak viscosity, cool viscosity and final viscosity of starch in 3 banana cultivars were lower than in potato. Banana starch had higher gelatinization temperature, which corresponded to the rich resistant starch in banana.

Table 2. Starch physiochemical properties for 3 banana cultivars and potato.

	Dajiao	Guang Fen	Baxi	Potato
Gelation initiation temperature (°C)	67.60	76.80	79.20	63.00
Gelation peak temperature (°C)	71.70	79.70	82.90	68.60
Gelation termination temperature (°C)	77.10	85.10	88.50	77.50
Pasting temperature (°C)	84.00	82.35	82.90	67.20
Peak viscosity (cP)	114.00	88.00	89.92	371.17
Cool viscosity (cP)	83.50	76.08	88.67	129.83
Final viscosity (cP)	126.75	98.00	123.67	172.67
Setback (cP)	43.25	21.95	35.00	42.84

Note: Starch was extracted from Dajiao (AAB gene group), Guang Fen (ABB gene group), Baxi (AAA gene group), and potato.

To explore the regulatory mechanism of starch degradation in banana fruit, 17 starch degradation-related genes were selected for quantitative real-time PCR analysis. The genes were classified into four groups based on their reaction products, including six starch phosphorylases, six a-amylases, four b-amylases, and one debranching enzyme. The ratios of the other samples compared to the 0 day samples were calculated and the Logs of ratios base 2 are presented in both 'Cavendish' and plantain. Up-regulated genes are shown in red and down-regulated genes are in green.

'Cavendish' and plantain fruits were harvested at the green maturity stage. After ethylene treatment, fruits were stored at 24°C and 90% moisture, and sampled at 0, 2, 3, 4, and 5 days. Resistant starch (RS) and non-resistant starch (non-RS) contents were determined using a Resistant Starch Assay Kit. Data presented are the average (kg kg^{-1} fresh weight) with SD of three replicates. Letters a, b, c, d, and e represent the significant differences with $P < 0.05$.

4. DISCUSSION

In commercial production, banana fruits are harvested at an unripe state and requires undergoing ripening in order to be edible after harvest. In the commercial process, fruits are harvested, and physiological and biochemical changes are controlled in ripening chambers/facilities to form unique flavors and quality. Starch, as the main carbon source stored in the fruit, is dramatically degraded during the ripening process for flavor substance synthesis and energy supply. In past decades, there have been two models of starch metabolism: one is based on the germinating cereal seed (Beck & Ziegler, 1989) and the other on the diurnal metabolism of leaves (Zeeman

et al., 2007). The banana fruit is likely to incline towards the seed model. In this process, starch is hydrolyzed into soluble sugars catalyzed mainly by four kinds of amylases, including b-amylases, a-amylases, starch phosphorylases, and debranching enzymes (Smith *et al.*, 2007).

5. CONCLUSIONS

This initial analysis of the difference in starch degradation in the fruit-ripening process between plantain and 'Cavendish' banana focuses on the role of starch structure and function of amylase enzymes in RS degradation. Both total starch and RS contents decreased in the two cultivars during ripening. Compared to 'Cavendish', plantain had a higher content of total starch and RS, a faster starch-degradation rate, and a lower decrease in RS proportion. Starch granules of 'Cavendish' were larger and more rounded, while smaller and ellipsoidal starch granules were observed in plantain, which degraded more easily. Also, the analysis of gene expression suggested that b-amylases had a key role in starch degradation in both cultivars. A number of genes were specifically up-regulated in plantain, including two a-amylases, one starch phosphorylase, and one starch debranching enzyme. These genes may be involved in the degradation of greater amounts of non-RS starch in plantain than in 'Cavendish' banana fruit.

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REFERENCES

Beck, E. & Ziegler, P. (1989). Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology, 33*, 431-454. https://doi.org/10.1146/annurev.pp.33.060182.002243

Englyst, H., Wiggins, H., & Cummings, J. (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst, *1272*. https://doi.org/10.1039/an9820700307

Fuentes-Zaragoza, E., Riquelme-Navarrete, M., Sánchez-Zapata, E., & Pérez-Álvarez, J. (2010). Resistant starch as functional ingredient: A review. *Food Research International, 43*, 931-942. http://dx.doi.org/10.1016/j.foodres.2010.02.004

Smith, A.M., Zeeman, S.C., & Smith, S.M. (2005). Starch degradation. *Annual Review of Plant Biology, 56*, 73-98. https://doi.org/10.1146/annurev.arplant.56.032604.144257

Soares, C. A., Peroni-Okita, F. H. G. A., Cardoso, M. B., Shitakubo, R., Lajolo, F. M., & Cordenunsi, B. R. (2011). Plantain and banana starches: granule structural characteristics explain the differences in their starch degradation patterns. *Journal of Agricultural and Food Chemistry, 59*(12), 6672-81. https://doi.org/10.1021/jf201590h

Zeeman, S., Smith, S., & Smith, A., (2007). The diurnal metabolism of leaf starch. *Biochemical Journal, 401*(1), 13–28. https://doi.org/10.1042/BJ20061393

Zeng, F. K., Zhao, X., Zhou, T. H., & Liu, G. (2012). Dual-wavelength colorimetric method for measuring amylose and amylopectin contents of potato starch. Modern Food Science Technology, 28(1), 119-122.

Zhu, T., Jackson, D. S., Wehling, R. L., & Geera, B. (2008). Comparison of amylose determination methods and the development of a dual wavelength iodine binding technique. *Cereal Chemistry, 85*(1), 51-58. https://doi.org/10.1094/CCHEM-85-1-0051