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ZmSBEIIb involves in resistant starch formation in maize seeds

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ABSTRACT

Resistant starch(RS) has various functions in controlling the glycemic index (GI), lowering concentration of cholesterol and triglycerides, inhibiting fat accumulation, preventing colonic cancer, reducing gall stone formation, maintaining intestinal tract healthy and enhancing the absorption of minerals. Elevated RS in food is an important and effective approach for public health. RS is also an important material for industries. The mechanism of RS formation is largely unknown. SBEIIb plays a central role in amylopectin biosynthesis and involves in regulating the branching profiles of starches in maize endosperm. Maize mutant ae1 is generated using a Mutator transposon I insertion in SBEIIb. In this paper, we found amylose content (AC) and RS in seeds of ae1are increased significantly than that of wild type. This show ae1 involves in RS formation in maize seeds. Novel maize lines with high RS content provide potential benefits for high RS content maize breeding. High RS content maize varieties possess high health care and industrial application value.

Key words: *ae1*, maize, resistant starch, *SBEIIb*.

INTRODUCTION

RS is also called enzyme resistant starch, definited as the starch and starch degradation products which cannot be digested and absorbed in the healthy small intestine of human[1]. RS provides functional properties in controlling GI[2], lowering concentration of cholesterol and triglycerides[3, 4], inhibiting fat accumulation[5], preventing colonic cancer[6], reducing gall stone formation[7], maintaining intestinal tract healthy[8]and enhancing the absorption of minerals[9].

The mechanism of RS formation is largely unknown. There are several factors affect the RS formation. It's reported that RS content is positive related to AC [10, 11]. Starch granule size and structure are related the RS content. Starch granule in potato is larger than that in cereals, the potato starch digested more slowly than that of cereals[12].Starch Crystalline structure can be classified into A type, B type and C type, according X-ray scattering pattern. The digestibility of the starch with B type less than A type, C type in the middle [13, 14].The chain length of amylose and amylopectin is another major factor affect the RS formation. RS increase according degree of polymerization(DP) of amylose (from 10 DP to 610 DP) by hydrothermal treatment with retention[15]. The effect of the chain length amylopectin on RS formation is unclear in detail. It reported that amylopectin starch debranched by pullulanase followed by heat-processing can increase RS content[16].It's due to long unbranched chains of amylopectin involve into RS formation[17].Other components in cell， such as protein, lipid, cellulose et al， can also effect RS content[17-19].Among them, Lipids is most important effect on RS formation. Lipids can decrease RS content significantly [17].Food additives and food processing technologies are another factors can affect RS content[20, 21].

Starch branching enzyme II (SBEII) is a key enzyme in amylopectin biosynthesis[22]. In maize (*ZeamaysL.*)， *SBEII* comprises two genes: *SBEIIa* and *SBEIIb*[23]. SBEIIa and SBEIIb may exert function by complex [24]. In maize amyloplast, SSIIa、SSIII、BEIIa and BEIIb formed 600KD complex and SSIIa、BEIIa and BEIIb formed 300KD

complex. In the maize amyloplaststroma, SBEIIb is the most abundant protein [25]. In maize endosperm, SBEIIb expression level is about 50 times higher than that of SBEIIa[26]. SBEIIb plays a central role in amylopectin biosynthesis in maize endosperm and can be phosphorylated by two distinct Ca^{2+} dependent protein kinase[27].

maize mutant *ae1*is generated using a Mutatortranspos on I insertion in *SBEIIb*[28]. In this paper, we found AC and RS in seeds of *ae1* is increased significantly than that of wild type. This show *ae1* involves in RS formation in maize seeds. Novel maize lines with high RS content provide potential benefits for high RS content maize breeding.

EXPERIMENTAL SECTION

Plant Materials

Mutant *ae1*seeds and W64A are obtained from Maize Genetics Cooperation - Stock Center. Wild type of *ae1* mutant is isolated form the F_2 population generated by the cross between *ae1* and W64A.

All the maize materials were grown in an experimental field of Yangtze University during the natural growing seasons. Maize grains were harvested about 40 days after heading, air-dried and stored at room temperature for 3 months before analysis. 10 endosperms of grains were ground to flour and used to measure the RS and AC.

Genotype of*ae1* mutant alleles

Genomic DNA was extracted from fresh leaves of each plant using the cetyltrimethylammonium bromide (CTAB) method[29].

PCR reaction was performed in 20mL total volume containing 2 mL 10×PCR buffer (100 mMTris-HCl pH 8.0, 15 mM $MgCl_2$,500 mMKCl, 1% TritonX-100), 0.2 mMdNTPs, 0.2mM primer set, 30-100 ng genomic DNA and 0.5 U Taq polymerase. The PCR products were separated on 1% agarose gel. The genotypes of W64A *and ae1* mutant were identified using genotyping primers shown in Table1.

Table 1 Primers used in this work

Primers for genotyping	
ae1F	AGGTGATGTAGGCGAGCTGT
ae1 R	ATTACGAGTTAAGAAGAGGCCGGTGT
Mu1 F	ACGGGAACGGTAAACGGGGACAGA
Primers for quantitative real-time RT-PCR	
UBCP F	AAATTGTGAGCGGCAGGGAA
UBCP R	GCATGGACCATACCCATTCA
CUL F	GAAGAGCCGCAAAGTTATGG
CUL R	ATGGTAGAAGTGGACGCACC
qae1 F1	TGCAGTCACCCAGAGCA
qae1 R1	TCAGCCAATGCTAAAACCCCA
qWx F	TGGGGAAAGACCGAGGAGAA
qWx R	TGGTCCGGAGAAGTATGGGT

Quantitative real-time RT-PCR

Immature ears were collected at 10 day after flowering (DAF). All samples were immediately frozen in liquid nitrogen, and then stored at -80°C until subsequent use. Total RNA was isolated from Immature ears tissues using Trizol (Ambion, Austin, TX, USA), followed by a DNase treatment (Turbo DNase, Ambion), and 1 µg total RNA were used for complementary DNA synthesis using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions.

qPCR experiments were conducted on the 7500 System (Applied Biosystems), using primers for quantitative real-time RT-PCR(Table1) and a reaction system with SYbGreen mix (Bio-Rad), according to the manufacturer's instructions. The thermal profile of the qRT-PCR reactions was 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The geometric means of *UBCP* and *CUL*, encoding an Ubiquitin carrier protein and *Cullin* respectively, were used as reference genes to normalize the expression levels.

Determination of RS content

RS content was measured according to AOAC method (2002.02)with a slight modification[30].100±1 mg milled maize flour(only endosperm) were accurately weighed and placed directly into screw-cap tubes (16 x 125mm). 500µL water was added into each tube, then boiled in electric cooker for 20 min and at warm keeping status at 50ºC for 10 min. Tubes were taken out and cooled to room temperature. KCl-HCl buffer (pH = 1.5) containing 6 IU/mg pepsin was added into each tube and the rice floury was ground and dispersed by a stirring rod, mimicking the chewing in mouth and warmed at 37ºCfor 1 h. Other procedures were carried out as described in the method AOAC(2002.02).

Determination of the amylose content

AC was determined according to the modified method of Perez[31]. 100±0.5 mg milled maize flour(only endosperm)were accurately weighed and wetted with 1mL of 95% ethanol in 100 mL volumetric flasks, mixed slightly. Samples in volumetric flasks added 9.0mL of 1MNaOH were boiled 10 min, then cooled to room temperature, distilled water was added to 100 mL.5mL of sample was taken out and put into a new volumetric flasks, added 50 mL distilled water, 1mL acetic acid and 1.5mL I_2 solution in turn. Then distilled water was added to 100 mL, standing for 20 min. The absorbency of sample was measured at 620 nm using a spectrophotometer.

RESULTS AND DISCUSSION

The structure of *SBEIIb* and the Mutational Site of *SBEIIb* in *ae1*Mutant

SBEIIb had N-terminal early set domain (NESD), Alpha amylase catalytic domain (AACD) and Alpha amylase, C-terminal all-beta domain(AAC). In *ae1*, the Mu1 insertion site is 598bp to the upstream of ATG in *SBEIIb*. Primers used in the genotype analysis were ae1F/ae1R and Mu1 F/ae1R primer sets (Fig. 1).

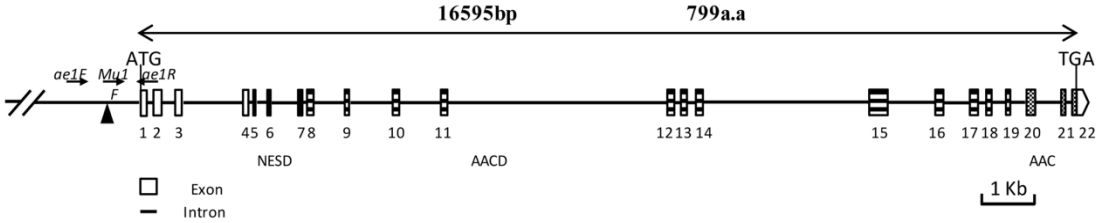

Fig.1.Genomic structure and the mutational sites of *SBEIIb* in *ae1* mutant with the indicated predicted protein domains. ATG and TGA indicate the initiation and termination codons, respectively. Boxes, lines, triangles and arrowheads indicate exons, introns, mutation sites and primer sites of genotype identification respectively. a.a., amino acid. NESD, N-terminal early set domain; AACD, Alpha amylase catalytic domain; AAC, Alpha amylase, C-terminal all-beta domain. Primers used in the genotype analysis were *ae1*F/*ae1* R and *Mu1* F/*ae1* R primer sets

Phenotypes of *ae1*and W64A

The *ae1* mutant did not have any visibly abnormal phenotype at the vegetative stage of plant growth and development. The seeds of *ae1* mutant did not have any visibly abnormal phenotype compared to wild type W64A(Fig.2A).RS content of *ae1* grains is 14.5%, increased greatly, compared to 6.47% of W64A grains(Fig.2B).AC content of *ae1* grains is 41.52%, increased greatly, compared to 24.83% of W64A grains (Fig.2C).

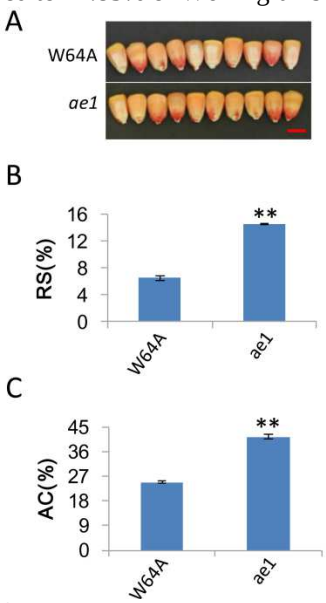

Fig.2.Morphologies, AC and RS content of seeds of ae1 and W64A. Upper panel is seeds of *ae1* Mutant, lower panel is seeds of wild type W64A, Bar = 1cm (*A*).Seed RS content in wild type W64A and *ae1*mutant(*B*).Seed AC content in wild type W64A and *ae1*mutant (*C*). Values are means±SE; n = 3. * indicate P < 0.05, ** indicate P <0.01

Genotypes of*ae1*and W64A

In *ae1*, the PCR reaction using ae1F/R primer pair had no product, while Mu F/R primer pair generated a879bp band (Fig.3). In wild type *W64A*, the PCR reaction using ae1F/R primer pair had a 1037bp band, while Mu 1F /1R primer pair had no product (Fig. 3).

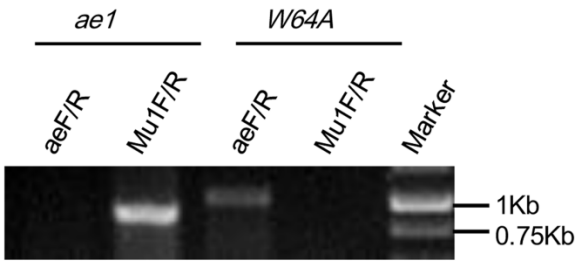

Fig.3.Genotypes of *ae1* and W64A. Analysis of genotypes by PCR uses ae1F/R primer pair and Mu F/R primer pair. PCR using ae1F/R primer pair has no product in *ae1* or a1037bp product in W64A. PCR using Mu F/R primer pair has no product in W64A or an 879bp product in *ae1*

The expression of *SBEIIb* and *Wx* in immature ears of W64A and *ae1*

SBEIIb expression levels in *ae1*were approximately 35% of that in the wild type immature ears(Fig. 4 A).Since AC in*ae1* increased significantly to 1.67 fold than that in wild type W64A, *Wx* expression levels was investigated in *ae1* and W64A.*Wx*relative expression level was increased significantly approximately to 574 fold in *ae1* compared to that in wild type W64Aimmature ears (Fig. 4B).

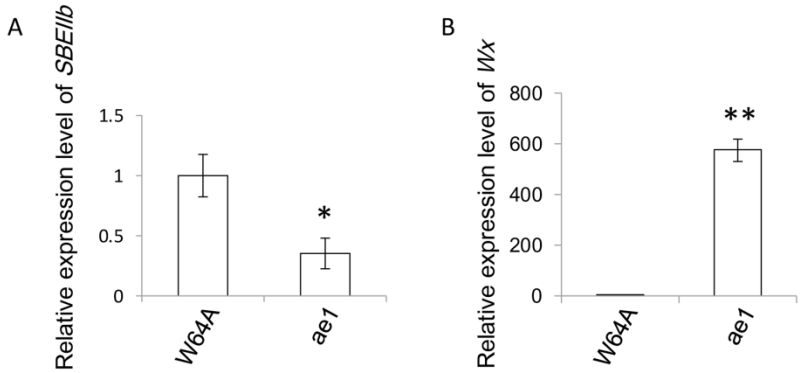

Fig.4.The expression of SBEIIb and Wx in W64A and *ae1*. Relative expression levels of *SBEIIb* in*W64A and ae1* immature ears were collected at 10 DAF (*A*). Relative expression levels of *Wx* in*W64A and ae1* immature ears were collected at 10 DAF (*B*). ** denotes significantly differences at P < 0.01. * denotes significantly differences at P < 0.05. Bars indicate the standard error of the mean of 3 plants

Starch molecules are biopolymers of anhydroglucose units linked bya-1,4 anda-1,6 glycosidic bonds. Starch iscomprised of amylose and amylopectin. Amylose is generally linear glucan polymers formed by anhydroglucose units linked major by a-1,4 glycosidic bonds and scarcely a-1, 6 glycosidic bonds. While amylopectin is the branched glucan polymers formed by anhydroglucose units linked by a-1,4 glycosidic bonds and more a-1,6 glycosidic bonds. *ae* mutant endosperms are glassy, tarnished and contains a higher proportion of amylose[32]. The amylopectin in *ae* mutant endosperm is longer than average chain length and has fewer branch points.

SBEII involves the a-1,6 linkages formation in starch, playing an important role in the formation of amylopectin[33]. High RS content in *ae1*, demonstrated the importance of *SBEIIb* in RS formation. Wx is the major gene control the AC in maize endosperm [34]. In*ae1*, we detect that *Wx* expression increased significantly and AC increased significantly compared to wild type W64A. AC is positive related to RS content [10, 11]. So*Wx* and *SBEIIb* together involved in RS formation in *ae1*. The main agronomic characters except the high RS content and maize grain quality were no significant difference in *ae1* mutants compare to their wild type, respectively.

AC and the molecular weight of amylose are major factors affecting the resistant starch content. During the starch gelatinization in the heating water, the crystal structure of starch is broken down, the amylose chains was dissolved and amylose molecules released into the water from the disintegrate starch granule. Then when the temperature was decreased gradually, the free curly amylose molecules closed to each other and form new double helix using intermolecular hydrogen bond. Many double helix amylose molecular form into micro crystal nucleus, eventually form into larger amylose crystal[35]. The larger amylose crystals prevent the amylase binding the starch molecular to access the glycosides in the crystal structure. So, the amylose crystal structure resistant amylase hydrolysis.

CONCLUSION

Our studies show that RS content and AC in maize endosperm of *ae1* are increased significantly. So, *ae1* has great

application value in breeding new maize cultivars with high RS content. Using high RS content maize as food will have great help to control GI and keep the health of diabetes. Now, the detail of molecular basis of RS formation and the regulation of starch biosynthesis in *ae1* are largely unknown. And the structure of RS in *ae1* is also largely unknown. So, the breeding of maize with high RS is still full of challenges.

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