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Characterization and Starch Properties of a Waxy Mutant in *Japonica* Rice Kitaake

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Abstract

A rice waxy mutant *M6* was generated from a *japonica* rice cultivar Kitaake through gamma irradiation. In this study, we characterized the mutant and analyzed its starch properties. The *M6* with milky opaque kernels had lower seed length, width, and weight than wild type. The cavity in the center of starch granule might be responsible for waxy appearance of *M6* mature kernels. Sequence analysis of granule-bound starch synthase I (GBSSI) gene showed that there was a 23 bp duplication inserted into the exon 2, generating one stop codon. No GBSSI protein was detected in the endosperm of *M6*. The isolated starch showed similar ratio of short and long branch-chains of amylopectin between *M6* and wild type, but the *M6* starch had no amylose. Both the *M6* and wild type had A-type starch, but the *M6* starch exhibited higher relative crystallinity than wild type starch. Compared with wild type starch, the *M6* starch had significantly high swelling power, gelatinization enthalpy and breakdown viscosity and low water solubility, gelatinization peak temperature, peak viscosity, hot viscosity, final viscosity and setback viscosity. The *M6* starch had significantly lower resistance to amylase hydrolysis than wild type starch.

Keywords: Rice waxy mutant; Endosperm starch; Molecular structure; Functional properties.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops and is the staple food for over half the world's population. Starch, the major storage carbohydrate in rice endosperm, consists of two distinct components: amylose and amylopectin. Amylose is a mixture of lightly branched and long-chain linear molecules, whereas amylopectin is a much larger molecule with a highly branched structure consisting of about 95% α-1,4 linkages and 4-5% α-1,6 linkages. According to the amylose content (AC), rice varieties are classified into waxy (0-5%), low AC (6-18%), intermediate AC (19-23%), or high AC (>23%) types [1]. Rice seeds with high amylose levels are usually associated with dry, hard, and poor glossy characteristics, which lower rice rating quality [2]. Therefore, control of the AC of starch is a major objective in rice breeding.

Amylose in the rice endosperm is mainly synthesized by granule-bound starch synthase I (GBSSI), which is encoded by the *Waxy* (*Wx*) gene located on chromosome 6 [3]. At least six *Wx* alleles have so far been identified: Wx^a, Wx^b, Wx^{mq}, Wx^{op}, Wx^{hp}, and *wx*. In nonwaxy rice varieties, Wx^a and Wx^b have been recognized as being distributed in the *indica* and *japonica* subspecies, respectively, and their expression levels are highly correlated with

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AC in the endosperm [4]. In low AC or waxy rice varieties, a mutation in the *Wx* gene drastically reduces synthesis of amylose. The leaky mutations in *Wx* alleles, such as Wx^{mq} from Milky Queen, Wx^{op} from the opaque endosperm mutants and Wx^{hp} from Yunnan landraces, control the low AC trait [5-7]. Sequencing of *Wx* promoter and 5' noncoding regions from 22 Bangladeshi rice cultivars shows that three of them with low to very low amylose lack the G/T splice site mutation [8]. In addition, a *wx* mutation in the 25 tropical waxy rice varieties shows that exon 2 has a 23 bp duplication in the coding sequence [9].

In rice, AC is one of the key components influencing the eating and cooking quality (ECQ). AC is controlled mainly by the expression levels of *Wx*, the activity of GBSSI and the binding characteristic of GBSSI with starch granule [4, 5]. Different mutations in *Wx* have different effects on starch properties. A tyrosine residue at position 224 of *Wx* correlates with the formation of extra-long amylopectin chains in cultivars carrying Wx^{a} [10]. Using site-directed mutagenesis, three amino acid substitutions of *Wx* transgenic rice lines show significant differences in GBSSI activities, AC and starch physicochemical properties [11]. An amino acid substitution in Wx^{hp} allele reduces the binding of GBSSI to starch granule and AC, affecting the pasting property of starch in rice seeds [5]. Therefore, it is necessary to investigate the effects of different *Wx* mutations on the properties of starch.

Waxy rice, also known as glutinous rice, is widely used for food products, such as glutinous rice crackers and glutinous rice wine in Thailand, Laos, and particularly in China. In Thailand, most of the waxy rice varieties are photoperiod sensitive landraces [9]. Being photoperiod sensitive landraces, waxy rice varieties are difficult to be popularized and applied in large areas. Compared with other rice varieties, the Kitaake variety has a life cycle of only nine weeks, four times a year, and is insensitive to photoperiod changes, which can greatly accelerate the functional genetic studies and new varieties breeding of rice [12]. In China rice market, a lot of waxy rice varieties are cultivated by ^{60}Co gamma irradiation, such as Funuo 101, Funuoyou 396, Guifunuo, Yangfunuo 1 and Yangfunuo 4. In this study, we isolated a rice waxy mutant from the japonica rice cultivar Kitaake through gamma irradiation. The characterization and starch properties of *M6* were investigated. The major objective of this study was to reveal the molecular mechanism of mutation and the structural and functional properties of starch in order to use the mutant effectively in waxy rice breeding program in the future.

2. Materials and Methods

2.1. Plant Materials

The waxy mutant *M6* was isolated from a ^{60}Co-irradiated mutant pool of *japonica* rice cultivar Kitaake. The Kitaake and *M6* were grown in a paddy field at Yangzhou University during the natural growing season. Mature seeds were harvested and used to isolate starch.

2.2. Scanning Electron Microscopy

Seeds were randomly selected for phenotypic analysis. To obtain cross sections, seeds were mounted on aluminum specimen stubs with adhesive tabs, coated with gold, and observed under an environmental scanning electron microscope (Philips XL-30) at 5 kV.

2.3. RNA Extraction and Sequence Analysis

Total RNA were extracted from seedlings of wild type and *M6* using an RNAprep pure Plant Kit (TIANGEN, Beijing). First strand complementary DNA (cDNA) was synthesized with oligo (dT18) based on a PrimeScript Reverse Transcriptase Kit (Takara, Japan). The wild type and *M6 GBSSI* cDNA sequence was cloned using primers 5'-ATGTCGGCTCTCACCACGTCCCA-3' and 5'-AGGAGAACGTGGCTGCTCCTTGA -3', and the PCR product was introduced into the pEASY-Blant Vector (Transgen, Beijing), and transformed into *E. coli* strain DH5α. The recombinant plasmid was sequenced with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA).

2.4. Protein Extraction and Western Blot Analysis

Mature seed endosperms were ground to powder in liquid N_2. The powder was then suspended in the extraction buffer consisting of 50 mM Tris/HCl, pH 8.0, 0.25 M sucrose, 2 mM DTT, 2 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride. After incubation on ice for 1 h, the homogenate was centrifuged for 20 min at 14,000 g, and the supernatants were transferred to new centrifuge tubes. Proteins were resolved by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. The antibodies used were anti-GBSSI rabbit antibody (Immunogen, Wuhan, China) diluted 1:5000, and anti-HSP82 (Beijing Protein Innovation) diluted 1:10000, and horseradish peroxidase-linked secondary antibody (Beyotime, Shanghai, China) diluted 1:5000.

2.5. Isolation of Starch from Brown Rice

The brown rice was soaked in distilled water at 4 °C for 24 h and extensively ground in a mortar. The ground sample was filtered through five layers of cotton cloth and filtered with 100-, 200-, and 400-mesh sieves, successively. The sample was centrifuged at 4000 g for 5 min and washed with distilled water. Starch was washed three times with water and twice with anhydrous ethanol. Finally, samples were dried at 40 °C and ground through a 100-mesh sieve.

2.6. Measurement of Starch Molecular Structure

Apparent amylose content (AAC) of starch was determined following the iodine colorimetric method described by Wang, *et al.* [13]. The molecular weight distribution of starch was analyzed using an Agilent Technologies gel-permeation chromatography (GPC) 220 system according to the method described by Lin, *et al.* [14].

2.7. X-Ray Diffraction (XRD) Analysis of Starch

Starch XRD patterns were obtained with an X-ray power diffractometer (D8, Bruker, Germany). All samples were treated in a desiccator with a saturated solution of NaCl to maintain a constant humidity (relative humidity=75%) for 7 days prior to XRD analysis. The relative crystallinity was determined as described by Wei, *et al.* [15].

2.8. Swelling Power and Water Solubility Determination of Starch

Swelling power and water solubility of starch were determined according to the method of Konik-Rose, *et al.* [16] with some modifications. Starch samples mixed with water (2%, w/v) was put in a 2 mL centrifuge tube and heated in a water bath at 95 °C for 30 min with regular gentle shock. The sample was cooled to room temperature and centrifuged at 8000 g for 20 min. The swelling power was the weight ratio of precipitated gel to dry starch.

2.9. Thermal Property Analysis of Starch

Thermal properties of starch granules were investigated with a differential scanning calorimetry (DSC) (200-F3, NETZSCH, Germany). Three milligrams of starch was mixed with 9 μL of distilled water, and sealed in an aluminum pan. The sample was then heated from room temperature to 130 °C at a rate of 10 °C/min.

2.10. Pasting Property Analysis of Starch

The pasting properties of starch were evaluated with a rapid visco analyzer (RVA) (RVA-3D, Newport Scientific, Narrabeen, Australia). Two grams of starch was dispersed in 25 mL distilled water and subjected to gelatinization analysis. A programmed heating and cooling cycle was used, where the sample was held at 50 °C for 1 min, heated to 95 °C at a rate of 12 °C/min, maintained at 95 °C for 2.5 min, cooled to 50 °C at a rate of 12 °C/min, and then held at 50 °C for 1.4 min.

2.11. Enzyme Hydrolysis Analysis of Starch

The starch was hydrolyzed by porcine pancreatic α-amylase (PPA) and *Aspergillus niger* amyloglucosidase (AAG). For PPA hydrolysis, ten milligrams of starch was suspended in 2 mL of enzyme solution (0.1 M phosphate sodium buffer, pH 6.9, 25 mM NaCl, 5 mM $CaCl_2$, 0.02% NaN_3, 50 U PPA (Sigma A3176)). For AAG hydrolysis, ten milligrams of starch was suspended in 2 mL of enzyme solution (0.05 M acetate buffer, pH 4.5, 5 U AAG (Sigma A7095)). The hydrolyses of PPA and AAG were conducted in a shaking water bath with continuous shaking (100 rpm) at 37 and 55 °C, respectively. After hydrolysis, starch slurry was quickly centrifuged (5000 g) for 5 min. The soluble carbohydrate in the supernatant was determined to quantify the hydrolysis degree using the anthrone-H_2SO_4 method.

2.12. Statistical Analysis

For sample characterization, at least three replicate measurements were performed. All data represent the means ± standard deviation (n=3). The results were analyzed using the Student's *t*-test to examine differences. Results with a corresponding probability value of $p<0.01$ were considered to be statistically significant.

3. Results and Discussion

3.1. Phenotypic Characterization of *M6*

Numerous mutants with defective endosperm were screened from the ^{60}Co-induced mutant library of *japonica* rice cultivar Kitaake and an opaque-kernel mutant was isolated and named *M6*. Throughout the vegetative growth stage, the mutant plants displayed no significant differences from the wild type plants. The mature grains of *M6* were phenotypically similar to its wild type (Fig. 1A), while the brown rice of *M6* was opaque and presented a milky-white appearance (Fig. 1B-D). Iodine staining is a sensitive and convenient method for the detection of amylose in various tissues. When the seeds of *M6* were cut transversely and stained with an iodine solution, a typical reddish color of waxy starch was revealed in the endosperm (Fig. 1C-F;) [17]. Scanning electron microscopy analysis of transverse sections indicated that the compound starch granules in both wild type and *M6* endosperm cells were irregularly polyhedral and densely packed, while some cavities were only observed in the center of the starch granule in *M6* (Fig. 1G, H). This phenotype in *M6* starch granule was similar to the low amylose materials such as Y268F and E410D [11]. Seed size and weight measurements showed that seed thickness was largely comparable between the wild type and *M6*, but the seed length, seed width and thousand seed weight were significantly reduced in the *M6* (Fig. 1I-L). These results suggested that the mutation in *M6* might affect the amylose synthesis in endosperm.

3.2. Molecular Analysis of *M6* Mutation

It is generally accepted that amylose synthesis is carried out by GBSS. Cereals contain two forms of GBSS, GBSSI and GBSSII, and GBSSI is responsible for amylose synthesis in storage tissues, such as endosperm [18]. Thus, the cDNA sequences of *GBSSI* were amplified by PCR and sequenced. Comparison of the sequences of wild type and the *M6* revealed that the *M6* allele carried 23 bp insertion (red font) in the exon 2 of *GBSSI* (Fig. 2A). The 23 bp insertion was the duplication of the 23 bp (black underline) in front of them and generated a premature stop codon that allowed translation of only the first 57 amino acids of the GBSSI protein (Fig. 2A, B). Thus, *M6* was a loss-of-function mutant in *GBSSI*. Subsequently, we used western blot to analyze GBSSI protein in rice endosperm. As shown in Fig. 2C, the GBSSI antibody specially recognized the endogenous GBSSI protein band in wild type but not in the *M6*. In summary, these results demonstrated that the mutation of *GBSSI* was responsible for the *M6* phenotypes.

3.3. Structural Properties of *M6* Starch

The AAC of wild type and *M6* starch as determined by iodine colorimetry is given in Table 1. The AAC of *M6* starch was 1.2%, which was significantly lower than that of wild type starch (14.3%). The molecular weight distribution of starch as determined by GPC is shown in Fig. 3A. In general, the GPC chromatogram of isoamylase-debranched starch exhibits three peaks. The Peaks 1 and 2 represent the short-branch chains (A and short B chains) and long-branch chains (long B chains) of amylopectin, respectively, and the Peak 3 is amylose [19]. The *M6* contained very low apparent amylose. Therefore, only two peaks (Peak 1 and 2) were detected in the GPC profile of *M6* starch. Whereas for wild type starches, three peaks were detected in its GPC profiles due to the simultaneous existence of amylose and amylopectin (Fig. 3A). The percentage of the peak area in GPC profile can reflect the molecular weight distribution of starch, and the area ratio of Peak 1 and Peak 2 can be used as an index of the extent of amylopectin branching; the higher the ratio, the higher the branching degree [20]. As is shown in Table 1, *M6* starch consisted of approximately 76.0% amylopectin short branch-chains and 24.0% amylopectin long branch-chains, and had 3.2% amylopectin branching degree, while wild type starch contained 65.0% amylopectin short branch-chains, 21.5% amylopectin long branch-chains, and 13.5% amylose, and had 3.0% amylopectin branching degree.

The XRD patterns of starches are presented in Fig. 3B. Both wild type and *M6* starches had the characteristics of A-type crystallinity with strong reflection peaks at about 15°and 23°, and an unresolved doublet at around 17°and 18° 2θ (Fig. 3B), which was in conformity with the characteristics of normal cereal starches [21]. The relative crystallinity of *M6* starch was 33.4%, which was higher than that of wild type starch (27.1%). This result was in accordance with that the relative crystallinity is negatively related to amylose [22].

3.4. Functional Properties of *M6* Starch

The swelling power and water solubility of wild type and *M6* starches at 95°C are shown in Table 1. The *M6* starch had higher swelling power and lower water solubility than wild type starch. The swelling power is a measure of the water-holding capacity of starch after being heated in water, cooled, and centrifuged, while the water solubility reflects the degree of dissolution during the starch swelling procedure [23]. Amylose is considered to contribute to the inhibition of water absorption and swelling of starch, whereas amylopectin tends to promote the process [24]. The *M6* starch had higher amylopectin content and lower AC than wild type starch, which might contribute to its higher swelling power. The lower water solubility of *M6* starch might be related to its lower AC, which could not leach out of the starch granules into the water.

The thermal properties of starch samples were determined by DSC, and their thermograms and thermal parameters are given in Fig. 4A and Table 2. Compared with wild type starch, *M6* starch exhibited lower gelatinization peak temperature and higher gelatinization enthalpy. The gelatinization peak temperature correlates positively with AC [25]. Starch with higher amylopectin contents can easily form more crystalline structures within granules; this type of starch also requires more energy to melt and uncoil the double helix structure during gelatinization [26]. In the present study, the *M6* starch had a lower AC and higher crystallinity, and therefore required lower gelatinization temperature and more energy for gelatinization than wild type starch.

The pasting properties of wild type and *M6* starches measured by RVA are presented in Fig. 4B, and their pasting parameters are given in Table 2. Compared with the wild type starch, *M6* starches had significantly different pasting properties because of their different molecular structure. The *M6* starch had significantly lower peak viscosity, hot viscosity, final viscosity, setback viscosity, and peak time than wild type starch. This finding confirms the suggestion that rice starch with a low amylose is more prone to gelatinization, in agreement with previous studies on maize [27]. In contrast, as for the breakdown viscosity, *M6* starch was significantly higher than wild type starch, possibly because amylose intertwines with amylopectin in wild type starch, which helped to maintain the integrity of the starch granules [28].

The time courses of PPA and AAG hydrolysis of starches are presented in Fig. 4C and Fig. 4D. A biphasic hydrolysis trend by PPA or AAG was observed in wild type and *M6* starches with an initial rapid hydrolysis of the amorphous region followed by a decreased hydrolysis, which was in agreement with previous studies [13]. The hydrolysis rate of *M6* starch by PPA or AAG was markedly higher than wild type starch. PPA hydrolyzes starch begins firstly from granule surface, and then it penetrates into granule interior and degrades starch from inside to outside, while AAG hydrolyses starch from the outer surface of the granule [29]. Susceptibility of starch to PPA and AAG attack is influenced by factors such as AC, amylose to amylopectin ratio, crystalline structure, granule

integrity, porosity of granules, and structural inhomogeneities [30]. In the present study, the low AC and cavity structure in *M6* starch led to that *M6* starch was enzymatically hydrolyzed faster than the wild type starch.

4. Conclusion

In conclusion, the *M6* was a loss-of-function mutant of *GBSSI*, which produced a waxy endosperm composed of amylose-free starch granules. Because of the differences in molecular structure between *M6* and wild type, *M6* starch contained higher relative crystallinity, swelling power, gelatinization enthalpy, and breakdown viscosity, but lower water solubility, gelatinization peak temperature, peak viscosity, hot viscosity, final viscosity, setback viscosity, and resistance to PPA and AAG hydrolysis than wild type starch. These findings could provide some practical information on the potential usefulness of the waxy mutant.

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References

[1] Bergman, C. J., Bhattacharya, K. R., and Ohtsubo, K., 2004. *Rice end-use quality analysis. In, Champagne, E.T.* USA: Rice Chemistry and Technology AACC, MN. pp. 415-472.

[2] Juliano, B. O., 1992. "Structure, chemistry, and function of the rice grain and its fractions." *Cereal Foods World*, vol. 37, pp. 772-779.

[3] Shapter, F. M., Eggler, P., Lee, L. S., and Henry, R. J., 2009. "Variation in granule bound starch synthase I (GBSSI) loci amongst Australian wild cereal relatives (Poaceae)." *J. Cereal Sci.*, vol. 49, pp. 4-11.

[4] Wang, Z. Y., Shen, G. Z., Gao, J. P., Snustad, D. P., Li, M. G., Zhang, J. L., and Hong, M. M., 1995. "The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene." *Plant J.*, vol. 7, pp. 613-622.

[5] Liu, L. L., Ma, X. D., Liu, S. J., Zhu, C. L., Jiang, L., Wang, Y. H., Shen, Y., Ren, Y. L., Dong, H., *et al.*, 2009. "Identification and characterization of a novel *Waxy* allele from a Yunnan rice landrace." *Plant Mol. Biol.*, vol. 71, pp. 609-626.

[6] Mikami, I., Aikawa, M., Hirano, H. Y., and Sano, Y., 1999. "Altered tissue-specific expression at the Wx gene of the opaque mutants in rice." *Euphytica*, vol. 105, pp. 91-97.

[7] Sato, H., Suzuki, Y., Sakai, M., and Imbe, T., 2002. "Molecular characterization of *Wx-mq*, a novel mutant gene for low-amylose content in endosperm of rice (Oryza sativa L.)." *Breeding Sci.*, vol. 52, pp. 131-135.

[8] Shahid, S., Begum, R., Razzaque, S., and Jesmin, S. Z. I., 2016. "Variability in amylose content of Bangladeshi rice cultivars due to unique SNPs in *Waxy* allele." *J. Cereal Sci.*, vol. 71, pp. 1-9.

[9] Wanchana, S., Toojinda, T., Tragoonrung, S., and Vanavichit, A., 2003. "Duplicated coding sequence in the *waxy* allele of tropical glutinous rice (*Oryza sativa* L.)." *Plant. Sci.*, vol. 165, pp. 1193-1199.

[10] Hanashiro, I., Itoh, K., Kuratomi, Y., Yamazaki, M., Igarashi, T., Matsugasako, J., and Takeda, Y., 2008. "Granule-bound starch synthase I is responsible for biosynthesis of extra-long unit chains of amylopectin in rice." *Plant Cell Physiol.*, vol. 49, pp. 925-933.

[11] Zhang, C. Q., Chen, S. J., Ren, X. Y., Lu, Y., Liu, D. R., Cai, X. L., Li, Q. F., Gao, J. P., and Liu, Q. Q., 2017. "Molecular structure and physicochemical properties of starches from rice with different amylose contents resulting from modification of OsGBSSI activity." *J. Agr Food Chem.*, vol. 65, pp. 2222-2232.

[12] Li, Jain, R., Chern, M., Pham, N. T., Martin, J. A., Wei, T., Schackwitz, W. S., Lipzen, A. M., Duong, P. Q., *et al.*, 2017. "The sequences of 1504 mutants in the model rice variety Kitaake facilitate rapid functional genomic studies." *Plant Cell*, vol. 29, pp. 1218-1231.

[13] Wang, P., Huang, J., Zhao, L. X., Chen, Y. F., and Wei, C. X., 2015. "Structural and functional properties of endosperm starch and flour from dicotyledon Mirabilis jalapa." *Starch*, vol. 67, pp. 328-337.

[14] Lin, L. S., Guo, D. W., Huang, J., Zhang, X. D., Zhang, L., and Wei, C. X., 2016. "Molecular structure and enzymatic hydrolysis properties of starches from high-amylose maize inbred lines and their hybrids." *Food Hydrocolloids*, vol. 58, pp. 246-254.

[15] Wei, C. X., Qin, F. L., Zhou, W. D., Yu, H. G., Xu, B., Chen, C., Zhu, L. J., Wang, Y. P., Gu, M. H., *et al.*, 2010. "Granule structure and distribution of allomorphs in C-type high-amylose rice starch granule modified by antisense RNA inhibition of starch branching enzyme." *J. Agr. Food Chem*, vol. 58, pp. 11946-11954.

[16] Konik-Rose, C. M., Moss, R., Rahman, S., Appels, R., Stoddard, F., and McMaster, G., 2001. "Evaluation of the 40 mg swelling test for measuring starch functionality." *Starch*, vol. 53, pp. 14-20.

[17] Zhang, M. Z., Fang, J. H., Yan, X., Liu, J., Bao, J. S., Fransson, G., Andersson, R., Jansson, C., and Sun, C., 2012. "Molecular insights into how a deficiency of amylose affects carbon allocation-carbohydrate and oil analyses and gene expression profiling in the seeds of a rice waxy mutant." *BMC Plant Biol.*, vol. 12, p. 230.

[18] Vrinten, P. L. and Nakamura, T., 2000. "Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues." *Plant Physiol.*, vol. 122, pp. 255-263.

[19] Song, Y. and Jane, J., 2000. "Characterization of barley starches of waxy, normal, and high amylose varieties." *Carbohyd Polym*, vol. 41, pp. 365-377.
[20] Wang, Y. J., White, P., Pollak, L., and Jane, J., 1993. "Characterization of starch structure of 17 maize endosperm mutant genotypes with Oh43 inbred line background." *Cereal. Chem.*, vol. 70, pp. 171-179.
[21] Huang, J., Shang, Z. Q., Man, J. M., Liu, Q. Q., Zhu, C. J., and Wei, C. X., 2015. "Comparison of molecular structures and functional properties of high-amylose starches from rice transgenic line and commercial maize." *Food Hydrocolloids*, vol. 46, pp. 172-179.
[22] Sevenou, O., Hill, S. E., Farhat, I. A., and Mitchell, J. R., 2002. "Organisation of the external region of the starch granule as determined by infrared spectroscopy." *Int. J. Biol. Macromol*, vol. 31, pp. 79-85.
[23] Carcea, M. and Acquistucci, R., 1997. "Isolation and physicochemical characterization of fonio (Digitaria exilis Stapf) starch." *Starch*, vol. 49, pp. 131-135.
[24] Cai, J. W., Man, J. M., Huang, J., Liu, Q. Q., Wei, W. X., and Wei, C. X., 2015. "Relationship between structure and functional properties of normal rice starches with different amylose contents." *Carbohyd Polym.*, vol. 125, pp. 35-44.
[25] Chung, H. J., Liu, Q. A., Lee, L., and Wei, D. Z., 2011. "Relationship between the structure, physicochemical properties and in vitro digestibility of rice starches with different amylose contents." *Food Hydrocolloids*, vol. 25, pp. 968-975.
[26] Singh, S., Singh, N., Isono, N., and Noda, T., 2010. "Relationship of granule size distribution and amylopectin structure with pasting, thermal, and retrogradation properties in wheat starch." *J. Agr. Food Chem.*, vol. 58, pp. 1180-1188.
[27] Yangcheng, H. Y., Blanco, M., Gardner, C., Li, X. H., and Jane, J. L., 2016. "Dosage effects of *Waxy* gene on the structures and properties of corn starch." *Carbohyd Polym*, vol. 149, pp. 282-288.
[28] You, S. Y., Oh, S. K., Kim, H. S., and Chung, H. J., 2015. "Influence of molecular structure on physicochemical properties and digestibility of normal rice starches." *Int. J. Biol. Macromol*, vol. 77, pp. 375-382.
[29] Balibrea, M. P., 2004. "Barcelona: del modelo a la marca." *Desacuerdos*, vol. 3, pp. 261-271.
[30] Blazek, J. and Gilbert, E. P., 2010. "Effect of enzymatic hydrolysis on native starch granule structure." *Biomacromolecules*, vol. 11, pp. 3275-3289.

Table-1. Apparent Amylose Contents (AAC), Molecular Weight Distributions, Swelling Powers (SP), and Water Solubilities (WS) of Wild Type and *M6* Starches

	AAC (%)	Molecular Weight Distribution				SP (g/g)	WS (%)
		ASB (%)	ALB (%)	AM (%)	ASB/ALB		
WT	14.3±0.3	65.0±1.3	21.5±0.1	13.5±1.2	3.0±0.1	17.2±0.1	5.9±0.3
M6	1.2±0.2**	76.0±0.5**	24.0±0.5**	ND	3.2±0.1	35.5±0.4**	3.2±0.3**

ASB, amylopectin short branch-chains; ALB, amylopectin long branch-chains; AM, amylose; ND, not detected.
Data are shown as means ± standard deviation (n=3) and compared with wild type by Student's *t*-test (**P<0.01).

Table-2. The Parameters of Thermal and Pasting Properties of Wild Type and *M6* Starches

	Thermal Parameter					Pasting Parameter					
	T_o (°C)	T_p (°C)	T_c (°C)	ΔT (°C)	ΔH (J/g)	PV (mPa s)	HV (mPa s)	BV (mPa s)	FV (mPa s)	SV (mPa s)	PT (min)
WT	62.0±0.2	69.8±0.2	77.2±0.1	15.1±0.1	10.0±0.2	1817±1	1632±6	185±4	1946±1	314±7	6.8±0.1
M6	61.7±0.3	68.5±0.4**	77.0±1.5	15.3±1.3	11.5±0.1**	1640±5**	841±8**	799±4**	974±13**	133±21**	3.7±0.0**

T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; ΔT, gelatinization temperature range (T_c-T_o); ΔH, gelatinization enthalpy; PV, peak viscosity; HV, hot viscosity; BV, breakdown viscosity (PV-TV); FV, final viscosity; SV, setback viscosity (FV-HV); PT, peak time.
Data are shown as means ± standard deviation (n=3) and compared with wild type by Student's *t*-test (**P<0.01).

Fig-1. Phenotypic analyses of *M6*. (A, B) The morphologies of grains (A) and seeds (B) of wild type (above) and *M6* (below). Scale bar = 1 mm. (C, D) Transverse sections of representative wild type (C) and *M6* (D) dry seeds. Scale bar = 1mm. (E, F) Transverse sections of representative wild type (E) and *M6* (F) dry seeds with iodine-staining. Scale bar = 1mm. (G, H) Scanning electron microscope images of transverse sections of the wild type (G) and *M6* (H) dry seeds. Red arrows indicate the cavity in the center of starch granule. Scale bar = 5 μm. (I-K) Quantification of seed length (I), seed width (J), and seed thickness (K) of wild type and *M6* (n=30). (L) 1000-seed weight of wild type and *M6* (n=3). Data are shown as means ± standard deviation, and compared with wild type by Student's *t*-test (**P<0.01)

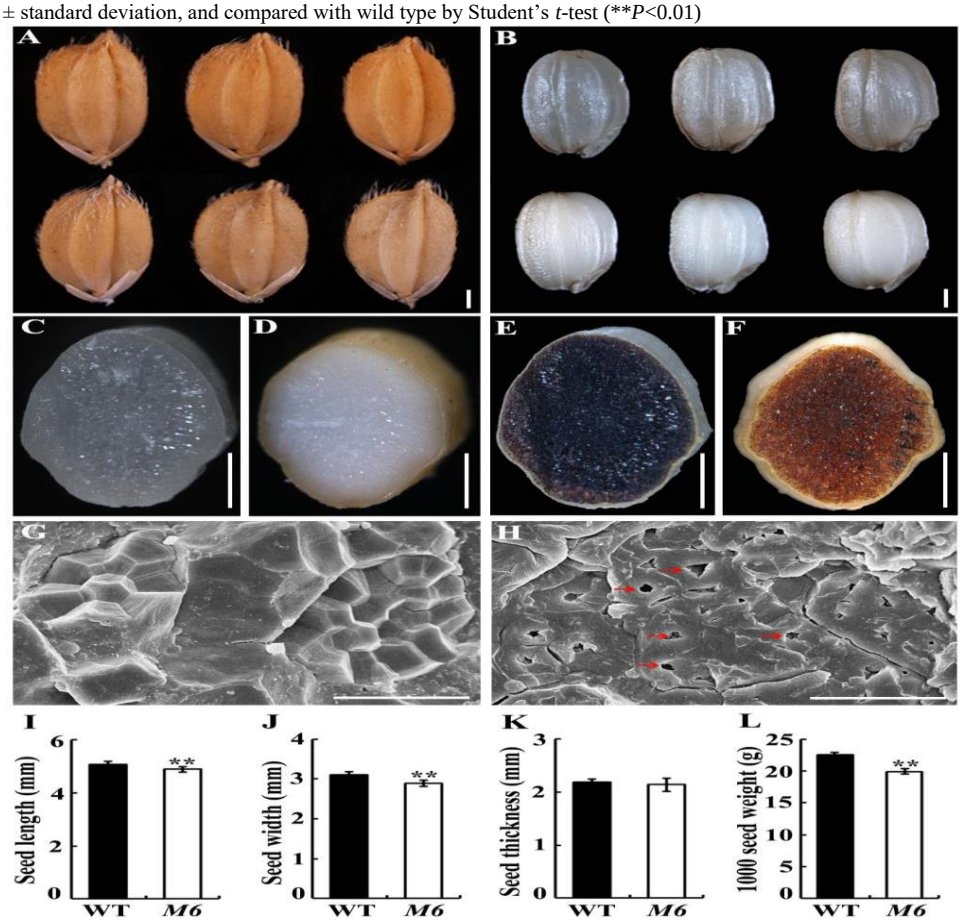

Fig-2. Molecular identification of *M6* mutation. (A) Structure of the *GBSSI* gene and positions of mutation site. The insertion of 23 bp in the exon 2 leads to frame shift and creates premature stop codon in *M6*. (B) Amino acid sequence of GBSSI in *M6*. (C) Western blot analysis of GBSSI in the mature seed of the wild type and *M6*. Anti-HSP82 antibody is used as a loading control

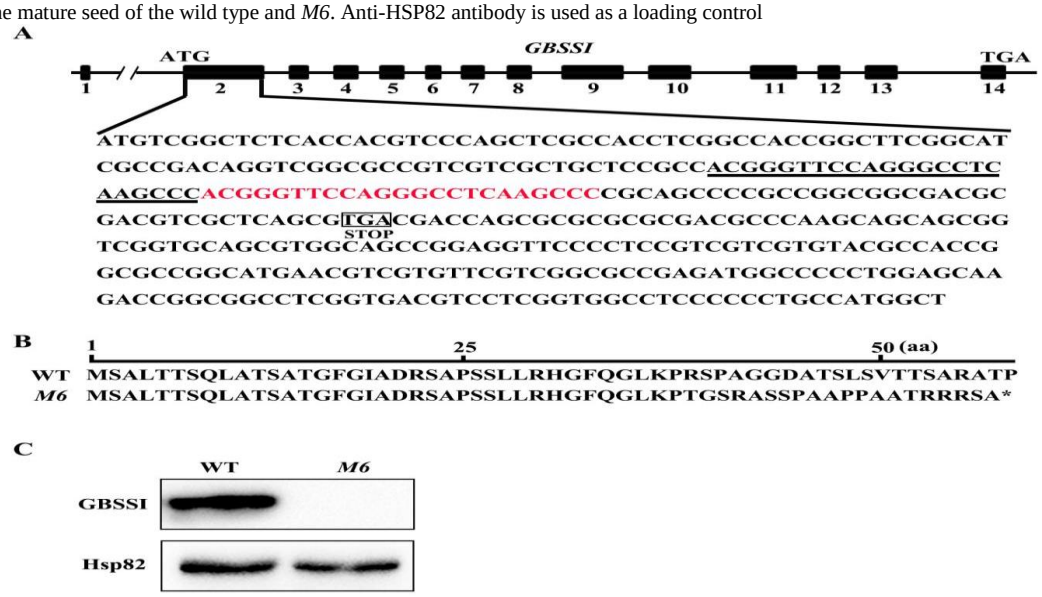

Fig-3. GPC Profiles (A) and XRD Patterns (B) of Wild Type and *M6* Starches. The Relative Crystallinity is Given in Parentheses

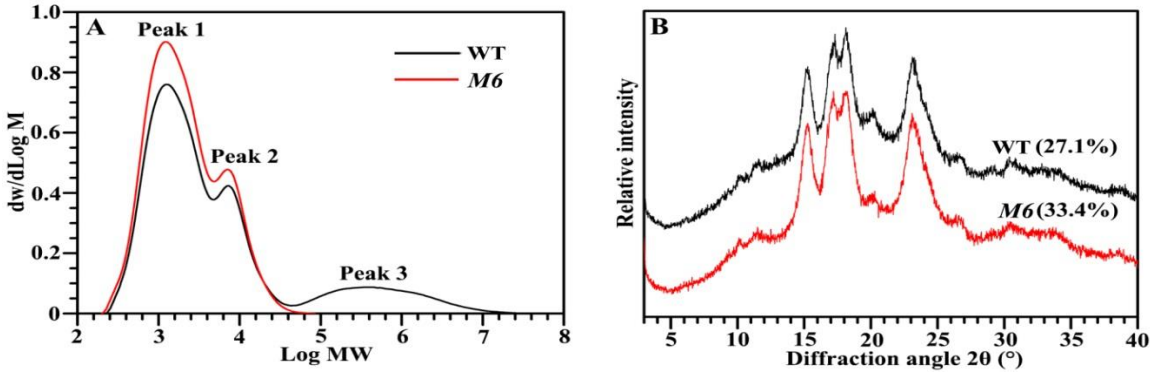

Fig-4. DSC the Rmograms (A), Pasting Profiles (B), PPA Hydrolyses (C), and AAG Hydrolyses (D) Of Wild Type and *M6* Starches