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%), 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: *Wxa*, *Wxb*, *Wxw*, *Wxp*, *Wxp′*, and *wx*. In nonwaxy rice varieties, *Wxa* and *Wxb* have been recognized as being distributed in the *indica* and *japonica* subspecies, respectively, and their expression levels are highly correlated with...
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 \( W_{x}^{\text{mil}} \) from Milky Queen, \( W_{x}^{\text{op}} \) from the opaque endosperm mutants and \( W_{x}^{\text{hp}} \) 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 \( W_{x} \) 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 \( W_{x}^{\text{h}} \) [10]. Using site-directed mutagenesis, three amino acid substitutions of Wx transgenic rice lines show significant differences in GBSSI activities, AC and starch physiochemical properties [11]. An amino acid substitution in \( W_{x}^{\text{h}} \) 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 \( ^{60}\text{Co} \) 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 \( M_{6} \) 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 \( M_{6} \) was isolated from a \( ^{60}\text{Co} \)-irradiated mutant pool of japonica rice cultivar Kitaake. The Kitaake and \( M_{6} \) 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 \( M_{6} \) 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 \( M_{6} \) GBSSI cDNA sequence was cloned using primers 5′-ATGTCGGCTCTCACCACGTCCCA and 5′-AGGAGAACGTGGCTGCTCCTTGA 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 CaCl₂, 0.02% NaN₃, 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-H₂SO₄ 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 ⁶⁰Co-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-chain branches (long B chains) of amyllopectin, 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 amyllopectin (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 amyllopectin branching; the higher the ratio, the higher the branching degree [20]. As is shown in Table 1, M6 starch consisted of approximately 76.0% amyllopectin short branch-chains and 24.0% amyllopectin long branch-chains, and had 3.2% amyllopectin branching degree, while wild type starch contained 65.0% amyllopectin short branch-chains, 21.5% amyllopectin long branch-chains, and 13.5% amylose, and had 3.0% amyllopectin 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° 20 (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 amyllopectin tends to promote the process [24]. The M6 starch had higher amyllopectin 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 amyllopectin 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 amyllopectin 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 amyllopectin 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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Table 1. Apparent Amylose Contents (AAC), Molecular Weight Distributions, Swelling Powers (SP), and Water Solubilities (WS) of Wild Type and M6 Starches

| AAC (%) | ASB (%) | ALB (%) | AM (%) | ASB/ALB | SP (g/g) | WS (%) |
|---------|---------|---------|--------|---------|----------|--------|
| 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 | 15.4±1.1 | 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 ($T_p-T_o$); $ΔH$, 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 = 1 mm. (E, F) Transverse sections of representative wild type (E) and M6 (F) dry seeds with iodine-staining. Scale bar = 1 mm. (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 Thermograms (A), Pasting Profiles (B), PPA Hydrolyses (C), and AAG Hydrolyses (D) Of Wild Type and M6 Starches.