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Grain Quality and Starch Physicochemical Properties of Chalky Rice Mutant

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Abstract: Rice mutants with altered starch components and properties are important genetic resources for grain quality and starch structure analysis. Accordingly, in the present study, two mutants of the transcription factor *OsbZIP09* were generated (*osbzip09a* and *osbzip09b*), and the rice grain quality and physicochemical starch properties of the mutant and wild-type lines were compared. The *OsbZIP09* mutants exhibit a chalky grain owing to loosely packed, small, spherical starch granules in the ventral region of the endosperm. Furthermore, grain-quality profile analysis showed that *OsbZIP09* deficiency leads to increased apparent amylose content but decreased gel consistency. Structural analysis of the mutant starches revealed that the mutant rice lines contain more amylopectin short chains and fewer intermediate chains, leading to lower crystallinity and lower gelatinization properties than those of the wild-type rice. Moreover, the *OsbZIP09* mutants rice presented a significantly higher pasting curve and corresponding parameters than the wild-type rice. The results from this work strongly indicate that the transcription factor *OsbZIP09* plays an important role in rice grain quality and starch fine structure modification, and extend our understanding of starch biosynthesis in rice endosperm.

Keywords: rice; *OsbZIP09*; grain quality; chalkiness; starch structure

Citation: Wang, C.-X.; Zhu, C.-C.; Lu, C.-Y.; Yang, Y.; Li, Q.-F.; Liu, Q.-Q.; Zhang, C.-Q. Grain Quality and Starch Physicochemical Properties of Chalky Rice Mutant. *Agronomy* **2021**, *11*, 1575. https://doi.org/10.3390/agronomy11081575

Academic Editor: Katherine Steele

Received: 2 July 2021
Accepted: 4 August 2021
Published: 7 August 2021

1. Introduction

Rice (*Oryza sativa* L.) is a significant cereal consumed worldwide, mostly in whole grains after cooking. Therefore, rice grain quality is a crucial factor when determining the economic value of a rice crop. In general, rice grain quality is defined in terms of grain physical appearance, softness, aroma, and nutritional profile [1,2]. Rice endosperm is composed mainly of starch, so the granular architecture, fine structure, and physicochemical properties of the starch also play important roles in determining rice grain quality [3,4].

Grain appearance, another crucial aspect of grain quality for consumers, is related to parameters termed chalkiness and transparency [5,6]. Rice chalkiness is a property of the opaque part of the endosperm and is caused by loosely arranged starch granules. Generally, chalky grains present white cores, white bellies, and white backs. Rice transparency is related to the moisture level of the kernel and the cavities within starch granules [6,7]. Accordingly, many studies have considered chalkiness by focusing on the starch synthesis processes [8–10]. However, the mechanisms regulating chalk formation and starch biosynthesis in rice seeds are not currently well understood.

Starch, the major component of rice endosperm, is a branched glucose polymer with (1→4)-α linear links and (1→6)-α branch points [11]. It typically comprises two polymeric

forms: amylopectin and amylose. Amylopectin is a highly branched large glucan polymer with a high number of short-chain branches, while amylose is a linear polymer with a low number of long-chain branches [12,13]. Generally, amylopectin is arranged in a double-helical structure and forms crystalline layers, while amylose forms a single helical complex with lipid molecules in amorphous layers [14,15]. Starch fine structure is a significant determinant of rice grain quality, and specific physicochemical properties of starch, such as apparent amylose content (AAC), gel consistency (GC), and gelatinization temperature (GT), as well as its pasting properties, are widely established parameters used to evaluate rice eating and cooking quality [16,17].

Numerous enzymes are directly involved in rice starch biosynthesis, including ADPGlc pyrophosphorylase (AGPase), soluble starch synthase (SSS), granule bound starch synthase I (GBSSI), starch branching enzyme (SBE), and starch debranching enzyme (DBE) [17–19]. The functions of these enzymes are well understood; however, the network of mechanisms by which starch synthesis is regulated is still a matter of ongoing debate. Recent studies on the regulation of starch biosynthesis have revealed a wide range of related metabolic signaling processes. For example, the transcription factors OsbZIP58, RSR1, OsNAC20, OsNAC26, and NF-YB1-YC12-bHLH144 are all implicated in starch synthesis and endosperm development in rice [20–23]. Therefore, mutations of the genes encoding these transcription factors change the appearance of the endosperm and alter the characteristics of the starch, affecting rice grain quality. Accordingly, elucidating the structural and compositional causes of variations in the physicochemical properties of starches is crucial if we are to understand the functions of the related genes.

To obtain a more accurate understanding of starch metabolism, the functions of its related genes need to be studied, so that their roles in regulating starch biosynthesis and thus rice grain quality may be determined. Accordingly, both forward and reverse genetics approaches are useful tools for investigating the roles of genes. For instance, in a recent study, we generated two mutant rice lines by editing the gene that encodes the OsbZIP transcription factor *OsbZIP09* using the CRISPR/Cas9 system. Our results indicate that this transcription factor is involved in regulation of seed germination through its attenuation of the ABA pathway [24]. Furthermore, it is reasonable to assume that, like other OsbZIPs (e.g., *OsbZIP58*), *OsbZIP09* also plays an import role in regulating grain appearance as well as starch composition and morphology.

The objective of this study was to investigate the effects of *OsbZIP09* null mutants on rice grain appearance, and morphological and structural changes in starches. The detailed morphological and physicochemical starch properties of the mutant rice will aid understanding of the role of *OsbZIP09* in the regulation of rice grain quality formation.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Japonica rice (*Oryza sativa* L.) cultivar zhonghua 11 (ZH11) was the wild type and its two mutants, *osbzip09a* and *osbzip09b*, were obtained by gene editing of two specific target sites in the first exon of *OsbZIP09*. Sanger sequencing of the CRISPR/Cas9 target sites detected two types of insertion in the *OsbZIP09*, which are believed to knockout *OsbZIP09* by shifting the open reading frame (Supplementary Figure S1). The homozygous T_1 mutants *osbzip09a* and *osbzip09b* were subjected to detailed phenotypic characterization.

For rice grain quality analyses, the rice lines were planted in the same field at the experimental farm in Yangzhou University, Yangzhou (Jiangsu Province, 32°23′ N) under management conditions during summer months from May to November of 2020. The soil in the experiments was classified as Gleysol and all the rice lines were planted in plots with three replicates. The plot size was 10 cm × 5 cm. Rice seedlings were transplanted with spacing of 20 cm × 10 cm. Nitrogen (220 kg ha^{-1}), phosphorus (40 kg ha^{-1}), and potassium (54 kg ha^{-1}) were applied during the whole growing season. Field management and disease and pest control followed standard procedures to prevent yield loss during the

growth period. All the rice seeds were harvested at maturity from 10 plants in the middle of each plot and air dried.

2.2. Sample Preparation

Mature seeds were air dried, dehusked with an SY88-TH rice huller (Shuanglong, Incheon, Korea), and polished using a grain polisher (Kett, Tokyo, Japan). Some of the polished grains were used for grain appearance quality analysis and some were used for flour and starch preparation.

Rice flours were generated using a FOSS 1093 Cyclotec Sample Mill (Tecator, Höganäs, Sweden) with a 0.5 mm sieve. Rice starches were prepared using an alkaline protease method as described by Zhu et al. [25]. The lipids in the starch were removed by washing with CHCl3/methanol (30 mL; 1:1, *v*/*v*). Specifically, following incubation at 42 °C in a shaker at 250 rpm for 3 h, the resulting starch slurry was centrifuged at 3000× *g* for 10 min. The starch sediment was then washed with 85% ethanol five times prior to drying in a convection oven at 37 °C for 48 h.

2.3. Scanning Electron Microscopy

Rice grains from ZH11 and the two mutants were randomly selected for direct camera imaging or scanning electron microscopy (SEM) analysis. In brief, grains were broken naturally and the cross-sections were observed by SEM (Philips XL-30), as described previously [26]. To observe the isolated starch, a ~5-mg sample was dispersed in 200 µL ethanol and vortexed. The starch slurry was pipetted onto a specimen holder and held for 15 min at room temperature. The dried samples were coated with gold using a sputter coater (Leica EM SCD500, Wetzlar, Germany) and examined using field emission scanning electron microscopy (FE-SEM, S-4800II, Hitachi, Tokyo, Japan). The starch granular size distributions were calculated from analysis of 500 complete starch granules based on the SEM images and plotted using Image J software (http://rsbweb.nih.gov/ij (accessed on 10 March 2021)).

2.4. Analysis of Rice Grain Quality

To evaluate grain appearance, an appearance detection analyzer (MRS-9600TFU2L, MICROTEK, Hangzhou, China) was used to determine chalky rice rate (CR) and chalkiness degree (CD). Specifically, 100 head rice grains were selected at random per entry and assessed using the appearance detection analyzer. The above measurements were repeated in triplicate. Crude protein content (PC) was calculated from the nitrogen content of the corresponding rice flour using a Kjeltec2300 nitrogen determination instrument (Foss Tecator, Hoganas, Sweden). The nitrogen content was converted to protein content by multiplying by a coefficient of 5.95. The total starch content (TSC) of the rice flours was determined using a total starch assay kit (K-TSTA, Megazyme; Wicklow, Ireland). The apparent amylose content (AAC), gel consistency (GC), and crude protein content values of the rice flours were determined as described by Zhang et al. [26]. The pasting properties of the rice flours and starches were subjected to rapid viscosity analysis (RVA) (Techmaster, Newport Scientific, Warriewood, Australia) according to Zhu et al. [25]. The parameters determined using RVA were peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown viscosity (BDV), setback viscosity (SBV), peak time (P_{time}), and pasting temperature (PT).

2.5. Measurement of Thermal Properties

Gelatinization properties were investigated using a DSC 200 F3 differential scanning calorimetry (DSC) apparatus (Netzsch Instruments NA LLC, Burlington, MA, USA) as described previously [26]. Briefly, 5 mg starch was weighed accurately into an aluminum pan and 15 µL deionized water was added. Samples were stored overnight at 4 °C and then at room temperature for 1 h before testing. Gelatinization was determined by heating the pan in the calorimeter from 20 to 120 °C at a rate of 10 °C min^{-1}. The DSC parameters

determined were onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and enthalpy change of gelatinization (ΔH).

2.6. Starch Fine Structure Measurement

The starch samples were debranched with isoamylase (EC 3.2.1.68, E-ISAMY; Megazyme, Bray, Ireland) and then analyzed using a high-performance anion-exchange chromatography (HPAEC) system (Thermo ICS-5000, Thermo Corp, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector, a guard column, a CarboPac™ PA200 analytical column, and an AS-DV autosampler according to previously published procedures for measuring the degree of polymerization (DP) of amylopectin [26].

2.7. Starch Crystalline Structure Analysis

To determine the supramolecular structures of the rice starches, a D8 ADVANCE type X-ray diffractometer (D8, Bruker, Germany) was used to perform powder X-ray diffraction (XRD) analyses as described previously [27]. Relative crystallinity (RC) was determined from the relationship Ic/(Ia + Ic), where Ia is the proportion of non-crystalline area and Ic is the proportion of crystalline area in the diffraction profile. Additionally, short-range molecular order near starch grain surfaces was investigated using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) (Perkin-Elmer Inc., Wellesley, MA, USA), as described previously [26]. In detail, spectra were corrected by a baseline in the region from 1200 to 800 cm^{-1} before deconvolution by using Resolutions Pro FTIR software. Absorbance values for peaks at 1047 and 1022 cm^{-1} were extracted from the spectra after correction, and the intensity 1047/1022 ratio was calculated to express the amount of ordered crystalline regions to amorphous regions near the surface of the starch granules. The above experiments were performed in duplicate.

2.8. Statistical Analysis

For sample characterization, three replicate measurements were performed (unless otherwise specified). All data represent mean values with standard deviations (mean ± SD). Data were subjected to one-way analysis of variance (ANOVA) analysis using the SPSS 16.0 statistical software program. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Analysis of Grain Quality Profiles

First, the effects of the *OsbZIP09* mutations on the appearance characteristics of milled grains were investigated. Overall, the appearance quality is significantly poorer for the *OsbZIP09* mutant lines. As shown in Figure 1, rice grains from both the *osbzip09a* and *osbzip09b* lines exhibit pronounced chalkiness, and both the *osbzip09a* and *osbzip09b* mutants have significantly higher CR values (over 65%) than that of the wild-type ZH11 (Table 1). These results indicate that, as expected, the components of the rice endosperm are affected by *OsbZIP09* mutation. Next, the major components and physicochemical characteristics of milled rice flours were measured and compared. As shown in Table 1, rice flours milled from *osbzip09a* and *osbzip09b* grains exhibit significantly increased AACs and lower GC than those of the wild-type ZH11 rice, which is consistent with the widely accepted idea that GC is negatively correlated with AAC in rice [7,16,28]. However, the PCs and TSCs of the three samples are not significantly different. There is extensive evidence demonstrating that AAC and GC are key physicochemical properties for rice sensory quality [16,17]. Therefore, the above data indicate that *OsbZIP09* mutation greatly affects rice eating quality.

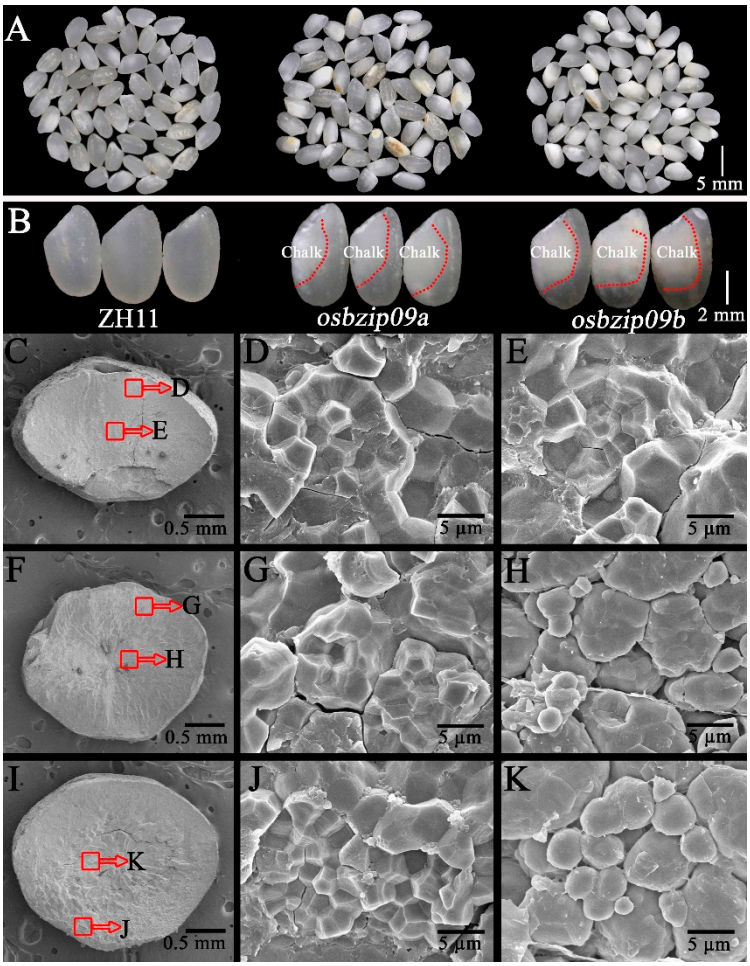

Figure 1. Grain appearance and SEM images of rice endosperm cross-sections. (**A**,**B**) represent milled rice grain appearance; (**C–K**) represent ZH11, *osbzip09a*, and *osbzip09b*, respectively; (**D**,**G**,**J**) represent the SEM micrographs of the transparent region of a rice endosperm; (**E**,**H**,**K**) represent the SEM micrographs of the chalky region of rice endosperm.

Table 1. Physicochemical characteristics and cooking properties of milled rice [a].

Lines	CR (%)	CD (%)	AAC (%)	GC (%)	PC (%)	TSC (%)
ZH11	18.86 ± 3.14 [b]	31.24 ± 4.53 [b]	15.01 ± 0.22 [b]	85.31 ± 6.13 [a]	7.02 ± 0.31 [a]	87.18 ± 0.74 [a]
osbizip09a	68.63 ± 5.67 [a]	69.58 ± 8.11 [a]	17.64 ± 0.36 [a]	72.26 ± 5.75 [b]	7.36 ± 0.24 [a]	86.12 ± 0.81 [a]
osbizip09b	65.24 ± 6.21 [a]	71.15 ± 6.05 [a]	17.28 ± 0.47 [a]	74.19 ± 4.72 [b]	7.41 ± 0.16 [a]	86.43 ± 0.69 [a]

A[a] Data are presented as mean ± standard deviation. For each column, values not displaying the same letter are significantly different ($p < 0.05$). AAC, apparent amylose content; CR, chalky rice rate; CD, chalkiness degree; GC, gel consistency; PC, protein content; TSC, total starch content.

Grain chalkiness is one of the most important factors affecting rice appearance, milling, and eating quality [29,30]. Indeed, grain chalkiness can be used as an indicator of abnormal endospermic starch synthesis. In addition to mutations of genes directly involved in starch biosynthesis, mutations in genes that code transcription factors involved in starch biosynthesis also affect endosperm development. For example, the mutation of *RSR1* and *OsbZIP58* both lead to marked grain chalkiness as a result of abnormal endosperm development [20,21]. Interestingly, *OsbZIP58* was also reported to inhibited the expression of some starch-hydrolyzing α-amylase genes, which were considered as important positive effectors of rice seed germination [31]. In fact, our previous studies have shown that *OsbZIP09* functions as a brake of the ABA pathway to attenuate the inhibitory effect of ABA on rice seed germination via dual strategies [24,32]. Additionally, we found that *OsbZIP09*

had no observable effect on rice agronomic traits such as plant height, heading date, and 1000-grain weight [32]. Thus, the above results suggest that *OsbZIP09* plays an import role in rice endosperm development and thus modulates starch metabolism and starch-related phenotyping.

3.2. Analysis of Starch Granular Structure

To investigate the differences in rice grain transparency and chalkiness, SEM was employed to observe the starch structures of the *OsbZIP09* mutant and wild-type lines under similar moisture-content conditions. As indicated by the SEM micrographs of mature-grain transverse sections in Figure 1C–K, all the starch granules in the transverse sections of the non-chalky region are packed together tightly and have polyhedral shapes and smooth surfaces (Figure 1D,G,J). However, the starch granules in the chalky region of *OsbZIP09* mutation grains show irregular shapes and loosely packed starch granules with large air spaces. To investigate the effects of *OsbZIP09* mutation on starch accumulation, isolated starch samples were then subjected to SEM analysis. As shown in Figure 2A–C, starch granules from the wild-type rice have regular shapes with smooth surfaces, which is consistent with the results for the transverse sections. However, the rice starches from the mutant lines comprise more small round granules, and some of the starch granules have surface holes.

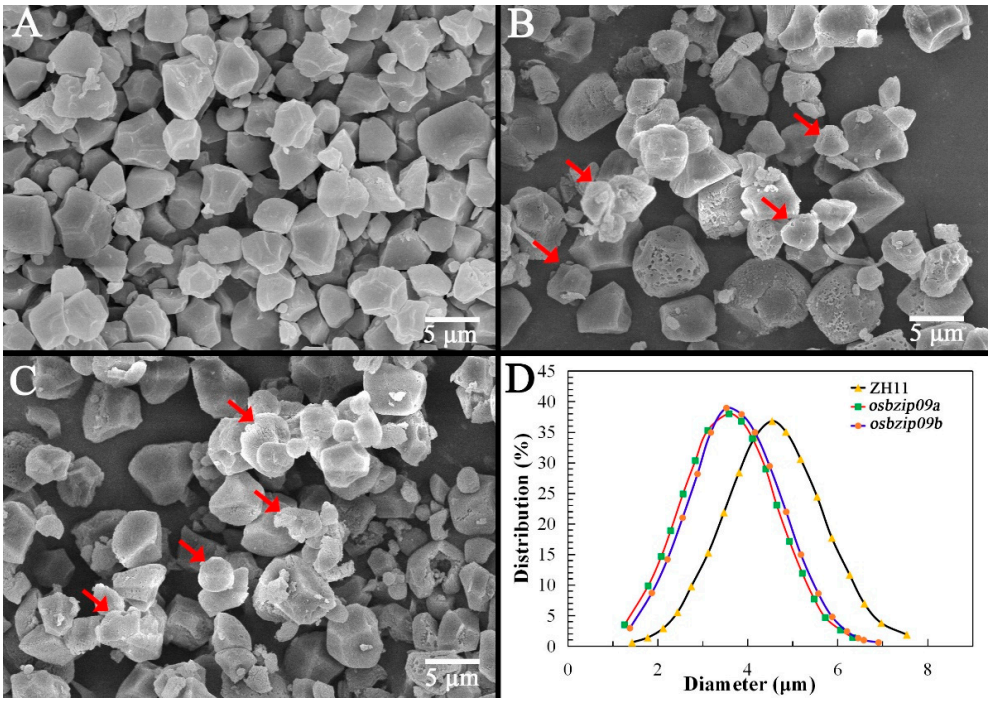

Figure 2. SEM images of starch and the corresponding starch granule size distribution. (**A**–**C**) represent SEM micrographs of purified starches from ZH11, *osbzip09a*, and *osbzip09b*, respectively; (**D**) shows the size distributions of the purified starches (n = 500).

Then, the starch granule size distributions were calculated based on the SEM images, revealing that the starches from both the *osbzip09a* and *osbzip09b* mutants exhibit a remarkable decrease in starch granule size compared with that of the wild-type ZH11 (Figure 2D). Moreover, there is a significant difference in the average particle size, and the mutant rice lines show significantly more decreased average granule diameters than the wild type. Similarly, studies of several chalky rice mutants have demonstrated that starch granular morphology is significantly affected in the chalky region of the endosperm, and that granular size is typically decreased in the mutant rice grains [21,22]. Our analyses further demonstrate that *OsbZIP09* mutation alters starch accumulation.

3.3. Starch Fine Structure Analysis

To determine putative changes in starch fine structure of the mutant rice, the chain-length distribution of the amylopectin therein was investigated by HPAEC. According to the classical amylopectin cluster model [33], amylopectin chains can be grouped into A chains (DP 6–12), B1 chains (DP 13–24), B2 chains (DP 25–36), and B3+ chains (DP $\geq$ 37). As shown in Figure 3A, there are significant differences between the polymerization profiles of the amylopectin in the mutants and the control cultivar ZH11. Specifically, compared with the control rice, the proportions of chains with DPs in the range 6–12 are significantly increased, whereas the proportions of chains with DPs in the range 13–24 are markedly decreased in both *OsbZIP09a* and *OsbZIP09b* rice starches (Figure 3B, and Supplementary Table S1). These results are similar to those of previous studies, where *OsbZIP58* mutants were observed to generate more short amylopectin chains with DP 6–12 and fewer intermediate chains with DP 13–21 [21]. The above results indicate that the alteration of amylopectin chain-length distribution is caused by *OsbZIP09* mutation.

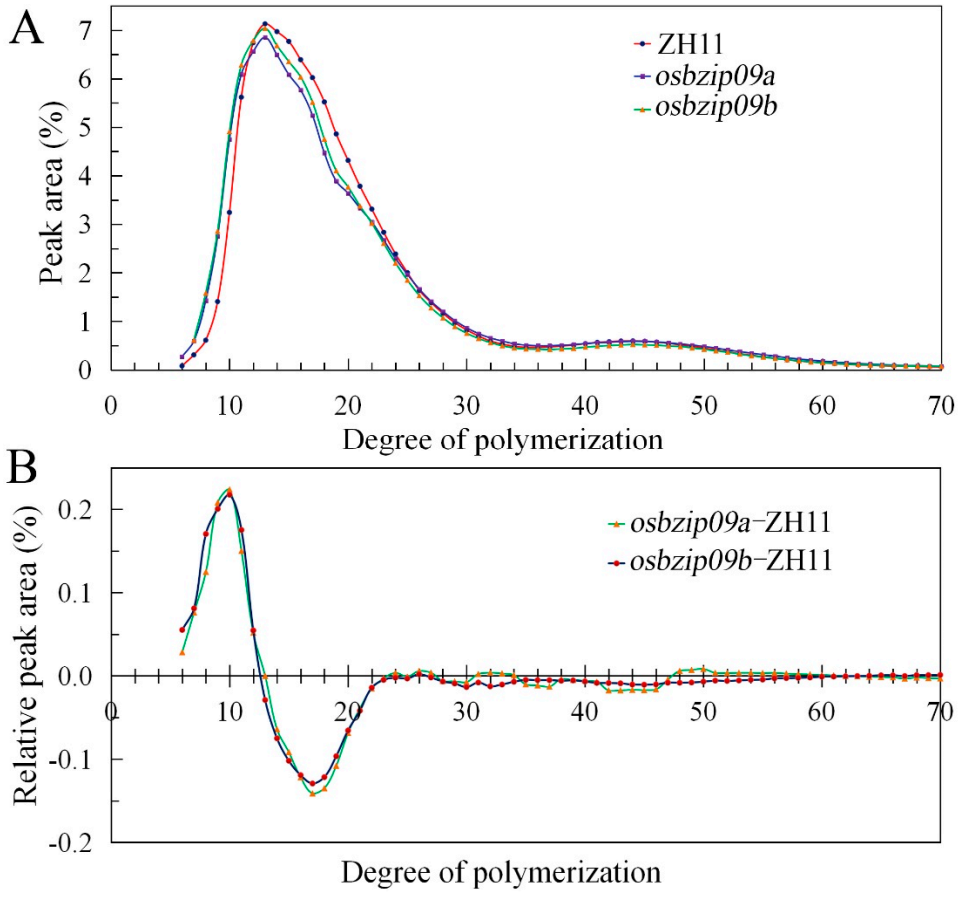

Figure 3. Chain-length distribution of starch from different rice lines. (**A**) indicates the distribution of polyglucans from the endosperm of ZH11 and its mutant lines *osbzip09a* and *osbzip09b*; (**B**) represents the differences in the chain-length distributions between wild-type ZH11 and the mutant lines *osbzip09a* and *osbzip09b*.

Thus, the altered composition and structure of the starch indicates that the transcription factor *OsbZIP09* modulates not only amylose but also amylopectin synthesis, especially the α-1,4 chain elongation of amylopectin, which involves several concerted reactions catalyzed by distinct starch synthesis enzymes, such as soluble starch synthase I (SSI) and soluble starch synthase IIa (SSIIa). It is well known that SSI works to extend the amylopectin short chains to those with DPs of 8 to 12, generating short A chains [34,35], whereas SSIIa is involved in the synthesis of intermediate B1 chains with DPs ranging from

12 to 24 [36]. In fact, the regulatory network involved in starch metabolism is complex, redundant, and compartmentalized at the subcellular level, and is responsive to both internal hormones and the external environment [37]. For example, RSR1 was found to negatively regulate the expression of type I starch synthesis genes, and RSR1 deficiency results in the enhanced expression of starch synthesis genes in seeds [20]. Considering that many transcription factors, e.g., *OsbZIP33* and *OsbZIP58*, regulate the expression of starch synthesis genes [21,31,38], we propose that *OsbZIP09* also plays an important role in starch synthesis regulation. However, confirming this hypothesis will require further study.

3.4. Comparison of Rice Starch Pasting Properties

Pasting properties are important parameters in the evaluation of rice grain quality, as they can be used to predict the texture of the corresponding cooked rice [39]. As shown in Figure 4A, rice starches from both *osbzip09a* and *osbzip09b* rice exhibit significantly higher RVA curves than the wild-type ZH11. In terms of RVA parameters, the PKV, HPV, BDV, and CPV values of starch from the mutant rice strains are all significantly higher than those of the ZH11 rice starch (Supplementary Table S2). However, there are no significant differences in SBV, P_{time}, and PT.

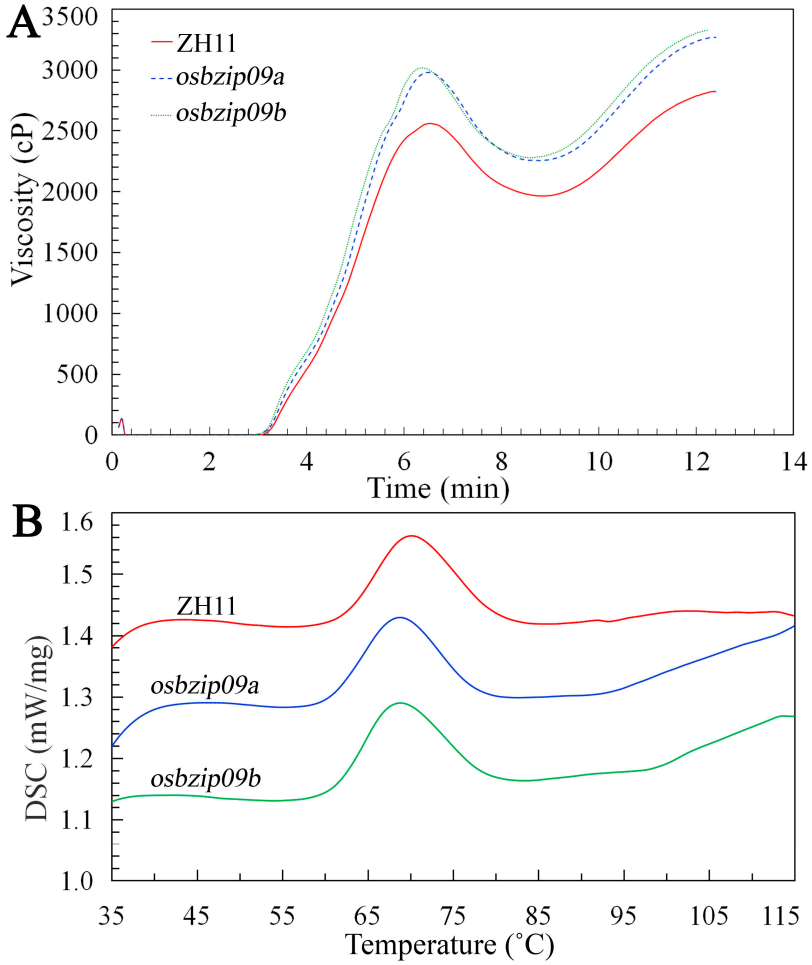

Figure 4. Pasting and thermal properties of endosperm starch from different rice lines. (**A**) shows the starch RVA curve; (**B**) shows the endothermic curves of the starches as determined by DSC.

Previous studies have found that many factors, such as starch granule size and starch fine structure, affect pasting profiles [40,41]. Generally, rice starch comprising large starch granules will exhibit higher viscosity [40]. However, in the current study, contrary to the starch size-distribution data, the samples with relatively small starch granules exhibit high

RVA curves, indicating that starch fine structure plays an important role in determining starch pasting profiles. Indeed, studies on the mutation of certain starch-biosynthesis-related genes have shown that changes in starch fine structure can lead to dramatic changes in starch pasting profile [42,43]. There is convincing evidence that the pasting properties of starches are related to the internal molecular structure of the amylopectin therein, and that the number of B1 chains is negatively correlated with PKV, HPV, BDV, and CPV [14,41]. Furthermore, related studies have revealed that the small amylopectin molecules within starch granules are more easily leached from the granule and thus increase viscosity [44]. Taken together, the higher number of amylopectin short chains (DP 6–12) as well as the reduced intermediate chains (DP 13–24) will cause more leached amylopectin during RVA measurement, thereby causing higher viscosity.

3.5. Thermal Properties of Starch

Gelatinization involves the uncoiling and melting of the ordered starch structure, which is mainly formed by amylopectin [45]. Accordingly, in order to determine the effects of *OsbZIP09* mutation on starch gelatinization profiles, DSC was employed. The DSC parameters are presented in Table 2. As shown in Figure 4B, both *osbzip09a* and *osbzip09b* rice starches exhibit a slight shift of endothermic peak, which indicates that the mutant rice lines have lower gelatinization temperatures than the wild type. We found that starches from the two mutants exhibit significantly lower gelatinization parameters, including T_o, T_p, T_c, and ΔH, compared with that from ZH11. Similarly, it has been previously reported that deficiency of certain starch-synthesis-regulation genes, e.g., *RSR1* and *NF-YB1*, can lead to a lower gelatinization temperature and significantly lower gelatinization parameters [20,22].

Table 2. Thermal and crystallinity parameters of rice starches from different rice lines [1].

Lines	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J G^{-1})	RC (%)	1047/1022 cm^{-1}
ZH11	62.01 ± 0.13 [a]	70.45 ± 0.49 [a]	80.45 ± 0.49 [a]	10.21 ± 0.27 [a]	28.16 ± 0.24 [a]	0.84 ± 0.02 [a]
osbizip09a	60.30 ± 0.28 [b]	68.40 ± 0.43 [b]	77.45 ± 0.35 [b]	8.31 ± 0.25 [b]	26.54 ± 0.34 [b]	0.78 ± 0.01 [b]
osbizip09b	60.88 ± 0.39 [b]	68.90 ± 0.28 [b]	78.20 ± 0.42 [b]	8.32 ± 0.31 [b]	26.72 ± 0.21 [b]	0.77 ± 0.02 [b]

[1] Data presented as mean ± standard deviation. Values with the same letter in a column of the same treatment are not significantly different ($p < 0.05$). T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; ΔH, enthalpy of gelatinization; RC, relative crystallinity calculated from XRD; 1047/1022, the 1047/1022 cm^{-1} absorbance ratio calculated form FTIR.

It is well accepted that gelatinization is predominantly influenced by amylopectin branch chain-length distribution, exhibiting a positive correlation with the number of amylopectin long chains [15,45]. It also has been reported that the presence of short amylopectin chains (DP 6–12) lowers GT, whereas high levels of long amylopectin chains (e.g., DP 13–24) can increase GT [14,45]. As a result, the enrichment of amylopectin short chains (DP 6–12) in *OsbZIP09* mutant rice starch causes the reduction in starch gelatinization properties observed here. In addition, the gelatinization parameters reflect the proportion of crystallites associated with the molecular order. Additionally, the endothermic peak in the DSC profile reflects the loss of double helices in amylopectin, and the total energy reflects the crystalline structure or molecular order [15]. The lower gelatinization enthalpy (ΔH) of *osbzip09a* and *osbzip09b* rice starches implies that less energy is required to melt the starch crystallinity in the *OsbZIP09* mutant relative to the wild type, which mainly depends on the fine structure of amylopectin. These results further demonstrate that the *OsbZIP09* deficiency contributes to a lowering of gelatinization properties compared to the wild type.

3.6. Starch Crystalline Structure

The long-range ordering of the starch structure was investigated using XRD. As shown in Figure 5A, the three samples show similar XRD patterns, with strong diffraction peaks around 2θ = 15°, 17°, 18°, 20°, and 23°, which represents an A-type diffraction pattern typical of most normal cereal starches [27,45]. However, the RCs calculated from

the XRD patterns differ between the three starch samples (Table 2). Starches from the *osbzip09a* and *osbzip09b* lines have significantly lower RCs than that from ZH11 rice (Table 2), which explains why the mutant rice starches have low gelatinization profiles; in short, the lower ΔH as determined by DSC is due to the endothermic transition upon loss of crystallites, which are mainly formed by ordered amylopectin structures [46]. Amylopectin is generally considered to be responsible for starch crystallinity, while amylose disrupts the crystalline packing of amylopectin [46]. It has been reported that the crystallinity degree was decreased with an increase in amylose content [7]. Thus, the enrichment of amylose content in *OsbZIP09* mutant rice might be an important cause of the lower RCs. In addition, it is evident that internal chain segments of amylopectin molecules influence starch crystalline properties [15]. It is thought that the minimum starch chain length required to form double helices is 10 monomeric units [47], and amylopectin short-branch chains decrease packing efficiency in the starch, lowering crystallinity and thus decreasing the gelatinization temperature [15,45]. Accordingly, the increased abundance of short amylopectin chains (DP 6–12) and decreased abundance of intermediate chains (DP 13–24) in the *OsbZIP09* mutant rice is the main reason for the reduced crystallinity of the starch.

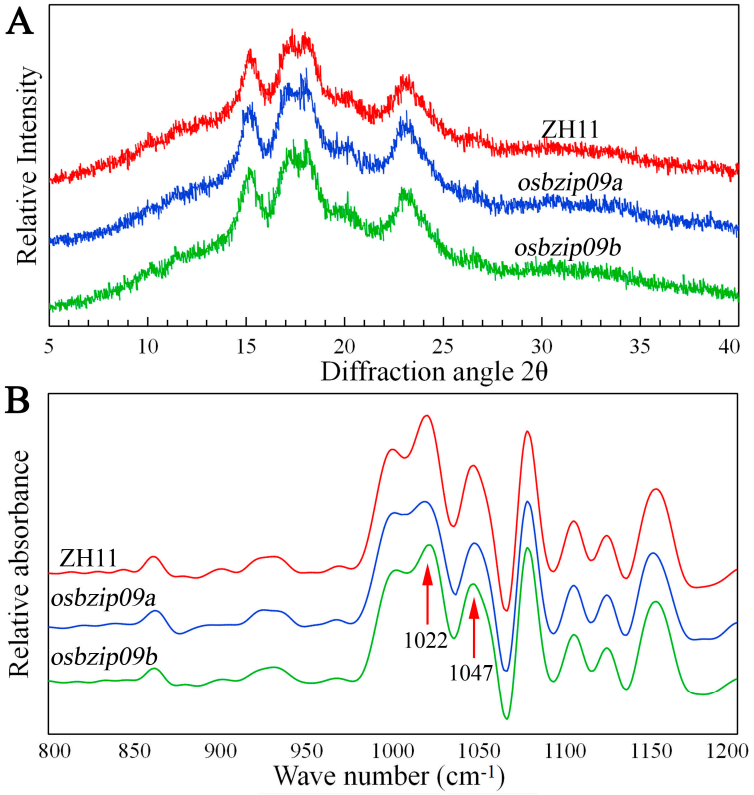

Figure 5. XRD patterns (**A**) and ATR-FTIR spectra (**B**) of rice starches from ZH11 and its mutant lines *osbzip09a* and *osbzip09b*.

In order to investigate the effects of *OsbZIP09* mutation on short-range starch structure (helical order) near granule surfaces, ATR-FTIR was performed. The signal intensity ratio for the absorbances at 1047 and 1022 cm^{-1} provides information on the relative amounts of ordered starch and amorphous starch [48]. As shown in Figure 5B, all starch samples exhibit similar FTIR spectra. However, the starches from the *osbzip09a* and *osbzip09b* rice exhibit significantly lower 1047/1022 cm^{-1} ratios than the wild-type ZH11, which is consistent with the XRD data (Table 2). Similar results have been reported for other rice starches, and ordered structure at the short-range level is a prerequisite for the presence of long-range order [27]. Studies have shown that in normal cereal starches, a low number of amylopectin short chains (DP 6–10) can form single helices rather than double helices [14,49]. Because

the 1045/1022 ratio mainly reflects the double helix content in starch, the lower helical order of the mutant starch is probably caused by the enrichment of amylopectin short chains.

4. Conclusions

OsbZIP transcription factors are key components in the ABA signaling pathway to mediate its regulation of downstream target genes and consequently ABA-triggered plant responses, including suppressing seed germination, enhancing plants' tolerance to stress [13]. Our previous studies have shown that *OsbZIP09* is involved in the regulation of rice seed germination through its attenuation of the ABA pathway [32]. However, knowledge about the effects of *OsbZIP09* mutation on rice grain quality and starch physicochemical properties is currently limited. The present investigation utilized two *OsbZIP09* mutant rice lines, *osbzip09a* and *osbzip09b*, to explore the roles of this gene in determining rice grain quality and starch structure. We have confirmed that *OsbZIP09* mutation leads to highly chalky rice grains that exhibit loosely packed, spherical starch granules in the ventral region of the endosperm. *OsbZIP09* mutation also leads to a lower average starch granule diameter in the endosperm. Importantly, the structural starch features determined using HPAEC revealed that the mutant rice lines contain more amylopectin short chains and fewer intermediate chains, leading to lower crystallinity than that of the wild-type rice. Furthermore, analysis of the pasting and thermal properties of the rice starches revealed that both the RVA and DSC characteristics are influenced significantly by the mutation of *OsbZIP09*, which might be attributed to the changes in the starch fine structure. These results strongly indicate that the transcription factor *OsbZIP09* also plays an important role in rice starch synthesis in the endosperm.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081575/s1, Figure S1: Schematic of the mutation sites in the *OsbZIP09* gene (A) and the phenotype of rice seeds (B,C), Table S1: Amylopectin chain distributions in different fractions of endosperm starch from different rice lines, Table S2: Pasting properties of starches from different rice lines.

Author Contributions: Conceptualization, C.-Q.Z. and Q.-Q.L.; methodology, C.-X.W.; software, C.-C.Z.; validation, C.-X.W., C.-C.Z., and C.-Y.L.; formal analysis, Y.Y.; investigation, Q.-F.L.; resources, Q.-F.L.; data curation, C.-C.Z.; writing—original draft preparation, C.-X.W. and Q.-F.L.; writing—review and editing, C.-Q.Z.; supervision, Q.-Q.L.; project administration, C.-Q.Z.; funding acquisition, C.-Q.Z. and Q.-F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Training Programs of Innovation and Entrepreneurship for Undergraduates, Programs of the Jiangsu Government (BE2020318-1, CX(20)3004, and PAPD), and the Yangzhou University high-end talent program.

Data Availability Statement: The datasets generated for this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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