

FORMATION CHARACTERISTICS OF ENDOSPERM STRUCTURES IN DIFFERENT RICE GENOTYPES

LIMIN YUAN*, RUNQIN LI, LIDONG FU, ZHIQIN WANG AND JIANCHANG YANG

Jiangsu Key Laboratory of Crop Genetics and Physiology / Co-Innovation Core for Modern Production
Technology of Grain Crops, Yangzhou University, Yangzhou 225009, China

*Corresponding author's email: lmyuan@yzu.edu.cn; Tel: 0514-87979317

Abstract

To explore the formation characteristics of endosperm structures in different rice genotypes, different rice genotypes of (*indica*, *japonica*, and glutinous) were used and grown in the paddy field. The endosperm structures in the grain during the filling period were investigated. The results showed that the compactness of amyloplast arrangement was positively correlated with grain filling percentage. The endosperm structure varied with the position within a grain. At maturity, the structure was the best in the back, the intermediate in the center, and the worst in the belly of a kernel. However, the filling was better in the center than in the back and in the belly from 5 to 10 days after flowering (DAF). The endosperm structure was different among genotypes. From 5 to 25 DAF, starch accumulation was the earliest in glutinous rice genotypes, followed by *indica* and *japonica* genotypes. Gaps and pores in endosperm were closely associated with rice transparency. The starch crystallinity in endosperm was negatively correlated with amylose content. Among the three genotypes, glutinous showed the highest crystallinity, followed by *japonica* and *indica* rice. The starch crystallinity in a grain was lower on a primary branch than that on a secondary branch. Among all grains, the second grain on a primary branch showed the lowest starch crystallinity. The results indicated that the starch structure of endosperm not only differ between rice genotypes, but also varies with the location of a grain on the panicle, and that it affects the grain-filling, transparency and amylose content of rice.

Key words: Rice genotypes; Grain filling; Endosperm structure; Starch granule; Crystallinity.

Introduction

Grain filling stage is a key stage influencing grain weight and quality of rice. The main component in rice grains is endosperm starch, and the structures of endosperm amyloplasts (size and arrangement of amyloplasts) are directly correlated with grain filling and rice quality (Wang *et al.*, 2012; Fu *et al.*, 2019). Previous studies indicated that the amyloplasts arranged tightly in the endosperm with large size and small difference in amyloplasts particle diameter, and the filling degree of grains was associated with better rice quality. Promoting the development of amyloplast structures in rice endosperm by genetic improvements and cultivation techniques is one of the important approaches for increasing the yield and quality of rice. The grain-filling rates and filling degrees vary with rice genotypes and the grain position in the same panicle of the same genotype (Wang *et al.*, 2012; Fu *et al.*, 2019). Normally, the filling rates of superior grains with the early flowering stage are high and the amyloplasts in endosperm are arranged compactly, the weight of superior grains was significantly higher than that of inferior grains (Peng *et al.*, 2011; Zhu *et al.*, 2021), whereas the grain filling rates of inferior grains with the late flowering date are low. The main manifestation was that the formation of endosperm structure of the early-flowering superior grains was earlier than the lately-flowering inferior grains (Wang *et al.*, 2020). Under the scanning electron microscope, amyloplasts of superior grains arranged tightly in the endosperm with large size and small difference on amyloplasts particle diameter, the remnants of endosperm cell were few and the gaps of amyloplasts were narrow. However, for inferior grains, the amyloplasts are arranged loosely in the endosperm,

the weight of grain is lower and the grain filling degree and the appearance quality are relatively poor (Yang *et al.*, 2003; Yang *et al.*, 2006; Peng *et al.*, 2017; You *et al.*, 2016; Zhao *et al.*, 2020). Chalkiness is the most important indicator of appearance quality in rice. When the rice grains had more chalkiness, the plumpness of rice grain decreased, the transparency of rice could be reduced, the taste of rice decreased, and the rate of head milled rice was low. The formation of chalkiness was closely related to dynamic of grain filling. Most of the previous studies on the grain filling characteristics among different grain positions focus on the comparison between superior grains and inferior grains (Lisle *et al.*, 2000; Ashida *et al.*, 2009), but the differences in morphology or structures of endosperm starch at different grain positions in panicles were seldom reported. In addition, the formation characteristics of endosperm structures in different rice genotypes have not been comprehensively explored. In the study, typical rice genotypes of *indica* and *japonica* and glutinous rice were used as the materials, the formation characteristics of endosperm structures and their relationship with plumpness in grain-filling stage in rice with different genotypes were analyzed in order to explore the morphological characteristics of endosperm structures which influences rice quality. The study of morphological structures would enrich the basic knowledge of structural science of the formation of high-quality rice and provide a basis for improving rice yield and quality through agronomic practices.

Materials and Methods

Experimental materials and treatments: The experiment was carried out in the experimental farm of

the Agricultural College of Yangzhou University. The used experimental materials were *indica* rice cultivars (Yangdao 4 and Yangdao 6), *japonica* rice varieties (Wuyunjing 7 and Huajing 2), and glutinous rice cultivars (Yangfunuo 4 (*indica* glutinous) and Yangjingnuo 1 (*japonica* glutinous)). The preceding crop was wheat and the soil texture were sandy loam. The tillage layer contained 2.04% organic matters, 106.2 mg kg⁻¹alkali hydrolysable N, 28.5 mg kg⁻¹ Olsen-P, and 93.6 mg kg⁻¹ exchangeable K. During the whole growth period, 420 kg hm⁻² of urea was applied with the ratio of basal fertilizer: tillering fertilizer: spikelet fertilizer = 4:2:4. The rice cultivars were sown from May 10 to 12 and then transplanted to the field from June 8 to 10. The spacing of plants and rows were 17 cm × 20 cm, two seedlings per hole. The area of each plot was 5.1 m × 4.2 m and 4 replicates were arranged for each treatment in random blocks. Field management was performed according to conventional high-yielding cultivation methods.

Measurement items and methods

Observations of rice endosperm structure: A total of 100-150 panicles that headed on the same day were chosen and tagged for each plot. The flowering date and the position of each spikelet on tagged panicles were recorded. Ten to fifteen tagged panicles from each plot were sampled in the 5th, 10th, 15th, 20th, 25th, 30th, and 35th days after flowering. The primary and secondary branches in the middle of the panicle were sampled and the grains sampled at the same position were combined together as a sample.

In the filling stage and mature stage, after the grains were separated from the panicles, scanning samples were prepared according to the method of Zhang *et al.*, (2019). The endosperm structures of grains were observed, photographed and recorded under the environmental scanning electron microscope (XL30-ESEM, PHILIPS, Amsterdam, Netherlands).

Endosperm starch of the grains of different rice genotypes based on X-ray diffraction analysis: The tested materials were Yangdao 4, Huajing 2 and Yangjingnuo 4. Firstly, 3-5 panicles were sampled from each variety in the maturity stage. Then, the primary branch and the secondary branch in the middle of the panicle were sampled according to different grain positions. After removing the glumes manually, the grains sampled at the same grain position were ground into powder with a mortar. Then powder was irradiated with Bruker D8 super speed XRD (X-ray diffraction) instrument. When the crystal lattice plane of the crystalline part of the starch sample and the X-ray met the Bragg condition, the diffracted X-rays from the crystal lattice plane overlapped in phase. Strong diffracted X-rays formed the 2θ angle with the irradiation X-rays.

In the obtained XRD spectra of endosperm starch of each test material, the positions of peaks were related to the crystal type and the peak width was related to the crystallinity. The narrower the peak was, the higher the crystallinity was. Therefore, the crystallinity could be

determined by the relative peak height or peak area in Topas-3 software (Warren *et al.*, 2016). The crystallinity of starch crystals could be calculated with the method of Retval and the figures were plotted with Origin software.

Statistical analysis

The results were analyzed for variance using SAS statistical analysis package (version 6.12; SAS Institute, Cary, NC, USA). Means were tested by least significant difference at $P = 0.05$ level (LSD_{0.05}).

Results and Analysis

Formation of endosperm structures of different rice genotypes: The morphological changes of endosperm structures of grains were obvious during the filling process. Endosperm structure varied with genotype and grain position.

The endosperm structure in 5 DAF was shown in Fig. 1. The superior grain started to accumulate starch and the size of amyloplast in the endosperm cells ranged from 0.68 to 8.63 μm (Fig. 1 (A1-D3)). The starch accumulation in superior grain started significantly earlier than inferior grains (Fig. 1 (E1-E3)).

In this study, different grain positions showed the differences in the morphological structures of endosperm including starch accumulation time, amyloplast number, amyloplast size, and amyloplast arrangement. The trends were consistent among two cultivars of different genotypes. Scanning electron microscopy images also indicated the characteristics of starch accumulation in 5 DAF. Starch accumulation was the earliest in genotypes of glutinous rice (Fig. 1 (C1-C3, D1-D3)), followed by genotypes of *indica* rice (Fig. 1 (A1-A3)) and genotypes of *Japonica* rice (Fig. 1 (B1-B3)). Glutinous rice had the roughest surface of starch granules and the thickest membrane.

The endosperm structure in 10 DAF was shown in Fig. 2. The amyloplasts in endosperm cells were exposed and loosely arranged in superior grains. Amyloplasts in the back and center were relatively tight compared with that in 5 DAF and amyloplasts in the belly were loosely packed (Fig. 2 (A3, B3)). The size of amyloplasts varied greatly. Starch accumulation of the two glutinous rice genotypes exceeded that of *indica* and *japonica* rice in 10 DAF. Amyloplasts on the back of glutinous rice grains were exposed mostly, arranged tightly, and squeezed into polyhedron, but more remnants of endosperm cells, such as cell wall, also existed. Micropores with a diameter of about 250 - 300 nm were observed on the cell wall. The endosperm structure in belly was slightly loose and there were still a considerable proportion of small amyloplast (Fig. 2 (C3, D3, E3)). The differences in the endosperm structure between the second grain and the first grain of the middle panicle branch of each genotype were not significant, indicating that the inferior grains of the different genotype cultivars selected in this study were filled well in 10 DAF.

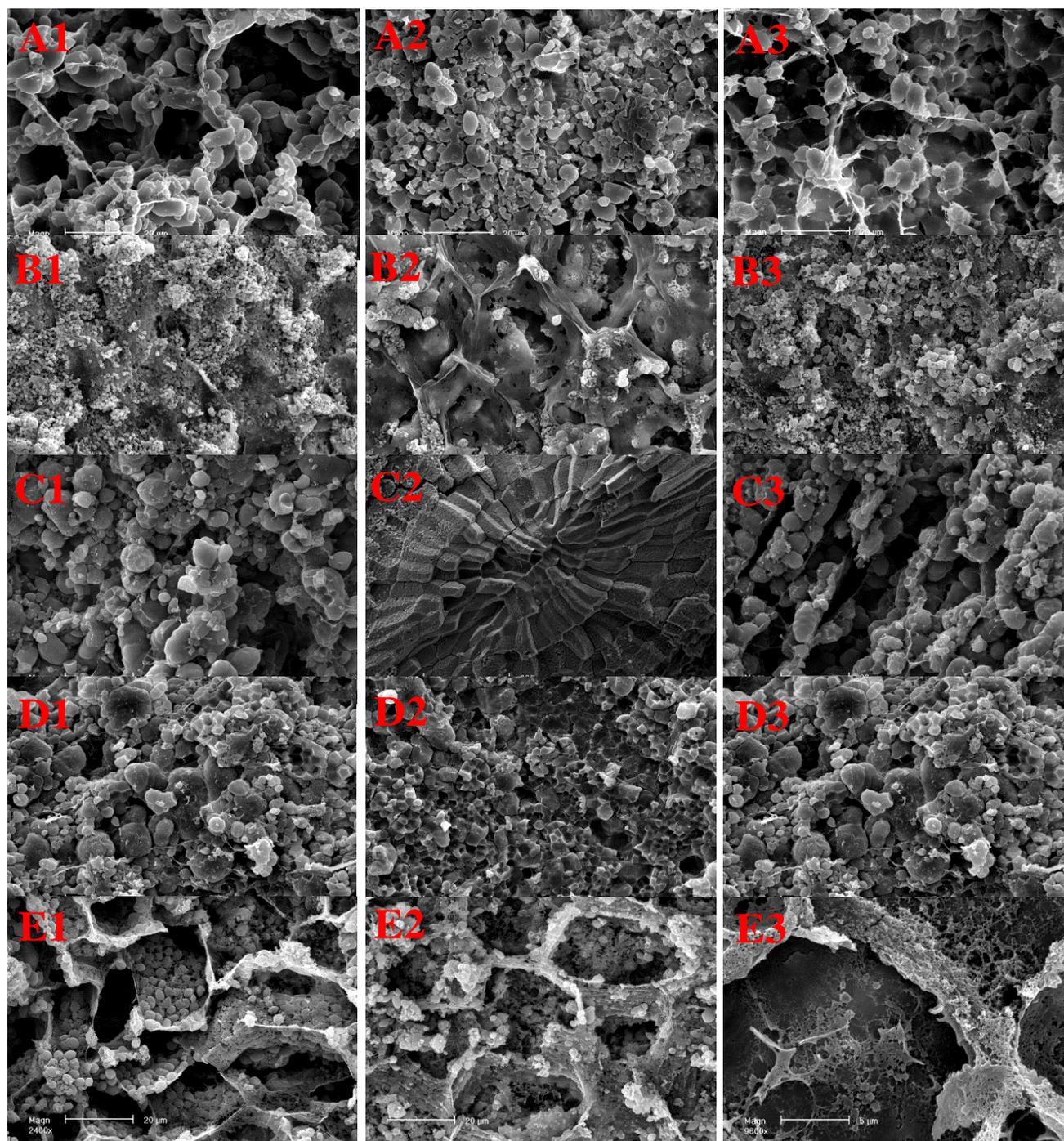


Fig. 1. Endosperm structure at 5 day after flowering (DAF) in different rice genotypes.

A: Yangdao4; B: Huajing 2; C: Yangfunuo 4; D: Yangjingnuo 1; A1, A2, A3-D1, D2, D3 show rice endosperm structure of the back, the center and belly of superior grain at 5 DAF in different genotypes, respectively; E1: endosperm structure of 2nd grain of Yangdao4 at 5 DAF; E2: endosperm structure of 2nd grain of Yangdao6 at 5 DAF; E3: endosperm structure of 2nd grain of Huajing2 at 5 DAF.

The endosperm structure in 15 DAF was shown in Fig. 3. Endosperm was further enriched, as indicated by the increased sizes of amyloplasts and decreased gaps. The amyloplasts in the back of superior grains were tightly arranged and the starch granules were polyhedral in shape with more cell remnants (Fig. 3 (A1-D1)). Amyloplasts in the center were relatively tight (Fig. 3 (A2-D2)) and the belly were loose (Fig. 3 (A3-D3)). In the grains with the late flowering period, the amyloplasts in the belly and center were loosely arranged, but also the endosperm on the back (Fig. 3 (E1-E3)). The cultivars of

all genotypes showed no significant differences. The glutinous rice cultivars Yangfunuo 4 and Yangjingnuo 1 showed micropores in the membrane (Fig. 3 (C2, C3, E2)) and the gaps between starch granules were also obvious (Fig. 3 (C2)). More small amyloplasts were observed in the belly and amyloplasts exposed occasionally had no intergranular gap. Different genotypes of rice showed significant differences in the filling process of the endosperm structure in this period. The endosperm structure of glutinous rice was better than that of *indica* rice and *japonica* rice.

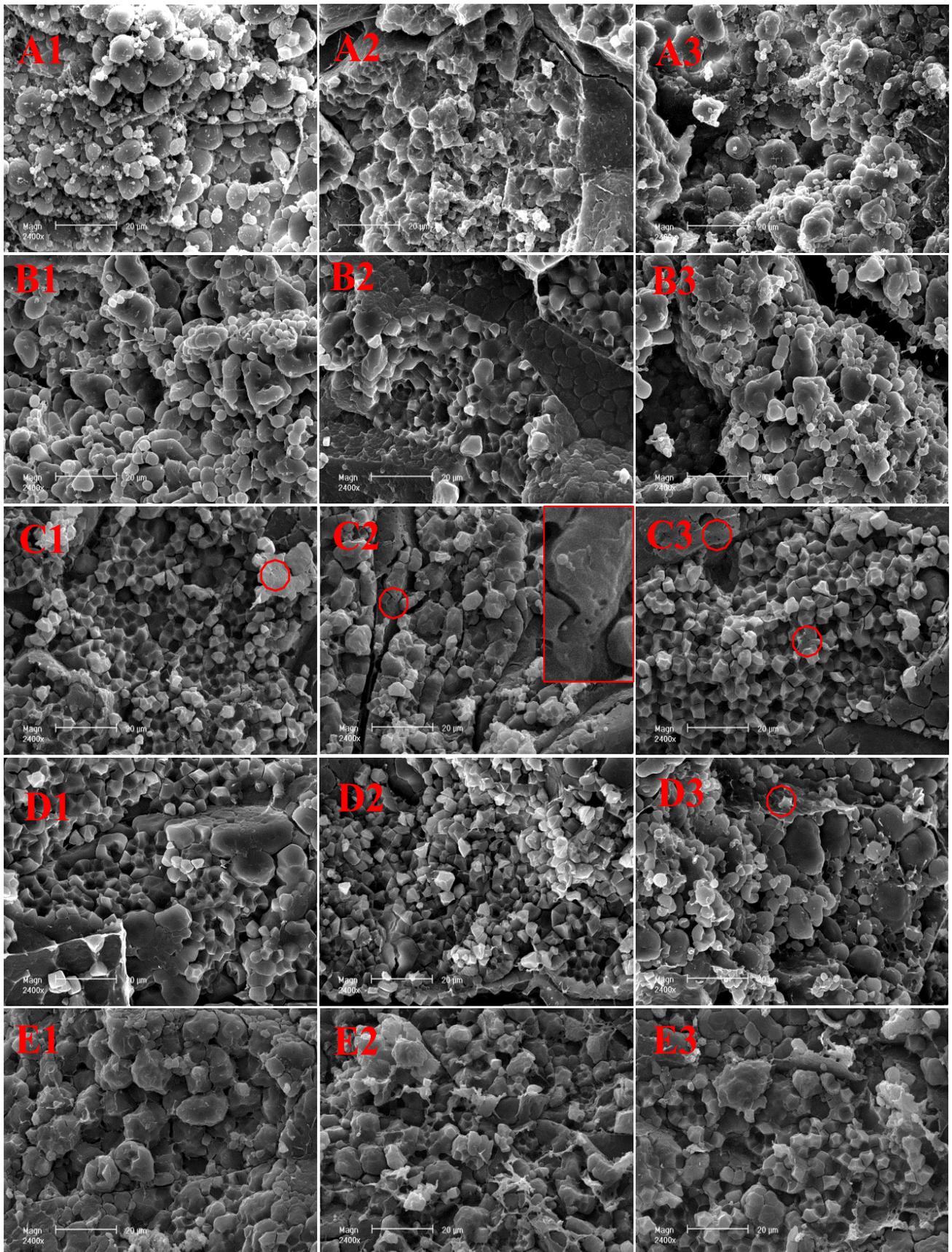


Fig. 2. Endosperm structure at 10 day after flowering (DAF) in different rice genotypes. A: Yangdao4; B: Huajing 2; C: Yangfunuo4; D: Yangjingnuo 1; E: Yangfunuo 4; A1, A2, A3-D1, D2, D3: endosperm structure of the back, the center and belly of superior grain at 10 DAF in different genotypes, respectively; E1-E3: endosperm structure of the back, the center and belly of 2nd grain at 10 DAF of Yangfunuo4; C2-the red box in the upper right corner is a magnification of \bigcirc , indicate the pore over the membrane.

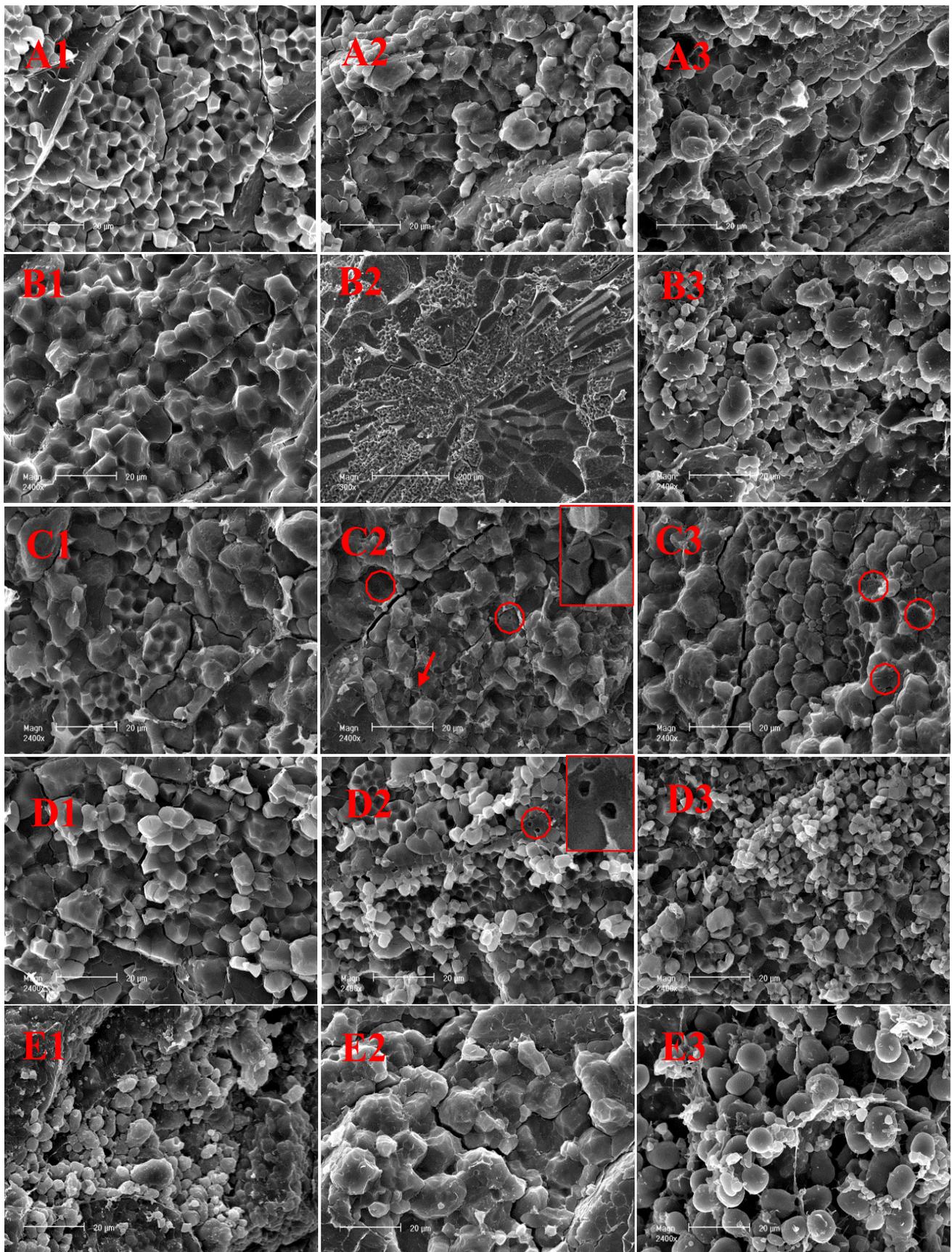


Fig. 3. Endosperm structure at 15 day after flowering (DAF) in different rice genotypes. A: Yangdao 6; B: Huajing 2; C: Yangfunuo 4; D: Yangjingnuo 1; E: 2nd grain; 1, 2, 3: the back, the center and the belly of superior grain at 15 DAF, respectively; E1: the back of 2nd grain at 15 DAF of Yangfunuo 6; E2: the center of 2nd grain at 15 DAF of Yangfunuo 6; E3: the belly of 2nd grain at 15 DAF of Huajing2; \blacktriangleright is the intergranular space of starch, as the red box in the upper right corner in C2 shown; \circ is the pore over the membrane, see upper right corner of C2 and D2 for details.

The endosperm structure in 20 DAF was shown in Fig. 4. The arrangement of amyloplasts in the back (Fig. 4 (A1-D1)) and the center (Fig. 4 (A2-D2)) of superior grains were tight. The difference in size was significantly reduced and the amyloplasts arrangement in the belly was relatively loose (Fig. 4 (A2-D2, A3-D3)). In the grains with the late flowering period, amyloplasts were loosely arranged in the belly and a certain proportion of small amyloplasts also existed (Fig. 4 (E2)). The amyloplasts arrangement in the back was relatively loose (Fig. 4 (E1, E3)). The amyloplasts in endosperm of *indica* rice, *japonica* rice and glutinous rice had basically the same arrangement in 20 DAF, but the starch plumpness in the belly of the glutinous rice grain was better than that of *indica* rice and *japonica* rice. The intergranular gaps in the amyloplasts of the two glutinous rice cultivars were more obvious (Fig. 4 (C3, D1-D3)).

The endosperm structure in 25 DAF was shown in Fig. 5. Amyloplasts of *Indica* and *japonica* genotypes, in the back (Plate V-A1, B1) and the center (Fig. 5 (A2, B2)) of the superior grains were tightly arranged, and amyloplasts in the belly were slightly loose (Fig. 5 (A3, B3)). The amyloplasts in the back and center of the second grain of the primary and secondary branches were still loose (Fig. 5 (E1-E3)). The inferior grains of glutinous rice had a compact endosperm structure in all parts of grains in 25 DAF (Fig. 5 (C1-C3, D1-D3)), and amyloplasts in the center were smaller than those in the back.

The endosperm structure in 30-35 DAF-the maturity period was shown in Fig. 6. In this period, the differences between different parts of the endosperm structure (the differences mainly refer to the filling in the belly) were further reduced and the differences between the different positions in a grain on a panicle were further reduced (the differences mainly refer to the filling of grains with the late flowering period). In the mature grains that flowered earlier on the primary branch of the central panicle, the amyloplasts in the back were tighter and even adherent, and cell remnants were significantly reduced. The size of amyloplast in the center and belly was larger than that in 20 DAF and the gaps between amyloplasts became smaller.

The endosperm amyloplasts of mature grains had significant differences in size and loose arrangement, commonly known as 'chalkiness', which is the morphological characteristic of poor rice quality (Lisle *et al.*, 2000). In this study, chalkiness generally occurred in the belly and rarely in the back of inferior grains of different cultivars, such as Yangdao 4, Wuyunjing 7, Yangfunuo 4, and Yangjingnuo 1 (Fig. 6 (E3)). However, in inferior grains, the second grains on the secondary branches of the middle panicle of Yangdao 6, had chalky characteristics in the belly, back and center of the endosperm (Fig. 6 (E1, E2)). In this study, the chalky phenomenon was also observed in the belly of the endosperm of superior grains of Huajing 2 in the mature period (Fig. 6 (B3)), indicating that the characteristics of the chalky endosperm structure in mature grains depended on grain position, branch position, and genetic characteristics of the cultivar.

The two glutinous rice cultivars (Yangfunuo 4 and Yangjingnuo 1) had significant intergranular gaps and pores in the endosperm amyloplasts (Fig. 6 (C1-C3, D1-D3))

and the number and the size of pores were large. This is a unique feature of glutinous rice. According to the measurements, the diameter of the pores in starch granules was 0.7 - 1.34 μm and the gaps between the starch granules in the amyloplasts were as long as 3.98 μm and its width even reached 1.14 μm .

The plumpness data of different rice cultivars (Table 1) showed that the plumpness of inferior grains of each cultivar was not as good as that of superior grains. Glutinous rice had the best plumpness, followed by *japonica* rice and the plumpness of *indica* rice (Yangdao 6) was the worst. Scanning electron microscopy images showed that the endosperm structure in the belly of the grains of Huajing 2 with the late flowering period was also slightly loose in the mature stage (Fig. 6 (B3)). The endosperm structure of the back and center of the grains with the late flowering period in the middle panicle in the mature stage of Yangdao 6 was loose (see Fig. 6 (E1, E2); in 20 DAF namely so, see Fig. 5 (E1, E2)). The endosperm structures of the two glutinous rice cultivars were tight, indicating that the difference in endosperm structure was consistent with the difference in the plumpness among different rice cultivars (Table 1).

Analysis of X-ray diffraction patterns of endosperm starch in different rice genotypes:

The XRD spectra of endosperm starch of each test material was shown in Fig. 7. The peak positions indicated that the endosperm starch types of Yangdao 4, Huajing 2, and Yangjingnuo 1 were intermediate types between Type-A starch and Type-B starch.

Topas-3 software was used to further analyze the XRD spectra of the endosperm starch of each test material, and the Retval's method was used to calculate the crystallinity of endosperm starch crystals of each test material. Table 2 showed the crystallinity of the endosperm starch of different grains on the panicles of the *indica*, *japonica* and glutinous genotypes. As for the same cultivar, the crystallinity of endosperm starch varied significantly among different grain positions. The differences were uncertain, but the differences showed a certain feature that the crystallinity of the grain on the primary branch was significantly lower than that on the secondary branch. The significance of difference was decreased in the following order: Yangdao 4 > Huajing 2 > Yangjingnuo 1. The second grain of the primary branch of the two conventional rice panicles had the lowest starch crystallinity, indicating that the amylose content of the grain on the primary branch of the panicle was higher than that on the secondary branch. In most cases, the second grain on primary branch in the upper and middle panicle had the highest amylose content. The results also showed that starch crystallinity was negatively correlated with amylose content. Starch crystallinity was also correlated with flowering date to a certain degree. Our results proved that the amylose content of superior grains was higher than that of inferior grains (Tran *et al.*, 2011; Li *et al.*, 2017).

Indica rice had the lowest crystallinity, followed by *japonica* rice, and glutinous rice had the highest crystallinity. Starch crystallinity was significantly negatively correlated with the amylose content of rice grain (Table 3).

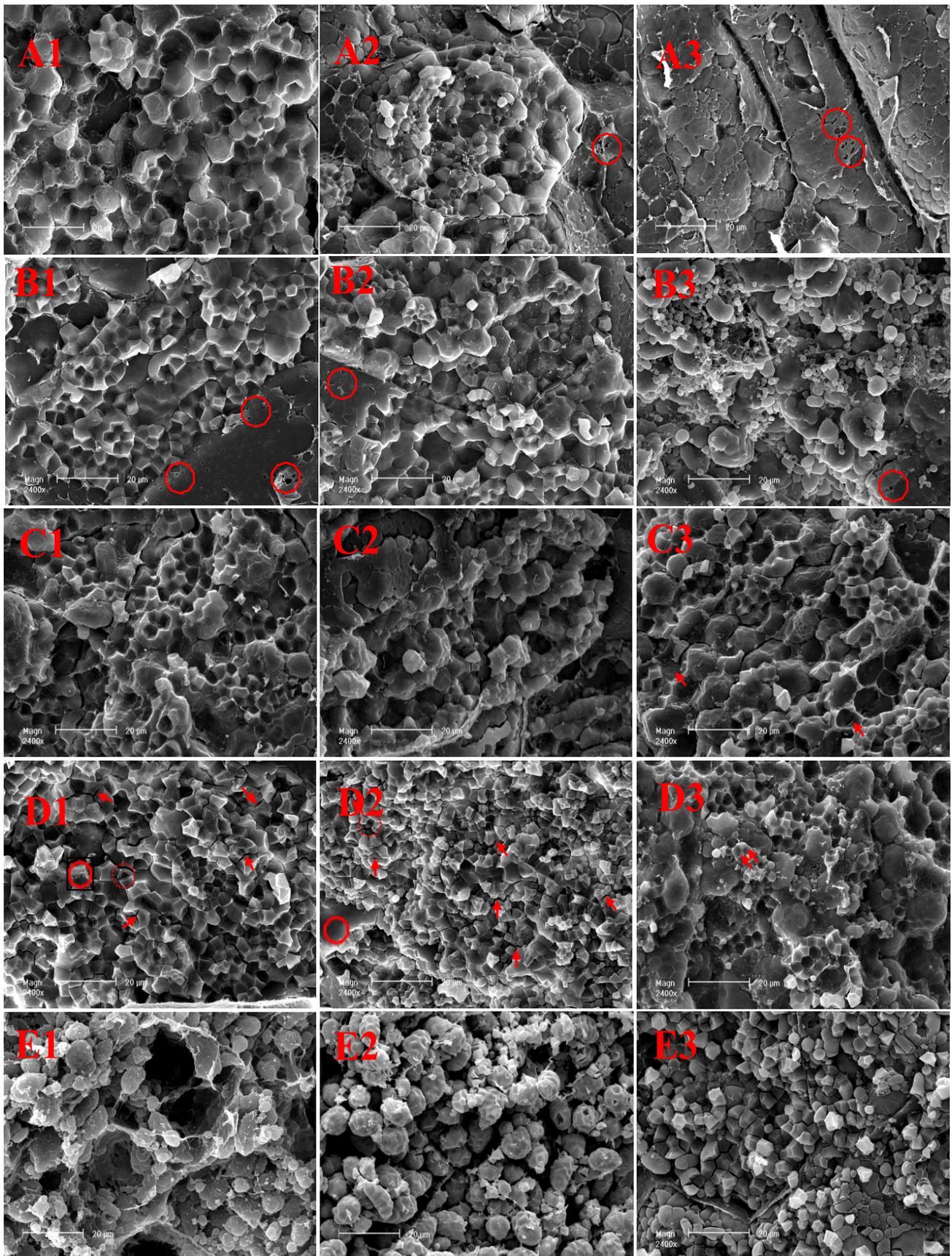


Fig. 4. Endosperm structure at 20 day after flowering (DAF) in different rice genotypes.

A: Yangdao6; B: Huajing2; C: Yangfunuo4; D: Yangjingnuo1; 1, 2, 3: the back, the center and the belly of superior grain at 20 DAF, respectively; E: 2nd grain at 20 DAF of secondary branch; E1: the back of Yangdao6 at 20 DAF; E2: the belly of wuyunjing7 at 20 DAF; E3: the back of Yangjingnuo1 at 20 DAF; ○ indicate micropores in starch granules; ➤ is the intergranular space of starch; ○ is the pore over the membrane.

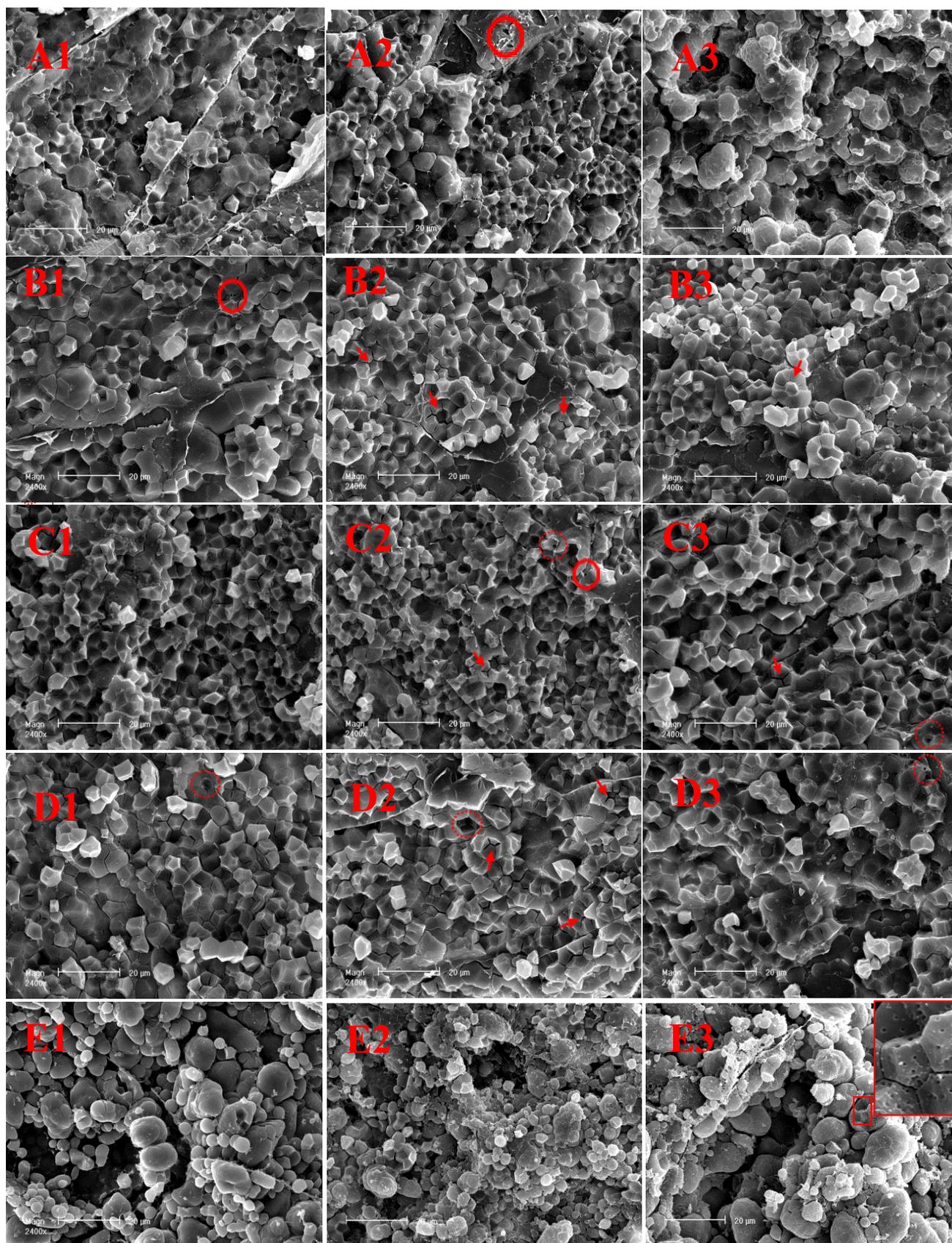


Fig. 5. Endosperm structure at 25 day after flowering (DAF) in different rice genotypes A: Yangdao 6; B: Huajing 2; C: Yangfunuo 4; D: Yangjingnuo 1; 1, 2, 3: the back, the center and the belly of superior grain, respectively; E1: the back of 2nd grain of primary branch of Yangdao6 at 25 DAF; E2: the back of 2nd grain of secondary branch of Yangdao6 at 25 DAF; E3: the belly of 2nd grain of secondary branch of Yangdao6 at 25 DAF, the red box in the upper right corner is a magnification of the small box, indicate micropores in starch granules; ○ indicate micropores in starch granules; ➔ is the intergranular space of starch; ○ is the pore over the membrane.

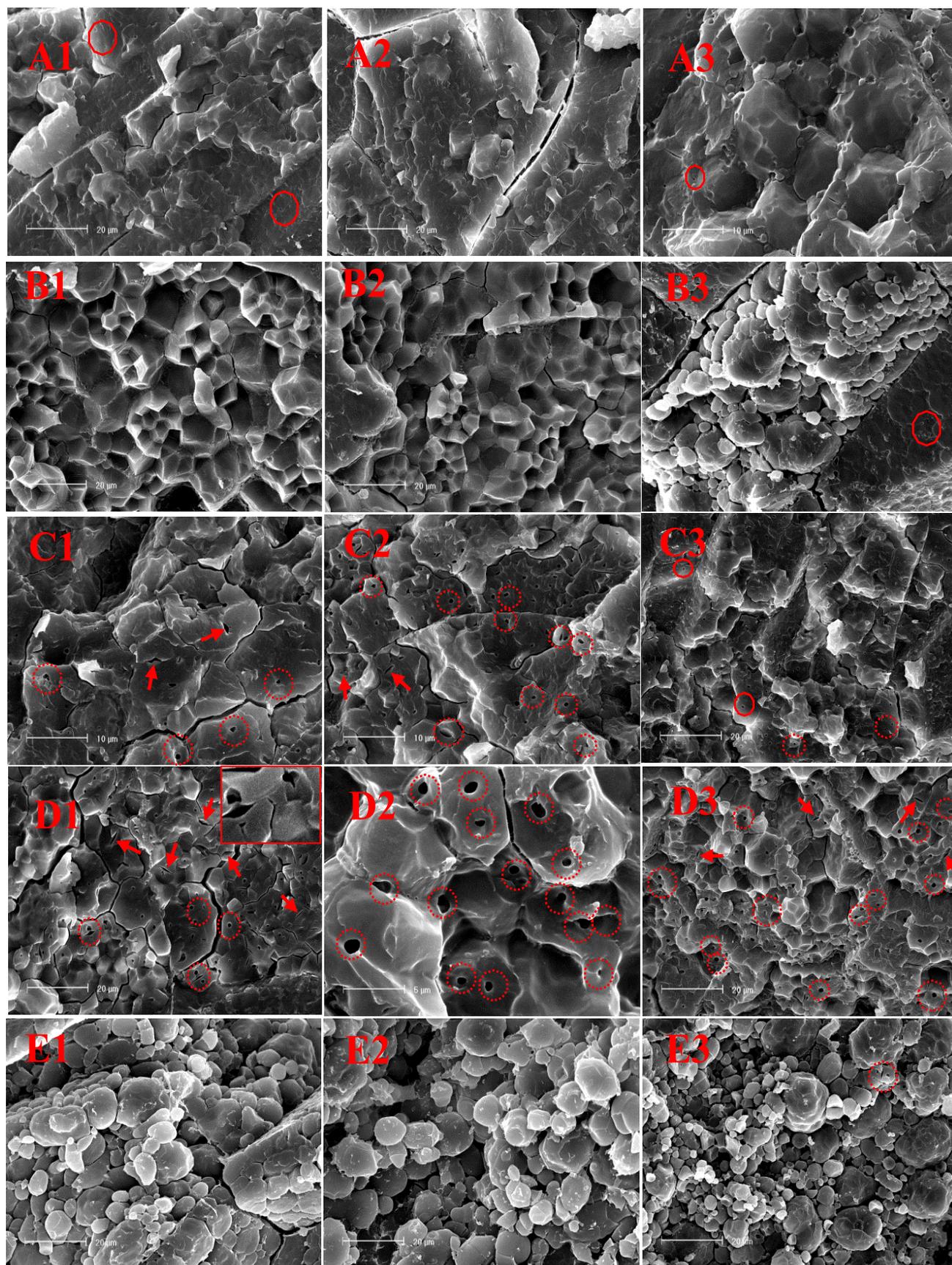


Fig. 6. Endosperm structure at maturity in different rice genotypes.

A: Yangdao 6; B: Huajing2; C: Yangfunuo4; D: Yangjingnuo1; 1, 2, 3: the back, the center and the belly of superior grain at maturity, respectively; E1: the back of 2nd grain of secondary branch of Yangdao6 at maturity; E2: the center of 2nd grain of secondary branch of Yangdao6 at maturity; E3: the belly of 2nd grain of primary branch of Yangjingnuo1 at maturity; ○ indicate micropores in starch granules; → is the intergranular space of starch, as the red box in the upper right corner in D1 shown; ○ is the pore over the membrane.

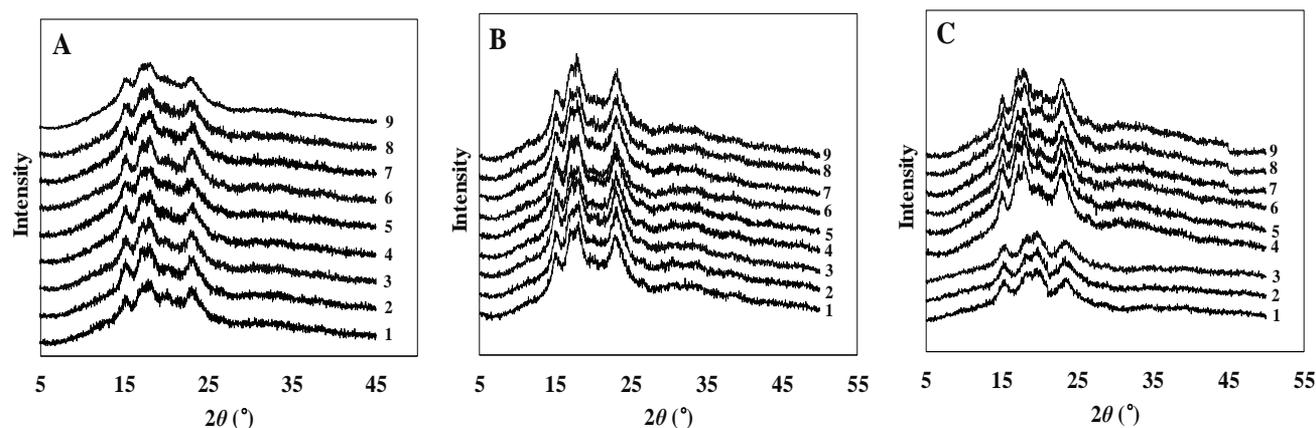


Fig. 7. A: X-ray diffraction patterns of endosperm starch of grains at different positions in a panicle of Yangdao 4. B: X-ray diffraction patterns of endosperm starch of grains at different positions in a panicle of Huajing 2. C: X-ray diffraction patterns of endosperm starch of grains at different positions in a panicle of Yangjingnuo 1. 1-6 represents the 1st grain-6th grain on the primary branch; 7-9 represents the 1st grain-3rd grain on the secondary branch.

Table 1. Grain filling percentage of different rice genotypes (%).

Genotypes	SPG	IPG	Mean
Yangdao 4	94.91b	93.74b	93.95b
Yangdao 6	92.11b	86.32c	89.22c
Wuyunjing 7	95.32b	92.13b	93.73b
Huajing 2	95.68ab	91.15b	93.42b
Yangfunuo 4	97.40a	94.20ab	96.10a
Yangjingnuo 1	98.30a	96.20a	97.10a

Values in the same column with different letters are significantly different ($p < 0.05$)

SPG: Superior Position Grain; IPG: Inferior Position Grain

Table 2. Crystallinity of endosperm starch at a different position within a panicle of different genotypes (%).

Grain positions	Primary branch			Secondary branch		
	Yangdao 4	Huajing 2	Yangjingnuo 1	Yangdao 4	Huajing 2	Yangjingnuo 1
1	21.12	30.77	30.49	26.59	32.34	31.40
2	20.79	23.29	31.51	30.58	33.16	31.72
3	21.88	25.55	31.88	32.59	32.93	33.93
4	24.07	29.23	29.03			
5	22.57	27.80	32.29			
6	22.55	28.36	25.92			
Mean	22.16	27.50	30.19	29.92	32.81	32.35

Table 3. Crystal percentages and amylose contents of endosperm starch of different genotypes (%).

Cultivar	Amylose content (%)	Starch crystallinity (%)
Yangdao 4	22.64a	24.75c
Huajing	15.97b	29.27b
Yangjingnuo 1	≤ 2.0 c	30.91a

Values in the same column with different letters are significantly different ($p < 0.05$)

Discussion

Formation characteristics of rice endosperm structures: Rice quality is closely related to endosperm structures (Lisle *et al.*, 2000; Fujita *et al.*, 2003; Satoh *et al.*, 2003; Cheng *et al.*, 2005; Inouchi *et al.*, 2005; Chun *et al.*, 2009; Patindol *et al.*, 2010; Tran *et al.*, 2011; Wang, 2015; Li *et al.*, 2017). Previous studies on endosperm structures mostly focused on the analysis of

rice quality and traits in the mature stage, but the dynamic changes of morphological characteristics during the formation of rice quality were seldomly explored. In this study, the dynamic changes of starch accumulation and endosperm structure formation during the rice quality formation process of two rice cultivars of three different genotypes were explored. The microstructure formation process of rice endosperm was displayed from the perspectives of morphology and anatomy. It is significant for understanding the internal mechanism of rice quality formation and improving breeding and cultivation of high-quality rice.

During the filling process, the morphological changes of the endosperm structure of grains included the increased quantity and size of amyloplasts, the decreased gaps, and the tighter arrangement. In this study, starch accumulation was observed in grains in 5 DAF. The formation of endosperm structures in different grain

positions was significantly different. The formation of endosperm amyloplasts in early-flowering grains was significantly earlier than that of late-flowering grains and amyloplasts gaps in early-flowering grains were also significantly smaller than those in late-flowering grains. Amyloplasts gaps were distributed according to the flowering order of spikelets.

In this study, in the grains at the same grain position in the same filling period, the formation of endosperm structures in glutinous rice was the earliest, followed by *indica* rice and the formation of endosperm structures in *japonica* rice was the latest. This differences in the formation of endosperm structure between different genotypes could be observed even in 25 DAF. The differences in the formation of endosperm between genotypes could be due to the differences in leaf photosynthetic capacities and root oxidation (Chen *et al.*, 2009; Gu *et al.*, 2017; Ye *et al.*, 2019; Chen *et al.*, 2021). This was consistent with the previous reports that the inferior grains of *japonica* rice had almost no dry matter accumulation in 5-10 DAF (Ishimaru *et al.*, 2003).

Relationship between endosperm structural characteristics and plumpness: The endosperm amyloplasts were characterized by the tight arrangement, small gaps and high plumpness (Wang, 2015). The plumpness data of rice cultivars of different genotypes (Table 1) showed that the plumpness of inferior grains of each cultivar was lower than that of superior grains. Among various rice genotypes, glutinous rice had the best plumpness, followed by *japonica* rice, and *indica* rice. Yangdao 6 had the lowest plumpness. The endosperm structure of late-flowering grains in the middle panicles of Yangdao 6 in the mature stage was slightly looser. The endosperm structure of glutinous rice was tight, indicating that the difference in endosperm structure between different rice genotypes in the mature stage was consistent with the differences in plumpness (Table 1).

In this study, the formation of endosperm structure was different in different position of grains. The endosperm structure of mature rice was better in the back than that in the center and the belly. In 5-10 DAF, the endosperm in the center was fuller than that in the back and belly. The results were different from the previous studies on the endosperm structure and did not seem to conform to the principle of nearby transportation of materials (Lisle *et al.*, 2000; Fujita *et al.*, 2003; Satoh *et al.*, 2003; Cheng *et al.*, 2005; Inouchi *et al.*, 2005; Chun *et al.*, 2009; Patindol *et al.*, 2010; Tran *et al.*, 2011; Li *et al.*, 2017). The differences might be interpreted as follows. Firstly, the photosynthetic product was transported to grains in the form of sucrose through phloem and the long transport route of filling materials determined that there was a sucrose concentration gradient inside grains (Zhu *et al.*, 2003). Secondly, the sucrose concentration in the center was the lowest, thus more sucrose from the surrounding accumulated in the center. Thirdly, due to the small size of center cells, the sucrose concentration

required for starch synthesis was reached more earlier to form amyloplasts. Fourthly, from the perspective of biological adaptability, the first formation of starch granules in the center was beneficial to the filling process of the entire grain. Otherwise, the formation of starch granules in the back or periphery would increase the filling resistance of materials into the inner endosperm cells, thus increasing the difficulty in filling the center of grains. Perhaps this is exactly the result obtained after the long evolution process of rice. Generally speaking, in 20 DAF, amyloplasts in the center of superior grains was tighter, but the size was small.

In 30-35 DAF-the mature stage, the differences among different position of the endosperm structure of a grain were further reduced. According to the principle of material transportation nearby, the back of the grain was filled before the belly. In 30-35 DAF-the mature stage, early-flowering grains were mainly filled in the belly, whereas late-flowering grains were filled in the core and belly as well as the back.

In 30-35 DAF-the mature stage, the differences among different grain location on a panicle were further reduced until the original filling rule was changed. Late-flowering grains had a slow filling rate and a long filling period. Inferior grains were still actively filled in 20-30 DAF, and starch accumulation in these grains could be always observed in 30-40 DAF. Therefore, in the late-flowering grains on the primary or secondary branches, starch accumulation or filling rate was not necessarily the worst, indicating that the filling trend of late-flowering grains could be reversed. This filling feature suggested that the cultivation and management of rice during the entire filling period was the focus. Therefore, the appropriate application of spikelet fertilizer, irrigation in the grain filling period, and delayed suspension of water to prolong the starch accumulation which might be conducive to the filling process of rice and the overall quality of rice.

Relationship between endosperm structural characteristics and grain transparency: Careful observations under a scanning electron microscope indicated that glutinous rice had three types of pores: pores in starch granules, gaps between single starch granules in amyloplasts, and micropores in membranous structures (from membrane or cell wall). The three types of pores did not appear in the early filling stage, but they appeared in the middle and late filling stages. According to microscopic observations, the surface of the early amyloplasts of glutinous rice was the roughest and the membrane was the thickest. We reasonably believed that the thick membrane concealed the holes that originally existed in starch granules until these holes appeared due to the dehydration and drying process in the later stage. According to our analysis, all rice cultivars experienced a process of water loss during the grain filling stage and the water loss process was not limited to glutinous rice. The endosperm of glutinous rice contained amylopectin and little amylose. The *Wx* gene of glutinous rice determined this trait. Amylose molecules were located in

the center of starch granules, whereas amylopectin was located in the periphery of starch granules (Nielsen *et al.*, 2002). Therefore, the starch granules of glutinous rice were different from those of *indica* and *japonica* rice at the beginning of formation. The holes in starch granules were not visible in the early stage of filling due to the thick membrane and the high water content. In the middle and late stages, especially in the mature stage, a large amount of the holes in starch granules were exposed due to the gradual water loss during the mature transition stage. It should be pointed out that in the two tested cultivars of *indica* rice and *japonica* rice, pores were also observed. The pores in *indica* and *japonica* rice were micropores of membranous structures (membrane or cell wall). These micropores were also observed in glutinous rice. Therefore, the micropores on this membrane structure were the common feature of different rice cultivars and the diameter of micropores was small and shallow. Compared with the other two forms of pores in glutinous rice (the holes on starch granules with the diameter of 0.7-1.34 μm), the gaps between starch granules in amyloplasts (3.98 $\mu\text{m} \times 1.14 \mu\text{m}$) were different in position, quantity and size.

If the degree of plumpness was good, the amyloplasts in endosperm were tightly arranged and the endosperm appearance was transparent. When the plumpness was poor, the amyloplasts in endosperm were loosely arranged and the endosperm appearance was not transparent. However, the opacity of glutinous rice did not mean that the amyloplasts in endosperm was poorly filled. The opaque appearance of glutinous rice was caused by too many voids in the endosperm and air refraction. The amyloplasts in endosperm of glutinous rice were also loosely arranged and poorly filled, but the phenomenon mainly occurred in the belly and center of inferior grains. Therefore, the space caused by poorly filled amyloplasts in the endosperm were the secondary structural characteristics responsible for the opaque appearance of glutinous rice. In the mature endosperm of glutinous rice, amyloplasts contained a large number of pores, as previously reported (Li *et al.*, 2014; Lu *et al.*, 2018). The pores in the starch granules and the obvious gaps between starch granules were two types of unique pores of glutinous rice and responsible for the opaque appearance of glutinous rice.

In scanning electron microscope images, under conventional cultivation conditions, the microporous structures with a diameter of about 0.2 μm appeared on starch granules in the belly of the 2nd grain on the primary branch of Huajing 2 and in the center of the 4th grain on the secondary branch of Yangdao 6. The microporous structures were most likely to occur in the belly of endosperm adjacent to the embryo because even in the seed storage period, the embryo breathed and consumed energy. The nearby belly of endosperm provided energy, so that the nearby amyloplasts were consumed and decomposed. Thus many small holes were formed in the endosperm. The phenomenon was also common in the process of seed germination. Under weak light, high temperature, and low-temperature stress, holes were

formed in starch granules (Satoh *et al.*, 2003; Tang *et al.*, 2019). Interestingly, under conventional cultivation conditions, in the belly of late-flowering grains on the secondary branches of the middle and lower panicles of conventional rice (Fig. 5 (E3)), the phenomenon often appeared in 20 DAF, it indicated a certain obstacle to amyloplasts development. Therefore, the obstacle is worthy of further study.

The formation of pores in the endosperm of rice grains was related to the content of amylose/amylopectin, the location of a grain on the panicle and environmental factors.

Relationship between starch crystallinity and amylose content:

In recent years, the XRD technology has been applied in starch research at home and abroad, especially in the studies on the industrial processing properties of starch (Tian *et al.*, 1991; Buléon *et al.*, 1998; Nielsen *et al.*, 2002; Iturriaga *et al.*, 2004; Ovando-Martínez *et al.*, 2011; Yang *et al.*, 2014; Chun *et al.*, 2015; Zhou *et al.*, 2020). The contents of amylose and amylopectin were the most important factors determining the cooking and eating quality. This study found that the crystallinity of starch was negatively correlated with the content of amylose. In addition, the crystallinity of starch was correlated with the flowering sequence to a certain degree. However, glutinous rice contained little or none amylose and its starch crystallinity was not much higher than that of conventional *indica* and *japonica* rice. The results indicated that the relationship between starch crystallinity and amylopectin content was not a simple one-to-one corresponding relationship. The relationship between starch crystallinity and cooking and eating quality, the optimal starch crystallinity range, and the corresponding relationship between starch crystallinity of cultivars with similar amylopectin contents maybe hot spots in the future research on rice quality.

Conclusion

The formation characteristics of endosperm structures of rice (starch granule size, properties, arrangement, starch crystallinity, etc.) were related to both genotypes and the location of grains on the panicle. The degree of grain filling was affected by the density of amyloplasts arrangement. The transparency of rice could be reduced by gaps and pores in the endosperm. The crystallinity of endosperm starch was closely related to amylose content. The grain plumpness and rice transparency could be improved by improving the arrangement of endosperm amyloplasts (increasing the degree of arrangement compactness) and reducing gaps and pores inside the endosperm. The crystallinity of endosperm starch could be reduced by increasing the amylose content of rice.

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