Chalkiness Characters and Scanning Electron Microscope Observation of Rice Grain Endosperm of *Japonica* Varieties in Southern Henan

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Abstract Chalkiness characters affect not only the grain appearance, milling, eating and cooking qualities but also the grain nutritional quality in rice, thus it is one of the most important traits in rice. It is very important for us to investigate the relation of the chalkiness formation and the development of endosperm structure and starch granule of different rice varieties. Here, we have investigated the chalkiness characters such as chalkiness rate, chalkiness degree and chalkiness area in 15 *japonica* rice varieties from southern Henan. Furthermore, the endosperm structure and starch granules of rice grain were also observed with scanning electron microscope. The results showed that the 15 *japonica* varieties have a significantly linear relationship between the chalkiness rate and chalkiness degree. Among the varieties, the biggest difference is the chalkiness rate, the second is the chalkiness area, and the last is the chalkiness degree. Moreover, there is a certain correlation between the distribution of starch granules, the arrangement of endosperm cells and the occurrence of grain chalkiness in the different rice varieties. For the same variety, the starch granules of chalky and non-chalky grains have obvious difference, while the starch granules from the transparent part of chalky rice and non-chalky rice do not have significant difference. The results would provide useful references for the improvement of grain quality in rice.

Key words Chalkiness, Scanning electron microscope, *Japonica* rice, Starch granule, Endosperm

1 Introduction

Oryza sativa L. is one of the most important grain crops in the world, and more than half of the population in the world and 2/3 of the population in China take rice as staple food[1-2]. With rapid development of national economy and continuous improvement of living level, people's demand on high-quality grain is also continuously increasing[3-6]. However, rice quality is an extremely complex trait, including appearance, milling, nutrient, eating and cooking qualities[2,7], while appearance quality is mainly decided by grain size, transparency and chalkiness[8-9]. Chalkiness characters not only affect the grain appearance, milling, nutrient, eating and cooking qualities[10-13], but also cause rice yield decline[14-15]. Therefore, chalkiness characters directly decide grain quality and market price of rice to a large extent. Chalkiness is one of main problems existed in rice production and consumption, especially in South China, rice always has the problem of high chalkiness rate and chalkiness degree[16]. Previous results showed that grain chalkiness characters of rice are controlled by multiple genes[3,17-18], have additive effect and interaction between additive effect and environment, and are affected by external environmental factors[10-11,19-24]. For example, high temperature induction[5,25], daily average temperature, diurnal temperature difference, sunshine and cultivation measures at grain filling stage all affect chalkiness characters of rice to different degree[26-29]. At present, a batch of genes affecting chalkiness characters of rice have been isolated and cloned by using natural population or mutant, such as *GW2*[30], *Chalk5*[19], *OsPPDKB*[31], *SSIIIa*[32-33], *GIF1*[34], *OsRab5a*[35] and *flo2*[36]. Chalkiness is white opaque part which is formed by aeration because of loose arrangement of starch granules when the development and enrichment of endosperm starch granules and protein of rice at grain filling stage are affected[29,37-38]. Different varieties and qualities of rice have the fixed starch granule morphology, and grain quality could be identified initially by scanning electron microscope (SEM)[16,39-41]. Based on prior researches, chalkiness characters of grain endosperm of the 15 *japonica* rice varieties were investigated in this paper, and SEM observation of grain endosperm cell and starch granule in rice was conducted to study the relationship between chalkiness characters formation of different *japonica* rice varieties and morphology and development of endosperm cell and starch granule. The results have paved the way for further studying chalkiness formation mechanism of *japonica* rice and cultivating high-quality new rice varieties.

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2 Materials and methods

2.1 Materials There were 15 *japonica* rice varieties for test, including 5 *japonica* rice varieties from Henan Province(Xiangnuo 1862, Xianggeng 33, Zhengdao 18, Xindao 18, Aiganxiangdaowan), 3 varieties of Liaoning Province(Yanfeng 47, Liaoxing 1 and Longyou 1875), 2 varieties of Jiangsu Province(Wuyungeng 23 and Nangeng 9108), 2 *japonica* rice varieties of Heilongjiang Province(Longgeng 31 and Longgeng 21), 2 varieties of Zhejiang Province (Zhegeng 88 and Xiushui 134) and Xinfeng 3 of Anhui Province. According to different growth periods of these varieties, they were sown in the same test field of Xinyang Academy of Agricultural Sciences in different batches of 2015, thereby guaranteeing that the 15 *japonica* rice varieties bloomed in last of August, and the temperature was relatively consistent at grain filling stage. Each variety was sown for two rows, and each row had 12 plants, and plant spacing was 16.5 cm × 26.4 cm. Conventional cultivation management of field was implemented from sowing to mature period of grain. After maturity, rice grain was tested after natural drying and conservation for three months at room temperature.

2.2 Methods

2.2.1 Determination of chalky traits. Chalkiness rate, chalkiness area and chalkiness degree of different *japonica* rice varieties were determined by the national standard GB/T17891-1999. 100 grains of full and complete polished rice from each variety were taken randomly, and the rice grain with chalkiness was picked out, which was repeated for three times, and the mean was the chalkiness rate of each variety. White belly rate and white core rate (the grain with white back was not observed) were determined by the method of chalkiness rate. 100 grains of full and complete polished rice were taken randomly, and the rice grain with white belly and white core was picked out, which was repeated for three times, and the mean was white belly rate and white core rate of each variety. 10 grains of chalky rice of each *japonica* rice variety were taken randomly, and the percentage of chalky part to whole rice grain area was estimated, which was repeated for three times, and the mean was chalkiness area. Chalkiness degree = Chalkiness rate × Chalkiness area. SPSS software was used for variance analysis of the measured data.

2.2.2 SEM observation. The chalky and non-chalky rice was taken from each *japonica* rice variety randomly. The blade back was used to knock middle part of the rice lightly, making it naturally split, and then the split site was cut off and made into 2 – 3 mm thickness of sample. A part was observed by ordinary optical microscope, and the other part was adhered to copper sample table by conductive adhesive. HUS-5GB type of vacuum coating apparatus was used to plate the section, and the sample was set under scanning electron microscope (Hitachi S-4800) to observe different parts of rice section and take a photo.

3 Results and analysis

3.1 Difference of chalkiness traits from different *japonica* rice varieties The measurement results of chalkiness traits from 15 *japonica* rice varieties were shown as Fig. 1. It was clear that chalkiness rate had the maximum difference among varieties, followed by chalkiness area, and the last was chalkiness degree. The chalkiness rate of Aiganxiangdaowan reached 93.1%, which was the highest. The chalkiness traits could not be observed from Xinfeng 3, and chalkiness rates of the 15 *japonica* rice varieties changed between 0 and 93.1%. Variance analysis showed that chalkiness rates of Yanfeng 47, Longyou 1875 and Nangeng 9108 had insignificant difference; chalkiness rates of Xindao 18, Longgeng 31 and Xianggeng 33 had insignificant difference; the difference of chalkiness rates of Longgeng 21 and Liaoxing 1 was insignificant; the difference of chalkiness rates of Xiushui 134 and Zhengdao 18 was insignificant. Chalkiness degree changed between 0 and 52.0%, and change trend among different varieties was very similar with that of chalkiness rate. Grain chalkiness of Zhegeng 88, Longgeng 21, Wuyungeng 23, Nangeng 9108, Liaoxing 1 and Longyou 1875 mainly occurred in belly, while chalkiness of other varieties mainly occurred in center part of rice endosperm. Chalkiness area changed between 0 and 63.0%, and the change trend was different from that of chalkiness rate and chalkiness degree. Chalkiness rates of Xianggeng 33 and Zhengdao 18 were not very high, but their chalkiness area surpassed 15.0%. It is noteworthy that chalkiness traits were not observed in Xinfeng 3. Chalkiness rate of Aiganxiangdaowan reached 93.1%, and its chalkiness area was 63.0%, while the chalkiness degree was 52.0%. Further analysis displayed that chalkiness rate showed obvious linear relationship with chalkiness degree, and chalkiness degree of the variety with high chalkiness rate was also higher.

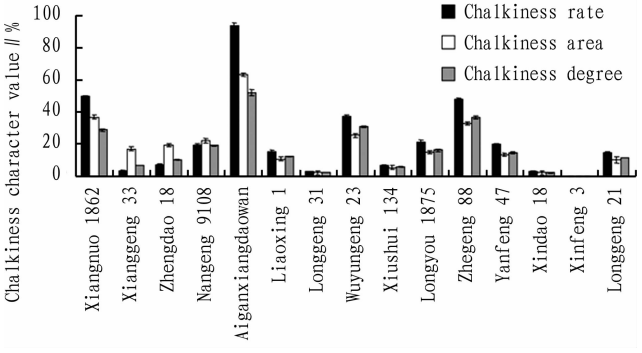


Fig. 1 Chalkiness characters of the 15 *japonica* rice varieties

3.2 Clustering of chalkiness traits of different *japonica* rice varieties The system clustering analysis of chalkiness rate (white belly rate and white core rate), chalkiness degree and chalkiness area of the 15 *japonica* rice varieties was conducted, and the results found that the tested *japonica* rice varieties could be divided into 4 classes(Fig. 2). Xindao 18, Longgeng 31, Xinfeng 3, Xiushui 134, Xianggeng 33 and Zhengdao 18 were clustered into Class Ⅰ, and their chalkiness rates changed between 0 and 7.4%. Chalkiness area changed between 0 and 19.2%, and chalkiness degree changed between 0 and 10.2%. Zhegeng 88, Wuyungeng 23 and Xiangnuo 1862 were clustered into Class Ⅲ, and their chalkiness rates changed between 47.7% and

49.5%. Chalkiness area changed between 25.3% and 36.7%, and chalkiness degree changed between 28.7% and 34.6%. Aiganxiangdaowan belonged to Class Ⅳ, and its chalkiness rate was 93.1%. Chalkiness area was 63.0%, and chalkiness degree was 52.0%. Longgeng 21, Liaoxing 1, *etc.* were clustered into Class Ⅱ, and their chalkiness rates changed between 14.4% and 19.6%. Chalkiness area changed between 10.1% and 22.2%, and chalkiness degree changed between 11.5% and 19.0%. Therefore, for 6 varieties of Class Ⅰ, their chalkiness rates were lower, and chalkiness degree and chalkiness area were smaller, while Aiganxiangdaowan of Class Ⅳ showed contrary chalkiness traits.

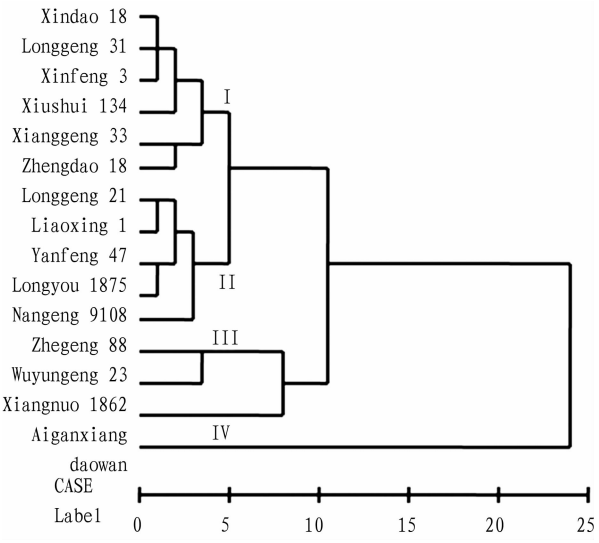
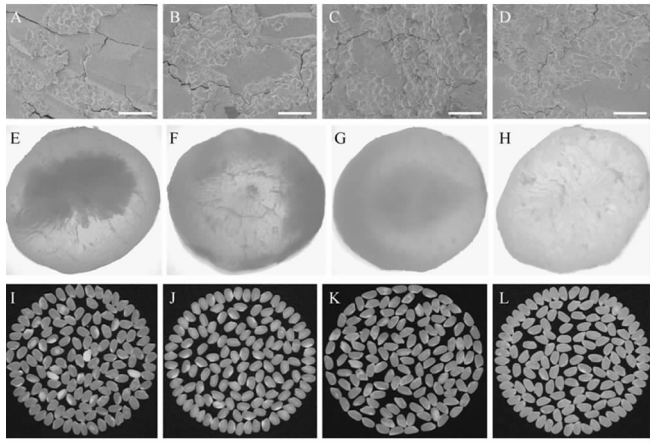


Fig. 2 Clustering of the chalkiness characters of the tested *japonica* rice varieties

3.3 Endosperm morphology of different *japonica* rice varieties The representative milled grains were selected, and ordinary optical microscope and SEM observation of endosperm morphology of 15 different *japonica* rice varieties was conducted from arrangement manner, morphology, layer number of rice endosperm cell and starch granule distribution of its cross section, and the results were shown as Fig. 3. Arrangement manner of endosperm cell at cross section mainly contained four classes. Class Ⅰ showed obvious radiation shape from middle cross section of rice endosperm to all around, and the radiation was longer. The variety dominated by white core belonged to the situation, such as Xianggeng 33, Xiangnuo 1862 and Aiganxiangdaowan (Fig. 3A, E, I). Class Ⅱ also showed obvious radiation shape from middle cross section to all around, but endosperm cell arranged radially was not straight, and some bent together. The variety dominated by white belly belonged to the situation, such as Nangeng 9108, Longgeng 21, Liaoxing 1, Wuyungeng 23 (Fig. 3B, F, J). Although Class Ⅲ showed radiation shape from middle cross section of rice endosperm to all around, the radiation was not obvious. The varieties with white core and white belly belonged to the situation, such as Yanfeng 47, Longyou 1875, Zhegeng 88 (Fig. 3C, G, K). Radiation shape could not be observed from middle part of cross section of Class Ⅳ rice endosperm, and the varieties with lower chalkiness rate or without chalkiness traits belonged to the situation, such as Xinfeng 3 and Xindao 18 (Fig. 3D, H, L). Under scanning electron microscope, it could see that the varieties dominated by white core had multilayer polygonal columnar cell, and there were many oval cell all around, such as Xianggeng 33, Xiangnuo 1862, Aiganxiangdaowan (Fig. 3A). The varieties dominated by white belly had irregular cell group, and multilayer polygonal columnar cells were at peripheral layer, such as Nangeng 9108, Longgeng 21, Liaoxing 1, Wuyungeng 23 (Fig. 3B). The varieties with white core and white belly had oval cell group, and polygonal columnar cells were at peripheral layer, such as Yanfeng 47, Longyou 1875 and Zhegeng 88 (Fig. 3C). Polygonal columnar cells, oval cells and irregular cells of the varieties with lower chalkiness rate or without chalkiness traits were not obvious, such as Xinfeng 3 and Xindao 18 (Fig. 3D).

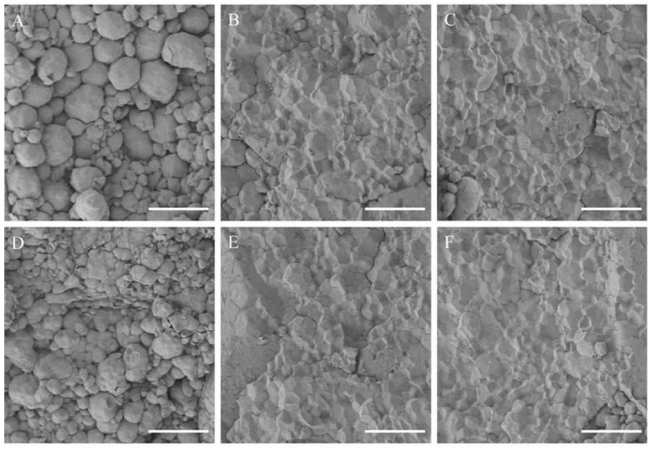
Electron microscope was used to observe distribution situation of starch granules at cross section of rice grains. It could be seen that the distribution of starch granules of chalky *japonica* rice were obviously uneven, and starch granules mainly concentrated in center part and dorsal abdominal diameter. The density of starch granule from different chalkiness types of *japonica* rice varieties was also different. Starch granule of *japonica* rice variety dominated by white core was mainly distributed in center part, while starch granule of *japonica* rice variety dominated by white belly was distributed in belly position of rice grain, and starch granule of *japonica* rice variety with white core and white belly was distributed in grain belly and center part, which was uneven. Starch granule of *japonica* rice variety without chalkiness or with very low chalkiness rate was obviously distributed in whole cross section, and the density of starch granule was larger (Fig. 3). The above results showed that arrangement manner of rice endosperm cells and distribution of starch granules had certain correlation with occurrence of chalkiness traits.



Note: A, E, I were Xianggeng 33; B, F, J were Longgeng 21; C, G, K were Longyou 1875; D, H, L were Xindao 18; Bar = 50 μm.

Fig. 3 Low magnification view of the transversely fractured mid-region of the tested rice

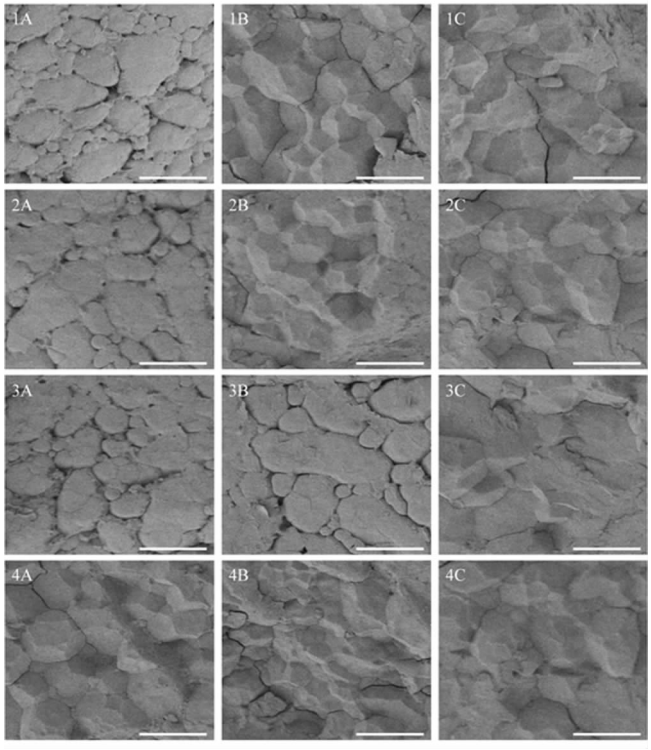
3.4 Starch granule morphology of chalky and non-chalky rice SEM observation of chalky and non-chalky rice from 15 *japonica* rice varieties was conducted. The results showed that starch granules at chalky part were spherical or ellipsoidal, with unobvious edge. The size of starch granule was different, with smaller diameter, and they accumulated with irregular shape. The space among starch granules became larger, with loose arrangement, and they were all single starch granule at free state(Fig. 4A, D). By contrasting transparent part of chalky rice grain with starch granule of non-chalky rice, it was found that there was not obvious difference, and most of starch granules had consistent size, and they were regular diamond or equal polygon, and had polyhedral edges. Moreover, the space among starch granules was very small, and the arrangement was very close (Fig. 4B, C, E, F). The above results showed that chalkiness formation was closely related to the shape and arrangement of endosperm starch granules. At the part with low chalkiness degree, its starch granule developed well, on the contrary, the development of starch granule was not good.



Note: A, D showed the starch grains in the chalky rice; B, E showed the starch grains in the transparent parts of chalky rice; C, F showed the starch grains in the non-chalky rice; Bar = 30 μm.

Fig. 4 Scanning electron microscopic observation on starch granules in endosperm cells of the chalky and non-chalky rice

3.5 Morphology of starch granule at different parts of chalky rice grain According to occurrence part of chalkiness in rice endosperm, chalkiness could be divided into three types: white belly, white core and white back. SEM observation on belly, core and back of chalky rice from 15 *japonica* rice varieties was conducted. It was found that the space among starch granules at center part of endosperm from Xianggeng 33, Xiangnuo 1862 and Aiganxiangdaowan was larger, with loose arrangement, and the development of starch granule was bad, while starch granule at endosperm belly and back developed better(Fig. 51A, 1B, 1C). Starch granule at endosperm belly of Nangeng 9108, Longgeng 21, Liaoxing 1, Wuyungeng 23 developed bad, while starch granule at endosperm core and back developed well(Fig. 52A, 2B, 2C). Starch granule at endosperm core and back of Yanfeng 47, Longyou 1875, Zhegeng 88 arranged loosely and developed bad, while the development of starch granule at back was better (Fig. 53A, 3B, 3C). Starch granule at endosperm core, belly and back of Xinfeng 3 and Xindao 18 developed well(Fig. 54A, 4B, 4C).



Note: 1 –4 were Xiangnuo 1862, Wuyungeng 23, Yanfeng 47 and Xinfeng 3 respectively; A showed starch grains in the center of the endosperm; B showed starch grains in the belly of the endosperm; C showed starch grains on the back of the endosperm; Bar = 10 μm.

Fig. 5 Scanning electron microscopic observation on starch granules in different parts of the tested *japonica* rice varieties

4 Conclusions and discussions

Chalkiness trait is an extremely important quality. Chalkiness affects not only appearance quality of rice but also milling, nutrient, eating and cooking qualities[10-13]. Morphology of starch granules in rice could be observed by scanning electron microscope, and rice quality could be identified preliminarily[16,39-41]. Seen from scanning electron microscope results of endosperm cells from 15 *japonica* rice varieties, starch granule distribution at cross section of the variety with higher chalkiness rate and chalkiness degree (Xiangnuo 1862, Aiganxiangdaowan and Zhegeng 88) was obviously uneven, and it was more obvious at center part of endosperm. For the variety with lower chalkiness rate and chalkiness degree, starch granule distribution at center part, belly and back of endosperm was even. By comprehensively analyzing chalkiness traits of endosperm from 15 *japonica* rice varieties and cell morphology at cross section, it was found that if starch granule distribution at cross section was even, chalkiness traits were not easy to occur in the variety, and chalkiness rate and chalkiness degree were lower. If starch granule distribution at cross section was uneven, the space among starch granules was larger, with loose ar-

rangement, and the development of starch granule was bad, so chalkiness traits were very easy to occur in the variety, and the chalkiness rate and chalkiness degree at corresponding position were higher. Therefore, distribution situation of starch granules at cross section of rice endosperm had the relationship with the generation of chalkiness traits.

By observing chalkiness traits of endosperm from 15 *japonica* rice varieties, it was found that morphology and arrangement of starch granule at different endosperm parts of the same variety were also different. Compared with transparent part or starch granule of non-chalky rice grain, starch granules at chalky part of endosperm mainly existed freely, and the space among starch granules was larger, so it could not further develop into starch granule complex with certain shape. If single starch granule could realize ordered arrangement and become complex starch granule with certain geometrical shape, chalkiness traits could not appear at the part. Ellipsoidal or spherical starch grains could not arrange orderly at chalky part, and the accumulation showed as disordered shape. Appearance embodiment of the ordered and disordered accumulation of starch grain was if rice endosperm had chalkiness and showed as transparent state. Previous studies found that the development of rice endosperm at the back was better than that of center part and belly, so chalkiness traits of back were rarely to occur[16,39,42-45]. In endosperm of the tested 15 *japonica* rice varieties, white back did not appear, and starch granule of back was regular diamond or equal polygon, with polyhedral edge, and the space among starch granules was very small, with tight arrangement, showing that the development of back starch granule was good. Maybe it was because that nutrients were transported firstly to the back of rice grain, and then center part and belly of endosperm, so the development of endosperm back was better than center part and belly. Therefore, chalkiness traits of rice endosperm were closely related to the development of starch granules.

Previous studies showed that chalkiness trait of rice grain was a complex number character, was commonly controlled by multiple genes, and was affected by environmental factors, especially temperature at grain filling stage significantly affected chalkiness traits[19-21,46-47]. Chalkiness traits had obvious difference among the tested 15 *japonica* rice varieties. By combining our previous results[7,48], it was found that chalkiness traits of endosperm were mainly controlled by inheritance. The tested 15 *japonica* rice varieties had better representation of ecological type. To decrease the influence of external environmental factors on chalkiness traits of the 15 *japonica* rice varieties as much as possible, the 15 *japonica* rice varieties were planted in the same tested field. Via prior pretest and the manner of batch sowing, it was ensured that they simultaneously bloomed in last dekad of August, 2015, and temperature was relatively consistent at grain filling stage. From first dekad of August, 2015 to first dekad of September, 2015, temperature was relatively higher in Xinyang City, which might cause that chalkiness traits of some *japonica* rice varieties could be more serious than that in origin production site. But external environmental conditions of the 15 *japonica* rice varieties at grain filling stage were basically same. The test results displayed that they showed similar some common chalkiness characters. However, the occurrence of chalkiness traits was a complex process, and the chalkiness formation may involve the relationship of "source", "storehouse" and "streams" in rice[47]. Their any change or uncoordinated relationship may cause or increase the formation of chalkiness traits in rice endosperm. By combining our previous results, it was thought that rice endosperm cell and the structure of starch granule at cross section decided appearance performance of chalkiness traits of endosperm, while endosperm structure was decided by the development of rice endosperm cell. All of those were controlled by inherited factors[48]. Therefore, the occurrence of chalkiness traits of rice endosperm was closely related to the distribution and development of starch granules, and the occurrence, development process, latter deposition mode and distribution situation of starch granules in rice endosperm may be very important. However, how to regulate prior occurrence, development process and latter deposition of starch granule in rice endosperm? How many genes join in the important process? These may be key problems further clarifying occurrence mechanism of chalkiness traits, which needs further research and exploration.

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