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Scanning Electron Microscopic Observation on Chalkiness of Rice Mutant *OsLHT1* Grains

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Abstract

Grain chalkiness greatly affects the grain appearance，milling，eating，cooking，and nutritional qualities，so it is one of the most important traits of grain qualities. To study the relationship between chalkiness of mutant of rice *OsLHT1* gene and the shape, structure and arrangement of endosperm cells and starch grains, the chalkiness rate, chalkiness degree and chalkiness area of the mutant of rice *OsLHT1* gene were investigated by field experiment, and the morphological structure of rice endosperm cells and starch grains were also observed by scanning electron microscope. Our results showed that the grain chalkiness character with the greatest difference between the tested mutant of *OsLHT1* gene and the wild *japonica* rice variety Zhonghua 11 is chalkiness degree, followed by chalkiness rate, and finally chalkiness area, and there is a significant correlation between chalkiness rate and chalkiness degree. Therefore, there is a close correlation between the arrangement of endosperm cells, the distribution of starch grains and the occurrence of grain chalkiness in the mutant of *OsLHT1* gene in *japonica* rice.

Keywords: *OsLHT1* mutant, grain chalkiness, endosperm, starch granule, scanning electron microscope

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. More than half of the world's population and two thirds of China's population take rice as their staple food (Chen et al., 2019). For a long time, China's rice breeding has taken yield as the main breeding goal, and paid less attention to rice quality. However, with the improvement of people's living standards, the demand for high-quality rice is increasing. Therefore, cultivating new rice varieties with high quality has become one of the important objectives of current breeding work. The quality of rice mainly involves processing quality, appearance quality, cooking and eating quality and nutritional quality. Processing quality can be reflected by such indicators as brown rice rate, milled rice rate and whole milled rice rate (Zhang et al., 2018; Ding et al., 2020; Wang et al., 2018). The cooking and eating quality and nutritional quality of rice are closely related to the protein content of rice, and the cooking and eating quality determines the acceptance of rice by consumers (Zhang et al., 2017). The appearance quality is an important character index to measure the commercial value of rice, which is mainly reflected by rice grain size, length width ratio, transparency, chalkiness rate and chalkiness (Zhang et al., 2012; Cheng et al., 2000). Chalkiness not only affects the appearance quality of rice, but also affects the grinding and processing quality, nutritional quality, cooking and eating quality of rice, and even leads to the reduction of the final yield of rice (Peng et al., 2019). Therefore, chalkiness directly determines the quality of rice and the market price to a large extent.

Chalkiness is one of the most important appearance quality traits, and also one of the key factors affecting the cooking and eating quality of rice (Peng et al., 2018). Chalkiness refers to the loose arrangement of starch and protein particles in rice endosperm due to the rapid filling rate, and the formation of a large number of voids after drying and water loss, resulting in light scattering and the formation of white opaque parts, including abdominal white, back white and heart white. It is the quality index most affected by the environment (Chen et al., 2013; Li et al., 2014). Rice with chalkiness has poor transparency, low head rice rate and poor commodity quality [13]. Reducing chalkiness is a key problem to be solved in rice quality improvement. Different varieties and qualities of rice have their fixed starch grain morphology, and the quality of rice can be preliminarily determined by scanning electron microscopy (Peng et al., 2014), thus providing a basis for identification for breeding high-quality rice.

Studies have shown that rice chalkiness is a quantitative trait jointly controlled by multiple genes (Qiu et al., 2014)., with additive effect and interaction effect between additive effect and environment, and affected by external environmental factors (Lv et al., 2021; Peng et al., 2021). For example, high temperature induction, daily average temperature, temperature difference between day and night, sunshine duration, cultivation measures and other factors in the filling period will have different degrees of influence on the chalkiness character of rice (Bai et al., 2021; Zheng et al., 2022; Yan et al., 2021; Cheng et al., 2000; Abayawickrama et al., 2017). At present, a large number of genes affecting rice chalkiness have been isolated and cloned by using natural populations or mutants, such as gw2, chalk5, osppdkb, ssiiia, gif1, osrab5a, FLO2, osssi, flo4, flo15, etc. (Bahuguna et al., 2017; Deng et al., 2021; Deng et al., 2018; Lu et al., 2018; Sheng et al., 2015; Zhu et al., 2020; Ying et al., 2019). It can be roughly divided into three categories: one is the main gene for rice chalkiness, and currently only chalk5 gene (Guo et al., 2020). One is that genes controlling other traits can also affect chalkiness. Such genes are usually expressed as one cause and multiple effects, such as gw2 gene controlling grain width (Tsuyoshi et al., 2017). Another is that genes involved in starch synthesis, such as FLO2, flo4, flo15, etc., can also affect chalkiness (Wang et al., 2015; Zhou et al., 2015; Luo et al., 2021; Tabassum et al., 2020). Previous studies have shown that *OsLHT1* gene is a protein gene regulating histidine transport in rice. It is expressed in important growth stages of rice, and the expression level is highest in roots and old leaves at tillering stage. Loss of function of *OsLHT1* gene will inhibit the growth of rice roots and stems, leaves will appear obvious brown spots, plants will show signs of premature senescence, and the 1000 seed weight and seed setting rate will be significantly reduced (Zhu et al., 2018; Li et al., 2017; Hu et al., 2019). However, it is not clear whether *OsLHT1* gene affects rice quality traits, especially chalkiness. Therefore, in this study, rice *OsLHT1* mutants were used as experimental materials to detect and analyze the Chalkiness Characters in their grains, providing reference for further study on the function of *OsLHT1* gene and its potential application in the breeding of new rice varieties.

2. Materials and Methods

2.1 Test Materials

The tested material was rice mutant *OsLHT1*, and the corresponding parent material was Zhonghua 11 (zh11). The rice mutants were derived from the rice mutant library of Huazhong Agricultural University.

2.2 Test Method

2.2.1 Field Planting

In the summer of 2021, the mutant *OsLHT1* plants and zh11 were planted in the same experimental field of Xinyang Normal University. The mutants *OsLHT1* and zh11 were planted in two rows, 12 plants in each row, and the plant row spacing was 16.5 cm × 26.4 cm. From sowing to seed maturity, the tested materials were managed by conventional cultivation in the field. After the seeds were mature, they were naturally dried and stored at room temperature for 3 months, and then the chalkiness characters were tested.

2.2.2 Chalkiness Character Determination

The chalkiness rate, chalkiness area and chalkiness degree of Japonica rice varieties were determined and analyzed according to the national standard GB/t17891-1999. Take 100 full and complete milled rice randomly from the mutant *OsLHT1* and zh11, count the chalky rice grains, repeat for 3 times, and take the average value as the chalkiness rate. The belly white rate, the heart white rate and the back white rate were measured according to the method similar to the chalkiness rate, that is, 100 full and complete milled rice were randomly taken, and the rice grains with belly white and heart white were counted, and repeated three times. The average value was taken as the belly white rate and the heart white rate. Similarly, 10 chalky rice grains were randomly selected from the mutant *OsLHT1* and zh11, and the percentage of chalky rice grains in the whole rice grain area was estimated.

Repeat three times, and the average value was taken as the chalky area. Chalkiness = chalkiness rate × Chalky area.

2.2.3 SEM Observation

Chalky rice grains and non-chalky rice grains were randomly selected from each japonica rice variety, and the middle part of the rice was tapped with the back of the blade to make it break naturally. Then, the broken part was cut off with a knife and made into a sample about 2-3 mm thick. One part is observed by ordinary optical microscope, and the other part is adhered to the copper sample table with conductive adhesive. Hus-5gb vacuum coater is used to deposit gold on the section, and then the sample is placed under scanning electron microscope (Hitachi s-4800) to observe different parts of the rice grain section and take photos.

2.2.4 Identification of Mutants

T-DNA is a piece of mobile DNA located on the Ti plasmid of agrobacterium tumefaciens or Agrobacterium hairy. The position and direction of insertion of T-DNA into plant chromosomes are random. After the exogenous DNA is inserted into the target gene, the nucleotide composition of the target gene changes, so it can be used to identify the homozygosity and heterozygosity of T-DNA integration of mutant plants. Primer sequence: PCR reaction system: 10 × Amplification buffer, 200 umol/L of four dNTP mixtures, 10pmol of primers, 0.1^{-2} ug of template DNA, 2.5 ug of Taq DNA polymerase, 1.5 mmol/l of Mg^{2+}, and double distilled water to 100 ul Reaction conditions: 94 °C, 1 min, denatured to release the double chain. Subsequently, at 55 °C for 1 min, the primers were renatured and combined with the template. 72 °C, 1 min 30 sec, extended amplification.

2. Results and Analysis

2.1 Identification of Rice Mutant OsLHT1

T-DNA is a piece of mobile DNA located on the Ti plasmid of Agrobacterium tumefaciens or Agrobacterium hairy (Wang et al., 2003). PCR technology can be used to determine whether the mutant produced by T-DNA insertion into *OsLHT1* gene is homozygous or heterozygous. There are obvious bands in Figure a, no bands in Figure B, and no bands in Figure C. since there is no band when T-DNA is inserted into the upstream primer and no band when T-DNA is inserted into the downstream primer, it can be judged that the *OsLHT1* gene mutant is homozygous.

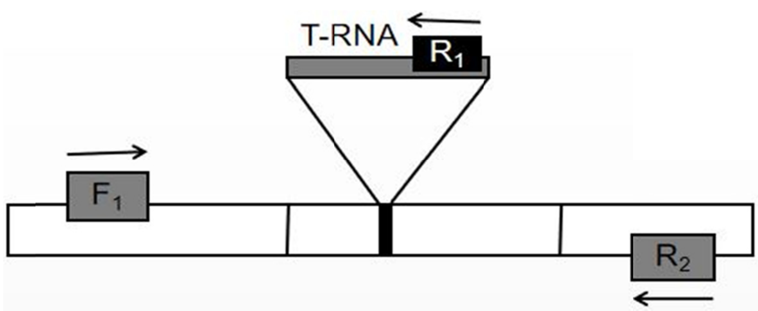

Figure 1. PCR identification of mutant *OsLHT1*

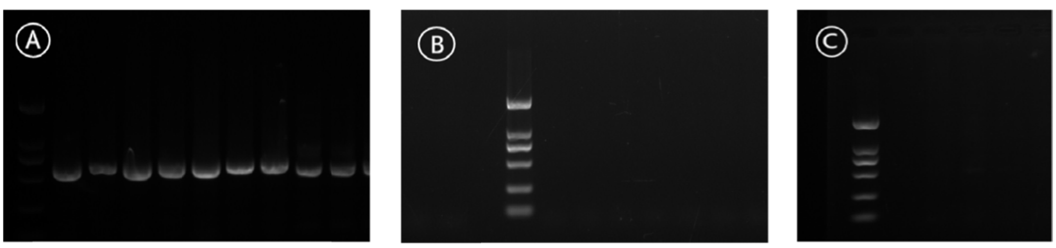

Figure 2. Judgment result of homozygote of *OsLHT1* gene mutant

Note. A: Upstream primers and T-DNA; B: Downstream primers and T-DNA; C: Upstream and downstream primer PCR.

2.2 Detection and Analysis of Grain Chalkiness of Mutant OsLHT1

The chalkiness character of mutant *OsLHT1* was significantly higher than that of wild-type zh11, the chalkiness degree reached 20.9%, the chalky grain rate reached 13.7%, and the chalky area reached 22.5%. The chalkiness degree and chalky grain rate were significantly higher than those of wild-type zh11, which were 90.0% and 98.5% of wild-type zh11, respectively. Chalky area was significantly higher than that of wild type zh11, which was 40.8% of that of wild type. After rice *OsLHT1* mutation, the amount of ventral White was significantly increased, and the amount of heart white was also significantly higher than that of wild-type zh11. The results showed that the mutation of rice *OsLHT1* gene led to a significant increase in the chalky area of rice, and the chalky grain rate was also much higher than that of wild-type zh11, thus the chalkiness degree was also significantly increased, which further indicated that there was an obvious correlation between chalkiness rate and chalkiness degree.

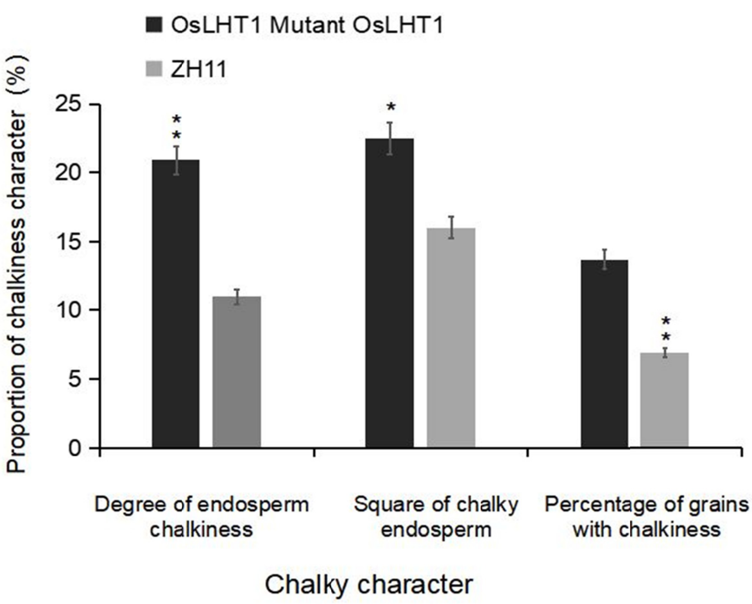

Figure 3. Measurement results of chalkiness characters of *japonica* rice variety Zhonghua 11 and mutant *OsLHT1*

Note. Significant differences at the levels of $^*P = 0.05$ and $^{**}P = 0.01$, respectively. All data are based on three biological replications and the significant differences are based on two-tailed *t*-test.

2.3 Endosperm Morphology of Mutant OsLHT1 Grain

Representative milled rice was selected to observe the endosperm morphology and structure of mutant *OsLHT1* by ordinary light microscope and electron microscope scanning from the aspects of the arrangement mode of rice endosperm cells, cell morphology, cell layers and the distribution of starch grains in the cross section. The results are shown in Figure 4. The arrangement of endosperm cells in the cross section can be divided into four categories. The first type shows obvious radiation from the middle of the cross section of rice endosperm to the periphery, and the radiation is long (Figures 4-A and 4-E). The second type is also radial from the middle of the cross-section to the surrounding, but the endosperm cells arranged radially are not straight, and some of them are curved and arranged together (Figures 4-B and 4-F). Although the third type radiates from the middle of the cross-section of rice endosperm to the periphery, the radiation is not obvious (Figures 4-C and 4-G). Type IV: no radial shape can be seen from the middle of the cross section of rice endosperm (Figures 4-D and 4-H). The chalkiness rate of mutant *OsLHT1* is generally high, and most of them are abdominal white. Compared with wild-type zh11, the arrangement of endosperm cells in the cross section of rice is mainly class II, and a few endosperm cells are class III. Under the scanning electron microscope, it can be seen that the mutant *OsLHT1*, which is dominated by abdominal white, has an irregular cell population and is surrounded by multilayered multilateral columnar cells (Figure 4-B). A small number of mutant *OsLHT1* with both heart white and abdominal white also have elliptical cell populations, and the periphery is multilateral columnar cells (Figure

4-C). The distribution of starch grains in the cross-section of rice was observed by electron microscope. It can be seen that the distribution of starch grains in chalkiness endosperm was obviously uneven, and the starch grains were mainly concentrated in the central part and the dorsal ventral diameter direction. The density of starch grains in endosperm was different with different chalkiness types. Compared with the wild-type zh11 with low chalkiness rate, the mutant *OsLHT1* was mainly ventral white, and the starch grains were mainly distributed in the ventral position of rice. A few mutant *OsLHT1* with both heart white and belly White had more and uneven starch grains in the belly and center of rice. The results showed that the endosperm cells of the mutant *OsLHT1* with high chalkiness rate were mainly arranged radially from the middle of the cross section to the periphery, and the starch grains were unevenly distributed, mainly concentrated in the center and the dorsal abdomen. The occurrence of chalkiness traits was mainly seen in this arrangement and distribution, which also indirectly proved that there was a certain correlation between the arrangement of endosperm cells and the distribution of starch grains and the occurrence of chalkiness traits.

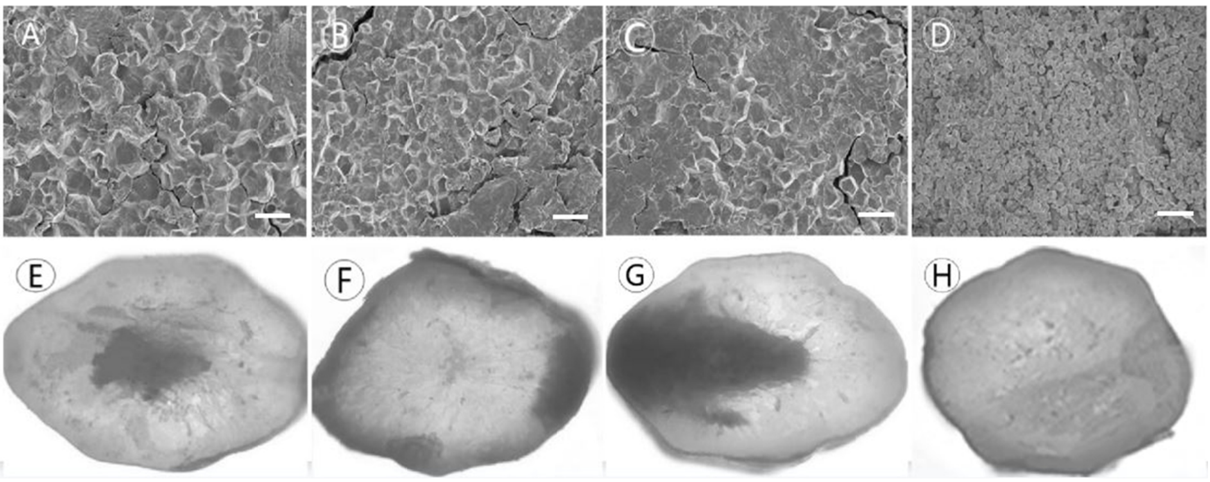

Figure 4. Morphological observation of upper endosperm cells in cross section

Note. A, E: ZH11 heart white; B, F: belly white of mutant *OsLHT1*; C, G: mutants *OsLHT1*, ZH11 with white background; D, H: ZH11 without chalkiness; A-D: Abdominal magnification of rice endosperm; Bar = 20 um.

2.4 Morphological Difference of Starch Grains Between Chalky and Transparent Parts of Chalky Rice of Mutant OsLHT1 Grain

The chalky and transparent parts of the chalky rice of the mutants *OsLHT1* and zh11 were respectively observed by scanning electron microscopy. The results showed that the starch grains in the chalky part of the mutant *OsLHT1* were spherical or ellipsoidal. Compared with the wild-type zh11 (Figure 5-A), the edges and corners of the starch grains were not obvious, the size of the starch grains was uneven, the diameter was small, and the starch grains were stacked irregularly. The gap between the starch grains became large and the arrangement was very loose, and almost all of them were single starch grains in free state (Figure 5-B). By observing the starch grains in the transparent part of mutant *OsLHT1* rice, it was found that there was no obvious difference between the starch grains of mutant *OsLHT1* and wild-type zh11 (Figure 5-C). Most of the starch grains had the same size and the shape was regular rhombus or isopolygon (Figure 5-D). The results showed that the starch grains of the chalky part of the mutant *OsLHT1* were loosely arranged, mostly round in shape, with obvious size difference and generally small in diameter. The shape of starch grains without chalkiness is polygonal, and the size is basically the same, and the arrangement is relatively tight. Therefore, it also showed that the formation of chalkiness was closely related to the shape and arrangement of starch grains in endosperm. The starch grains of the parts with low chalkiness developed well, whereas the starch grains of the parts with low chalkiness developed poorly.

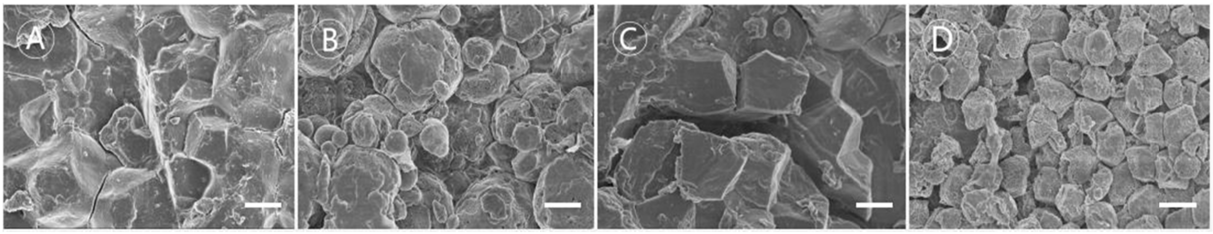

Figure 5. Scanning electron microscopic observation on starch granules in endosperm cells of the chalky rice

Note. A, D: The starch grains in the chalky rice; B, C: The starch grains in the transparent parts of rice; Bar = 4 μm.

2.5 Difference of Starch Grain Development in Different Parts of Chalky Rice of Mutant OsLHT1

According to the different positions of Chalkiness in rice endosperm, chalkiness can be divided into three types: belly white, heart white and back white. The scanning electron microscopic observation on the abdomen, center and back of chalky rice grains of the mutant *OsLHT1* and zh11 showed that the starch grains in the abdomen of the mutant *OsLHT1* endosperm were poorly developed, compared with the wild-type zh11 (Figure 6-2A and 2B) with good development in the middle of the endosperm, the starch grains in the center of the endosperm of the mutant *OsLHT1* had large gaps, loose arrangement and poor development, while the starch grains in the abdomen and back of the endosperm were well developed (Figures 6-1A and 1B). The results showed that the rice *OsLHT1* gene was mutated, the ventral White was significantly increased, and the arrangement of starch grains in the ventral was loose and irregular. The position of chalkiness was closely related to the arrangement of starch grains.

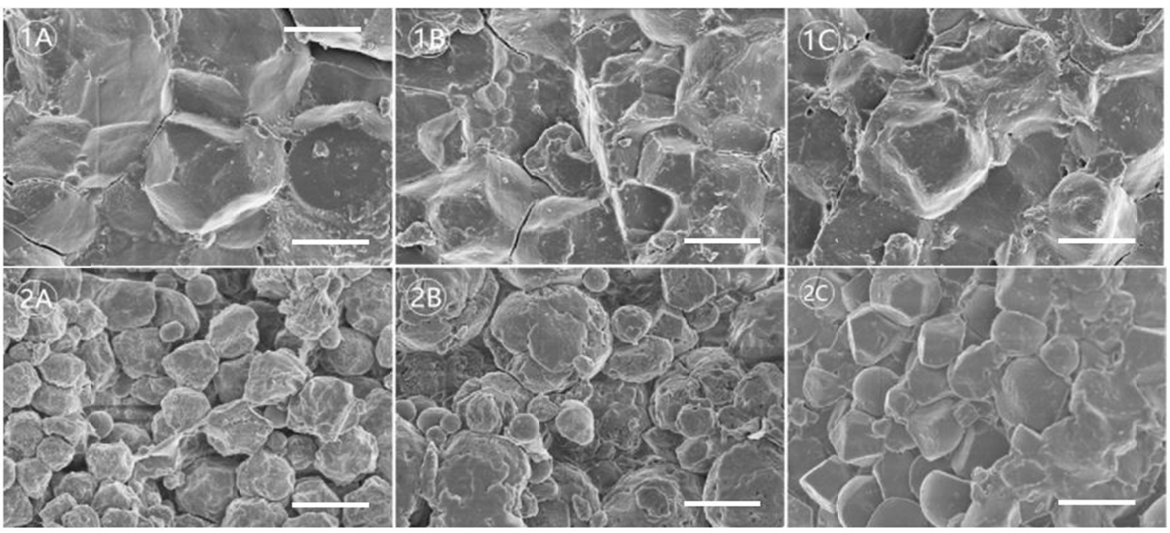

Figure 6. Scanning electron microscopic observation of starch grains in different parts of *japonica* rice variety Zhonghua 11 and mutant *OsLHT1*

Note. A, Starch grains in the center of the endosperm; B, Starch grains in the endosperm of the belly; C, Starch grains on the back of the endosperm; 1: ZH11; 2: Mutant *OsLHT1*; Bar = 5 μm.

3. Discussion

Chalkiness is a very important quality trait, because chalkiness not only affects the appearance quality of rice, but also affects the milling quality, nutritional quality, cooking and eating quality of rice. The morphological structure of starch grains in rice can be directly observed by scanning electron microscope, and the quality of rice can be preliminarily identified. According to the observation results of the endosperm cells of the mutant *OsLHT1* by scanning electron microscopy, after the mutation of the rice *OsLHT1* gene, the distribution of starch grains in its cross section was obviously uneven, especially in the central part of the endosperm. Previous studies have shown that if a single starch grain can achieve a composite starch grain with a certain geometric shape and orderly arrangement, chalkiness will not appear in this part [38-39]. Ellipsoidal or spherical starch grains cannot be arranged orderly in the chalky part, and the deposition is disordered. The deposition of such ordered and disordered starch grains is reflected in the appearance, that is, whether the rice endosperm is chalky and whether it is transparent. However, after the mutation of rice *OsLHT1* gene, the gap of starch grains increases, the arrangement is loose, and the development of starch grains is poor. Therefore, chalkiness characters are easy to be produced, and the chalkiness rate and chalkiness degree of the corresponding positions are also increased, which further indicates that there is a close relationship between the distribution of starch grains on the cross section of rice endosperm and the production of Chalkiness Characters.

By observing the chalkiness of the endosperm of the mutant *OsLHT1*, we found that after the mutation of the rice *OsLHT1* gene, the arrangement of the endosperm cells also changed from the central part to the surrounding, and the shape of the endosperm cells was mostly spherical, with different sizes. The ventral White was significantly increased, and most of the endosperm cells were arranged in this form, indicating that the arrangement of the endosperm cells had a certain correlation with the generation of the chalkiness of rice. Combined with our previous research results, it showed that the chalkiness of endosperm was mainly controlled by genetics. However, the occurrence of chalkiness is a complex process, and the formation of chalkiness may involve the relationship between "source", "sink" and "flow" in rice plants. Any change in any one of the three or the disharmony of their relationship may lead to or increase the formation of Chalkiness in rice endosperm (Jiang et al., 2018; Nevame et al., 2018; Huang et al., 2020).

According to the results of this study, we believe that the chalkiness degree, chalkiness rate and chalkiness area of rice increased significantly after the mutation of *OsLHT1* gene, and the increase of chalkiness degree and chalkiness area reached a very significant level. In the grain of the japonica rice mutant *OsLHT1*, the arrangement of endosperm cells showed a tendency of radiating from the center to the periphery, and the shape was mostly spherical, with different sizes. The distribution of starch grains is loose and irregular, thus forming a high chalkiness character. The results showed that the structure of endosperm cells and cross-sectional starch grains determined the appearance of chalkiness, and the endosperm structure was determined by the development of rice endosperm cells, all of which were controlled by genetic factors. Therefore, the occurrence of Chalkiness in rice endosperm is closely related to the distribution of starch grains in a certain position and the development of starch grains in that position. Thus, it will lay a foundation for studying the mechanism of chalkiness formation and cultivating high quality rice varieties in the future.

4. Conclusion

Chalkiness is one of the most important appearance quality traits, and also one of the key factors affecting the cooking and eating quality of rice. The chalkiness of rice is not only affected by environment, but also by genetic factors. In recent years, the development of rice functional genome has greatly promoted the research on the genetic mechanism of chalkiness and the mining of favorable genes. However, at present, the mechanism of Chalkiness of mutant *OsLHT1* and the relationship between gene and chalkiness are not clear. Through this experiment, it was found that the chalkiness rate, chalkiness degree and chalkiness area of rice *OsLHT1* gene were significantly higher than that of wild-type zh11, and the arrangement of starch grains was loose and irregular, and the starch development was also poor. Therefore, it can be speculated that *OsLHT1* gene mutation may lead to Chalkiness in rice. It also showed that the formation of Chalkiness in rice was closely related to the shape structure and arrangement of starch grains, so it could be used for the identification of rice quality.

Acknowledgements

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