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Scanning Electron Microscopic Observation of Endosperm Chalkiness Traits of *OsAAP1* Mutant in Rice Gains

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Abstract

In order to investigate the effect of *OsAAP1* gene mutation on chalkiness traits of rice endosperm and the microstructure of starch granules in endosperm, we used rice *OsAAP1* mutants as experimental materials, with the help of ordinary optical microscopes and scanning electron microscopes, the morphological structure and arrangement of chalkiness traits and starch granules in the endosperm of Zhonghua 11 rice were observed in rice *OsAAP1* mutants and controls. The results showed that the chalkiness rate, chalkiness area and chalkiness degree in rice endosperm of the *OsAAP1* mutants were significantly increased, and the morphological structure, arrangement and development of chalkiness traits and starch granules were closely related. Compared with the control Zhonghua 11, there were also significant differences in the shape, structure and arrangement of starch granules between chalky rice and non-chalky rice. However, there was no significant difference between the starch granules in the transparent part of chalky rice and non-chalky rice, and the starch granules in the areas with low chalkiness degree developed well. Thus, the *OsAAP1* mutation of rice could significantly increase the chalkiness traits of rice endosperm, and have an important impact on the morphology, structure and development of starch granules in its endosperm.

Keywords: *OsAAP1* gene, mutant, chalkiness trait, scanning electron microscopy, starch granule

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, with more than half of the world's population and more than two-thirds of China's population taking rice as their staple food (Kim et al., 2013; Tian et al.,2009). The world's annual rice planting area exceeds 15 million hectares, and the total output is nearly 60 million tons (Delseny et al., 2001). With the continuous improvement of people's living standards, consumers' demand for rice quality has also increased. The contradiction between the growing consumer demand and the relative lack of high-quality rice has become increasingly prominent (Peng et al., 2014; Fitzgerald et al., 2009; Peng et al., 2020). Rice quality is an extremely complex quantitative trait (Peng et al., 2020; Liu et al., 2010). It mainly consists of appearance quality, nutrition quality, processing quality, cooking and eating quality. The appearance quality of rice is mainly determined by grain shape or size, transparency and chalkiness (Zhang et al., 2007; Wan et al., 2008). Chalkiness of rice is the white opaque part formed by the loose arrangement of starch particles and protein bodies in endosperm during filling (Shi et al., 2002). According to the different positions of Chalkiness Characters on endosperm, it can be divided into belly white, back white and heart white. Chalkiness traits mainly refer to the presence or absence of chalkiness and the size of the occupied area in rice, which are usually expressed by 3 indicators, namely chalkiness rate, chalkiness area and chalkiness degree. Chalkiness rate refers to the percentage of chalky rice grains in the whole rice sample; Chalkiness area refers to the percentage of

chalky rice grains in the projected area of the whole grain when the chalky rice grains are laid flat; Chalkiness degree refers to the percentage of chalkiness area of chalky rice in the total area of rice grains in the sample (Liu et al., 2007). Chalkiness is an important indicator to evaluate the appearance quality of rice. Rice with chalkiness not only has poor appearance quality, but also is fragile during processing and fine milling. It has low amylose content, low head milled rice rate and high broken rice rate, which directly affects the commercialization and market value of rice. At the same time, the chalkiness rate and chalkiness area of rice not only affect its appearance quality, but also have a great impact on the processing quality, cooking and eating quality of rice. Therefore, analyzing the formation mechanism of chalkiness traits and reducing chalkiness traits of rice have important theoretical significance and application value for improving rice quality. It was previously reported that the chalkiness traits of rice were controlled by multiple genes (Peng et al., 2021; Tao et al., 2015; Qiu et al., 2015). there are additive effects and interaction effects between additive effects and environment, and they are affected by external environmental factors (Zhang et al., 2012; Liu et al., 2011; Li et al., 2014; Tsukaguchi et al., 2018; Peng et al., 2020; Mei et al., 2013; Yamakawa et al., 2008; Wan et al., 2005). At present, more than 80 rice chalkiness related QTLs or genes have been reported, of which *Chalk5* is the first gene cloned to control chalkiness traits in natural populations, and some are derived from previous research progress of genes cloned in mutants to control chalkiness traits, such as *GOTIB*, *Os-SMKI*, *FucT*, etc. *Chalk5* can increase chalkiness rate significantly (Li et al., 2011), especially increase the probability of rice belly white formation (Bai et al., 2021; Liu et al., 2021; Tang et al., 2022). The breakthrough of rice yield and the improvement of rice quality are closely related to the creation, exploration and utilization of rice mutants (Lv et al., 2021; Hu et al., 2021). The strategy of screening insertion mutations such as T-DNA insertion and transposon tag can accelerate the analysis of the genetic mechanism of important agronomic traits in rice. In this study, rice *OsAAP1* mutant and Zhonghua 11 were used as test materials to observe the chalkiness traits of rice *OsAAP1* mutant endosperm and the shape, structure and arrangement of starch granules in the mutant endosperm were also observed by scanning electron microscopy. Thus, our results will provide a theoretical basis for revealing the formation mechanism of chalkiness in rice and cultivating new rice varieties with high quality.

2. Materials and Methods

2.1 Test Materials

The test materials were the T-DNA insertion mutant (RMD mutant library) of rice *OsAAP1* gene and the corresponding parental control material Zhonghua 11.

2.2 Test Methods

2.2.1 Field Planting

On May 10, 2021, the test materials were sown in the rice experimental field of Xinyang Normal University. The mutant of rice *OsAAP1* gene and its control Zhonghua 11 were planted in 3 rows, 12 plants in each row, and the plant row spacing was 16.5 cm × 26.4 cm. From sowing, transplanting to the final maturity of seeds, rice materials are cultivated and managed in conventional fields. After the rice seeds are mature and harvested, they are dried naturally, threshed by a single thresher (5TS-150A), and placed at room temperature for 3 months before being tested.

2.2.2 Determination of Chalkiness Traits

Thresh the harvested rice *OsAAP1* mutant and Zhonghua 11, and then count the chalkiness rate and chalkiness area of the test materials after threshing. Calculate the chalkiness degree of rice *OsAAP1* mutant and Zhonghua 11 respectively according to the percentage of chalkiness area of chalkiness rice in the total area of rice grains in the sample.

2.2.3 Observation with Ordinary Light Microscope

Select 100 dried seeds, remove the rice husks, and randomly select 10 of them, including chalky rice, rice with white heart, white belly and white back, to make a sample with a thickness of about 0.2 mm; Place the sample material on the glass slide and observe it under the ordinary optical microscope.

2.2.4 SEM Observation

Fix the rice *OsAAP1* mutant of rice and the rice of Zhonghua 11 with tweezers, gently tap the back of the knife on the near middle of the rice grain to make it break naturally under pressure, and then cut the broken part with the knife to make a sample with a thickness of about 2 mm. Adhere it to the copper sample table with conductive adhesive, coat it with platinum on the Eiko-IB5 ion sputtering instrument, and place the sample under the S-4800 scanning electron microscope (Hitachi, Japan) to observe the back, abdomen and middle of the rice grain section,

Electron microscope images were obtained under high vacuum using an accelerating voltage of 30 kV.

3. Results and Analysis

3.1 Identification of OsAAP1 Mutant Genotype

T-DNA is a piece of mobile DNA located on the Ti plasmid of Agrobacterium tumefaciens or Agrobacterium hairy (Hu et al., 2011; Chen et al., 2012). *OsAAP1* genotype can be identified by PCR amplification using the combination of specific primers on T-DNA and primers upstream and downstream of *OsAAP1* gene. Wherein, the primer sequence upstream of *OsAAP1* gene (F): CGACCAACGTTTGATCATTC, *OsAAP1* gene downstream primer sequence (R): CAGAGCAAG AACCACTCCTT, T-DNAPrimer sequence （T-DNA）: AATCCAGATCCCCCGAATTA. The conditions of PCR reaction were as follows: pre-denaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 56℃ for 30~90 s, extension at 72℃ for 10 min after 32 cycles, and preservation at 4℃. Detection and analysis of the PCR products using 1.5% agarose gel electrophoresis. Electrophoretic detection of the mutant PCR product showed that after PCR reaction with primer f upstream of *OsAAP1* gene and primer combination of T-DNA, there was no target band in all lanes (Fig. 1-A); When the primer r downstream of *OsAAP1* gene was combined with T-DNA for PCR reaction, the target band appeared (Fig. 1-B); After PCR reaction with primer combinations upstream and downstream of the *OsAAP1* gene, there was no target band, that is, the obtained rice *OsAAP1* mutant was a homozygous genotype single plant.

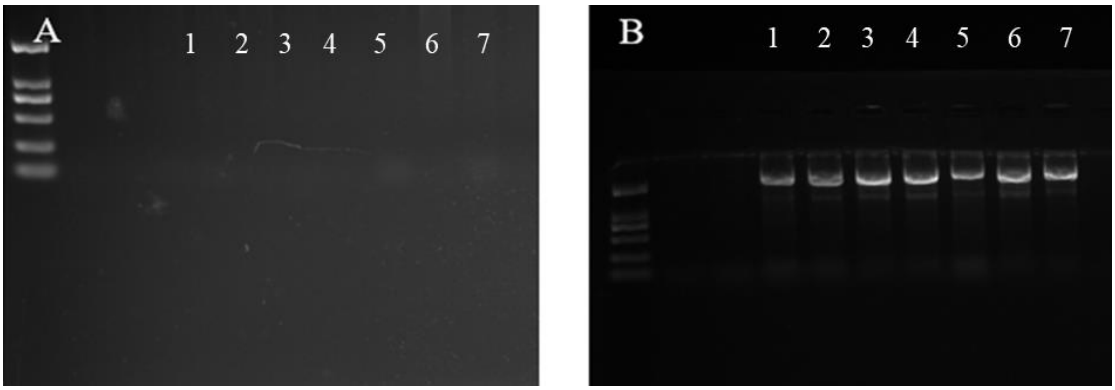

Figure. 1 Electrophoretic detection of PCR products of *OsAAP1* mutant

A: PCR products amplified by combination of upstream primer F and T-DNA primer; B: PCR products amplified by combination of downstream primer R and T-DNA primer; 1-7: Different individual plants of OsAAP1 mutant.

3.2 Detection of Chalkiness Characters of OsAAP1 Mutants

Compared with the control Zhonghua 11, the chalkiness degree was the largest difference among the *OsAAP1* mutants, followed by chalkiness degree, and the difference in chalkiness area also reached a significant level. The chalkiness rate of the *OsAAP1* mutant endosperm was 39.7%, which was significantly higher than that of the control Zhonghua 11 (6.9%); Chalkiness degree in endosperm of *OsAAP1* mutant was significantly higher than that of Zhonghua 11, reaching 8.6% ($P < 0.01$); The chalkiness area of the *OsAAP1* mutant was 21.8%, while that of Zhonghua 11 was 16.0% (Fig. 2). Compared with the control Zhonghua 11, the chalkiness area, chalkiness degree and chalkiness rate in the endosperm of rice *OsAAP1* mutant were significantly increased, that is, the mutation of rice *OsAAP1* gene can significantly increase the chalkiness character of rice.

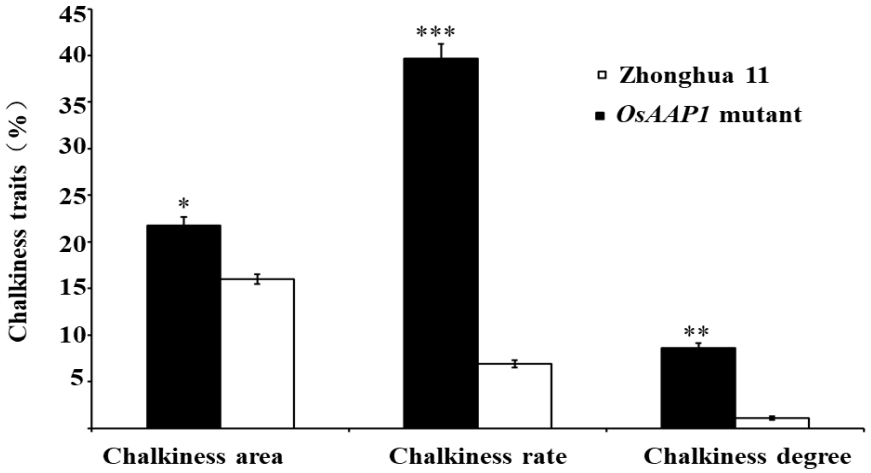

Figure 2. Statistics of chalkiness traits in endosperm of *OsAAP1* rice mutant

The data are based on three biological replications, error bars, s.e.m. Significant differences **P*<0.05, ***P*<0.01, *** *P*<0.001. Significant differences are based on two-tailed *t*-test.

3.3 Observation on Starch Granules of OsAAP1 Mutants

The cross-sectional starch granules of rice *OsAAP1* mutant were observed with the help of an ordinary light microscope. The results showed that among the rice *OsAAP1* mutant, there was also rice without chalkiness (Fig. 3-A); In *OsAAP1* mutant rice with chalkiness, there are both heart white (Fig. 3-B) and belly white (Fig. 3-C). Compared with Zhonghua 11, the parent material of the *OsAAP1* mutant, in the heart white part of the endosperm of the *OsAAP1* mutant, its Chalkiness granule have a tendency to emit and diffuse around (FIGS. 3-B and 3-E); In the ventral white part of the endosperm of the *OsAAP1* mutant, the ventral white area is significantly larger than that of Zhonghua 11 (Fig. 3-C and 3-F, which is consistent with the statistical result that the chalky area of the endosperm of the *OsAAP1* mutant is significantly higher than that of Zhonghua 11 (Fig. 2), that is, the increase of the chalky area of the endosperm of the *OsAAP1* mutant is mainly caused by the obvious increase of the ventral white area. It is worth noting that compared with the chalky free part of hua11 in the control, the chalky free part of the *OsAAP1* mutant of rice has basically the same starch granule distribution and arrangement, that is, the starch granules on rice are evenly distributed and relatively large in density, and both are well developed (Fig. 3-A and 3-D). The starch granules in the endosperm of *OsAAP1* mutant, which is mainly composed of heart white and belly white, are unevenly and obviously clustered in a certain part, and the aggregation range and dispersion part of starch granules are larger than that of the heart white and belly white starch granules corresponding to Hua 11 in the control. For example, the aggregation range of starch granules in the ventral white part of *OsAAP1* mutant obviously occupies a larger proportion than that of Zhonghua 11 (Fig. 3-C and 3-F), which makes the distribution of starch granules uneven, and is also the reason why the density of mutant endosperm is less than that of Zhonghua 11 in the control. Therefore, the uneven distribution and density change of starch granules on the cross section of endosperm of *OsAAP1* mutant directly affect the chalkiness character of rice; The differences in the distribution of starch granules in the cross-section of rice make it related to the formation of chalkiness.

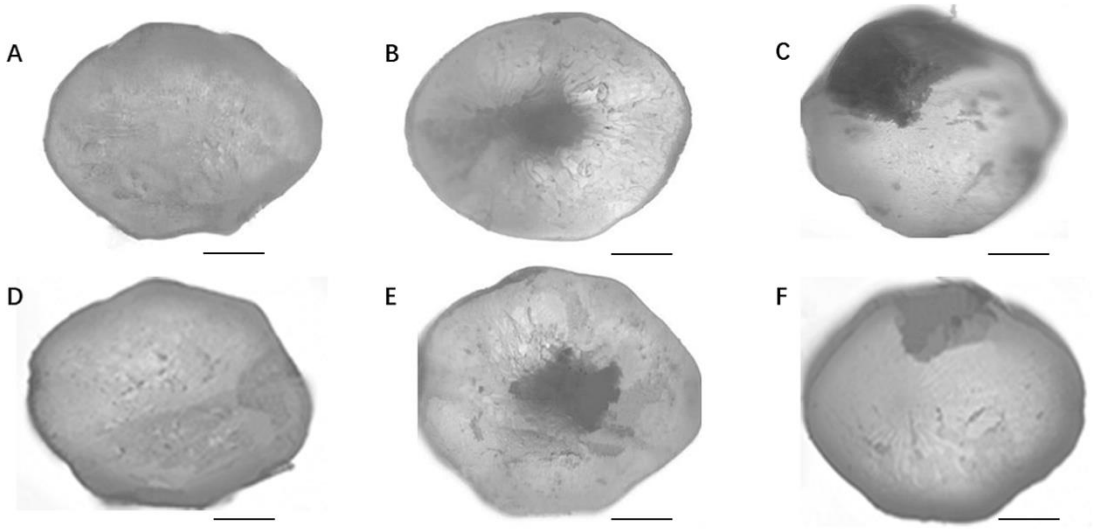

Figure 3. Distribution of starch granules on endosperm cross-section of rice

A-C: *OsAAP1* mutant endosperm with non-chalky, white core and belly white; D-F: Zhonghua 11 endosperm with non-chalky, white core and belly white; Bars=200 μm

3.4 Morphological Difference of Starch Granules Between OsAAP1 Mutant Chalky Rice and Non-chalky Rice

In order to further explore the microstructure of the starch granules of chalky rice and non chalky rice in rice *OsAAP1* mutant, the starch granules of rice *OsAAP1* mutant, chalky rice and non chalky rice were observed by scanning electron microscope. The results showed that there were significant differences in the arrangement of starch granules and the grain type of starch granules in the endosperm cross section of rice *OsAAP1* mutant (Fig. 4). In terms of starch granule morphology, the starch granules in chalky rice are spherical, elliptical and irregular polygonal. Especially in the chalky part of chalky rice (Fig. 4-A), the shape of starch granules is mainly composed of ellipses and circles; Compared with the starch granules in the transparent part of *OsAAP1* mutant chalky rice (Fig. 4-B), the arrangement of starch granules in the chalky part is more loose than that in the transparent part; Compared with the starch granules of non chalky rice composed of polygons (Fig. 4-C), the structural characteristics of starch granules with chalky parts are loose and not compact, while those without chalky starch granules are relatively compact. It is worth noting that the starch granules in the transparent part of the endosperm chalky rice of *OsAAP1* mutant are in the middle of the two (no chalky rice and chalky rice), and are composed of ellipses, circles and irregular polygons. Therefore, the formation of chalkiness in *OsAAP1* mutant rice is closely related to the shape and structure of starch granules.

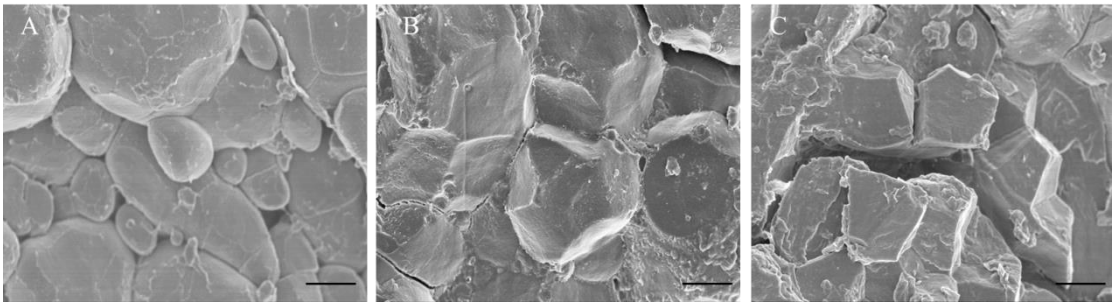

Figure 4. Electron microscopic observation of starch granules in chalky rice and non-chalky rice of *OsAAP1* mutant

A: Starch granules in chalky part of chalky rice; B: Starch granules in transparent part of chalky rice; C: Starch granules of non-chalky rice; Bar=10 μm

3.5 Observation on Starch Granule Development in Different Parts of Chalky Rice in OsAAP1 Mutant Grains

According to the different positions of chalkiness formation in rice endosperm, chalkiness traits can be divided into three types: heart chalkiness, belly chalkiness and back chalkiness. Scanning electron microscopic observation of different parts of rice *OsAAP1* mutant chalky rice showed that there were mainly four states. The first type is rice whose starch granules are well developed in the center, abdomen and back of the endosperm, that is, no loose starch granules are found in the endosperm (Fig. 5-1). When observed under scanning electron microscope magnification of 1000 times, 2000 times and 5000 times, the starch granules are rhombic or equi polygonal, with small or no gaps, and are closely arranged and relatively regular (Fig. 5-1). The second state is that the gap between starch granules and starch granules in the endosperm center of rice is large. Under the scanning electron microscope, it is observed that the starch granules in the chalky part of the chalky rice center are spherical or ellipsoidal, with large difference in size and irregular arrangement. Moreover, the arrangement of starch granules is loose, and the development of starch granules is relatively poor (Fig. 5-2), but the development of starch granules in the abdomen of the endosperm is better; The third state is that the abdominal starch granules of rice endosperm are spherical or ellipsoidal, with large differences in size, irregular and loose arrangement and poor development, but the starch granules in the center and back of endosperm are relatively well developed (Fig. 5-3); The fourth state is that the starch granules in the endosperm center and abdomen of rice are loosely arranged and poorly developed (Fig. 5-4). Through observation under scanning electron microscope of different multiples, it can be seen that the starch granules in the non chalky part of rice *OsAAP1* mutant of rice are rhombus or isopolygon, with small or no gaps, closely and regularly arranged, and the starch granules are well developed, which corresponds to the good appearance quality of rice; For chalky rice, the starch granules in the chalky part are spherical or ellipsoidal, with large difference in size, irregular and loose arrangement, poor development of starch granules, and unsatisfactory appearance quality of corresponding rice. Therefore, the formation of chalkiness in endosperm of *OsAAP1* mutant is closely related to the development of starch granules in different parts.

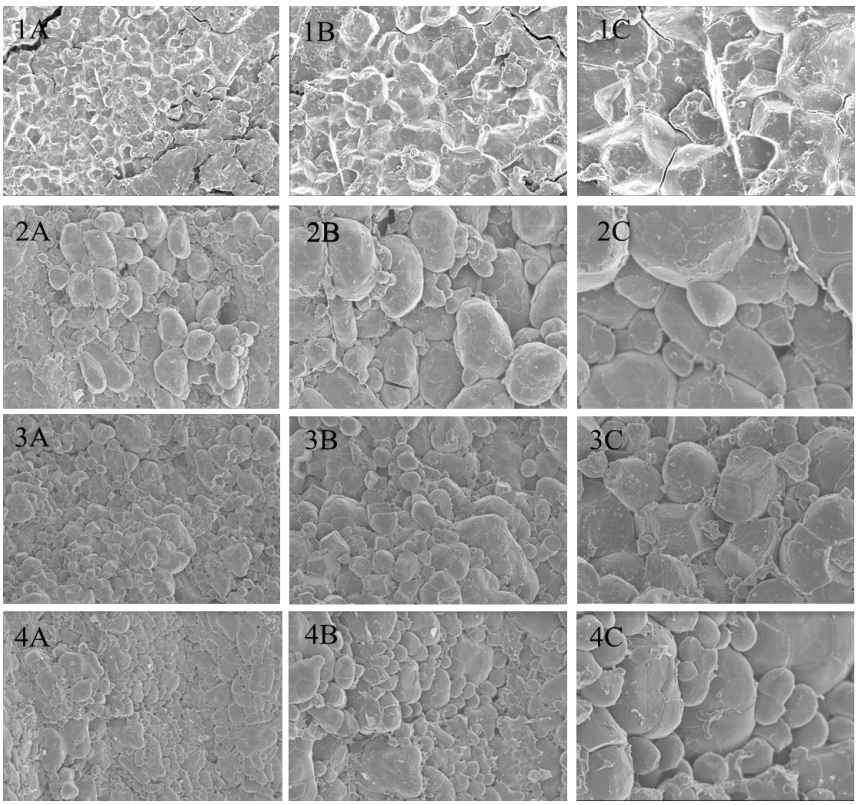

Figure 5. Scanning electron microscopic observation of starch granules in different parts of *OsAAP1* mutant in rice

1: Starch granules with non-chalky in endosperm; 2: Starch granules in the center of endosperm; 3: Starch granules in the abdomen of endosperm; 4: Starch granules on the back of endosperm; A-C: Scanning electron microscope magnified 1000 times, 2000 times and 5000 times

4. Discussion

4.1 Chalkiness and Starch Granule Distribution of Rice OsAAP1 Endosperm

Chalkiness is an important quality trait of rice, which not only affects the appearance quality and processing quality of rice, but also affects the cooking and eating quality of rice. The morphological structure of starch granules in rice endosperm can be directly observed by scanning electron microscopy, and the appearance quality of rice can be preliminarily identified (Liang et al., 1996; Kang et al., 2007; Chang et al., 2006). The chalkiness study preliminarily observed the endosperm of the *OsAAP1* mutant of rice by scanning electron microscopy. It was found that the starch granules in the cross section of the endosperm of rice with high chalkiness rate and chalkiness degree were obviously uneven; The starch granules are spherical or ellipsoidal in shape, with large difference in size, uneven diameter, irregular arrangement and loose arrangement. However, the starch granules without chalkiness or at positions where chalkiness is not obvious are rhombus or equipolygon in shape, with small or no gaps between the starch granules, which are arranged tightly and regularly. That is, the chalkiness character of the *OsAAP1* mutant of rice is closely related to the distribution of starch granules, especially with the morphological structure of starch granules in the cross section of rice. The corresponding chalkiness rate and chalkiness degree of the parts with more uniform distribution of starch granules in the cross section are low, so it is not easy to form chalkiness characters; If the starch granules on the cross section of rice endosperm are unevenly distributed, with large gaps and loose arrangement, the corresponding chalkiness rate and chalkiness area are higher, which is easy to form chalkiness traits. That is rice *OsAAP1* gene not only affects rice yield (Ji et al., 2020), but also *OsAAP1* mutant leads to a significant increase in chalkiness rate, chalkiness area and chalkiness degree of its rice endosperm (Fig. 2). The morphology and structure of starch granule distribution and arrangement in rice are closely related to its chalkiness traits (Fig. 3-4). Therefore, in the process of rice breeding, the appearance quality of rice can be improved by improving the *OsAAP1* gene.

4.2 Chalkiness and Starch Granule Development of Rice OsAAP1 Endosperm

With the increase of amylose content in rice, endosperm can form starch granules with different shapes. The composition, crystal structure and physical and chemical characteristics of these starch granules are obviously different, and they are distributed regionally in endosperm. Chalkiness is the white and opaque part of rice endosperm, which can be divided into belly white, heart white and back white according to its occurrence location (Hu et al., 2018). In the *OsAAP1* mutant of rice, both ventral white and heart white were observed in the endosperm. The significant increase in chalkiness area was mainly derived from the obvious increase in ventral white area in the *OsAAP1* mutant (Fig. 2-3). Some studies have shown that in cereal seeds, the content of resistant starch is positively correlated with the content of amylose, and the production of chalkiness is closely related to the development status of its seeds (Avella et al., 2002). Through scanning electron microscopic observation of different parts of rice *OsAAP1* mutant chalky rice, it was found that there were four different states of starch in its endosperm (Fig. 5): starch granules were closely arranged with starch granules, evenly distributed and well developed, and no chalkiness character appeared in its endosperm; If the distribution of starch granules is uneven, the gap between starch granules is large, and the arrangement is loose, the corresponding chalkiness rate and chalkiness area are high. If this part is poorly developed, it is easy to form chalkiness characters. Therefore, in the endosperm of rice *OsAAP1* mutant, there is an important relationship between the development of starch granules and the occurrence of chalkiness.

4.3 Reasons for Chalkiness Traits

Starch and protein are the main storage materials in rice endosperm, and the relative content of starch and protein affects the formation of chalkiness in rice (Rao et al., 2021). The chalkiness character of rice is a complex quantitative character. Its inheritance has maternal effect, endosperm effect and cytoplasmic effect, and is controlled by multiple genes, which is vulnerable to environmental factors (Lin et al., 2015; Zhou et al., 2009; Rao et al., 2020). At present, there are two main views on the mechanism of chalkiness formation: cell theory and assimilate flow path theory. Among them, the cell theory holds that the formation of chalkiness in rice is mainly due to the fullness but not compactness of endosperm starch granules. The fullness and not compactness of spherical or elliptical starch granules make the cells of endosperm starch granules have more gaps, which scatter when light is irradiated to form chalkiness. The theory of assimilated product flow and economics holds that the formation of rice endosperm is the process of organic assimilates produced by functional leaves being transported from the transport organization to the grain. The ability of functional leaves to produce substances through photosynthesis, the transport ability of assimilated substance transport organization and the size of the grain jointly restrict the accumulation of organic assimilates in the grain (Zhu et al., 2011). Regional and altitude conditions, meteorological conditions, soil conditions, fertilizer and water conditions, high concentration of CO_2,

high concentration of O_3 and high temperature all have certain effects on the formation of chalkiness (Wang et al., 2006; Zhu et al., 2020). That is, chalkiness is not only affected by internal factors, but also by external factors (Shi et al., 2020). It is an important direction of quality breeding in the future to explore how different factors affect the formation of chalkiness and reduce chalkiness, chalkiness rate and chalkiness degree by reducing chalkiness (Ruan et al., 2021; Zheng et al., 2022). The chalkiness character in the endosperm of the *OsAAP1* mutant of rice was significantly increased (Fig. 2). Further observation by scanning electron microscopy revealed that the size, distribution and arrangement of starch granules in the chalkiness part had changed significantly (Fig. 4), and the development status of starch granules in the white part was poor (Fig. 5), which revealed the cause of the chalkiness character in the endosperm of the *OsAAP1* mutant from a microscopic perspective. Whether the external environment and other factors also affect the chalkiness character of the endosperm of *OsAAP1*mutant needs to be further verified in the later stage.

5. Conclusion

The chalkiness rate, chalkiness area and chalkiness degree in rice endosperm of *OsAAP1* mutant were significantly increased; It was found that the chalkiness of *OsAAP1* mutant was closely related to the morphology, arrangement and development of starch granules in rice endosperm by scanning electron microscopy. That is, the mutation of *OsAAP1* gene in rice has an important adverse effect on the morphological structure and development of starch granules in rice endosperm, resulting in the emergence of chalkiness.

Acknowledgments

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