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OsAAP3 Gene Mutation Promotes the Chalkiness Character in Rice

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Abstract

Rice chalkiness is one of the most important appearance quality traits and has a great impact on its processing quality, nutritional quality, cooking, and eating quality traits. It has important theoretical significance and application value to clarify the genetic mechanism of rice chalkiness. To reveal the relationship between chalkiness of OsAAP3 mutant rice and the shape, structure and arrangement of endosperm cells and starch grains, which will lay a foundation for further research on the formation mechanism of chalkiness character of rice and the cultivation of new high-quality rice varieties. The results showed that the chalkiness, chalkiness rate, and chalkiness area of *OsAAP3* mutant rice significantly increased compared with the control ZH11, and there were significant variations in the arrangement, morphology, and cross-section starch grain distribution of the endosperm cells of the mutant rice: the white heart area of the mutant increased, and the white belly almost distributed in the edge of the whole rice cross-section. The chalky part and transparent part of the *OsAAP3* mutant chalky rice also have obvious variations in the shape, structure, and arrangement of starch grains, but there is no significant difference between the transparent part of the mutant chalky rice and the starch grains of the non-chalky rice. The starch grains in the endosperm center of the *OsAAP3* mutant of rice are well developed, but the starch grains in the abdomen and back are loosely arranged, and the starch grains are poorly developed. Therefore, the chalkiness character of *OsAAP3* mutant rice increased significantly. The chalkiness character of its mutant mainly occurred in the abdomen, and the rest occurred in the center of the endosperm of rice. Moreover, the arrangement of starch grains in *OsAAP3* mutant rice was loose, the grain shape was mostly irregular polyhedrons, and the starch development was poor.

Keywords: *OsAAP3* mutant; Chalkiness; Endosperm; Starch granules; 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 our population live on rice. At the same time, rice is also a model organism for crop genetic improvement and functional genomics research [1-3]. With the continuous improvement of people's living standards, high-quality rice is increasingly favored by consumers [4, 5]. However, rice quality character is a relatively complex biological character. It is generally believed that rice quality can be divided into four aspects: appearance quality, nutrition quality, grinding quality, and cooking and eating quality [6, 7]. The appearance quality is mainly composed of chalkiness character, grain type, and transparency, and the chalkiness character is mainly measured by chalkiness rate, chalkiness area, and chalkiness degree [8, 9]. Rice chalkiness refers to the white and opaque part of the rice endosperm. It is the white and opaque part formed by the loose and irregular arrangement of starch grains due to the influence on the development of starch grains in rice endosperm during filling [5, 10, 11]. Chalky rice not only affects appearance quality, but also is fragile during fine milling, with low head rice rate and high broken rice rate, thus affecting rice yield; There are many cracks in the cooked rice grains, and the rice is fluffy and hollow, which seriously affects the cooking and eating quality of rice [12, 13]. Therefore, the causes and influencing factors of rice chalkiness, as well as the technical measures to reduce rice chalkiness, have been a focus of rice quality genetic improvement.

Chalkiness in rice is a quantitative trait controlled by multiple genes. Its formation is not only controlled by genetic factors, but also affected by many other factors, such as starch synthesis, starch granule structure and arrangement, and external environmental factors [12, 14, 15]. At present, a large number of genes affecting rice chalkiness have been isolated and cloned from natural populations or mutants of rice, such as *OsPPDKB*, *GW2*, *flo2*, *OsRab5a*, and *Chalk5* [16-20]. Rice may mutate not only in the natural environment but also by artificial induction. Mutants are materials that can be inherited and mutated for certain traits. Several breakthroughs in rice yield in history are closely related to the discovery and utilization of mutants [21]. From the identification and utilization of semi-dwarf gene *sd1* and cytoplasmic male sterile gene variants, the second leap in rice yield has been achieved; the discovery of photo (temperature) sensitive genic male sterile gene, realizing the transformation of hybrid rice production from three lines to two lines [22]. Since the 1970s, artificial mutants created by Y-ray and chemical mutagenesis have been applied in plant genetics and breeding, and the later development of T-DNA insertion, transposon tagging, and other methods of insertion mutation and selection of mutants has greatly accelerated the pace of mutant creation [23]. Previous studies have shown that the *OsAAP3* gene belongs to the *AAP* family of *OsAATs*, located on chromosome 6, with high expression in leaves, followed by seeds and roots, and the lowest expression in stems; The expression effect of the *OsAAP3* gene is the same at different stages of ear development, but there are differences in the expression effect of *OsAAP3* gene at different stages of seed development. The expression amount at the middle stage of seed development is higher than that at the early and late stages of seed development [24]. The high expression of the *OsAAP3* gene in rice plants can improve its grain yield [25], that is, the *OsAAP3* gene is closely related to rice yield.

At present, it is not clear whether the *OsAAP3* gene is related to rice quality, especially the chalkiness character of rice grains. However, the chalkiness character in the seeds of ZH11 and its *OsAAP3* mutant is significantly different, which suggests that the *OsAAP3* gene may be related to the chalkiness character in rice seeds. In this study, the chalkiness characters in the seeds of ZH11 and its *OsAAP3* mutant were compared and analyzed, providing a reference for further research on the chalkiness characters of the mutant *OsAAP3* and the cultivation of new rice varieties. Therefore, by studying the formation of the chalkiness character of rice mutant *OsAAP3* and the morphology, structure, arrangement, and development of endosperm cells and starch grains, this study will lay a solid foundation for further in-depth research on the formation mechanism of chalkiness character of mutant *OsAAP3* and the cultivation of new rice varieties with good quality.

2. Materials and Methods

2.1. Test Materials

The test material is rice *OsAAP3*mutant, and the corresponding parent material is ZH11. Rice mutants come from the Rice Mutant Bank of Huazhong Agricultural University.

2.2. Test Method

2.2.1. Field Planting

In the summer of 2021, the rice *OsAAP3* mutant plant and ZH11 will be sown in the same experimental field in the rice experimental base of Xinyang Normal University. The mutant *OsAAP3* plant and ZH11 will be planted in 2 rows, 10 plants in each row, with a row spacing of 16.5 cm × 26.4 cm. The test materials were conventionally cultivated and managed in the field from sowing to seed maturity. After the seeds were matured, they were naturally dried and stored at room temperature for 3 months before being tested for chalkiness.

2.2.2. Determination of Chalkiness Character

The chalkiness grain rate, chalkiness area, and chalkiness degree of rice are determined according to the national standard GB/T17891-1999: 100 complete milled rice of each variety are randomly selected, and the number of chalkiness grains is calculated. Repeat 3 times, the average value is the chalkiness grain rate. The rate of abdominal and white heart granules was determined by the same method. Take 10 chalky rice grains randomly from *OsAAP3* mutant and ZH11, estimate the percentage of the chalky part of each grain in the whole area, repeat 3 times, and take the average value as the chalky area. Chalkiness=chalky grain rate × chalky area. Finally, SPSS 20.0 was used for statistical analysis of the measured data.

2.2.3. Scanning Electron Microscope Observation

Select chalky rice and non-chalky rice randomly from the two varieties, tap the middle of the rice with the back of the blade to make it naturally break, and then cut off the broken part with a knife to make a sample of 2-3 mm thick. One part was observed with an ordinary optical microscope, and the other part has adhered to a copper sample table with conductive adhesive. The section of the rice grain was gold plated with a HUS-5GB vacuum coater, and then different parts of the section of the rice grain were observed and photographed under a scanning electron microscope (Hitachi S-4800).

2.2.4. Identification of *OsAAP3* Mutants

The T-DNA primer (P: AATCCAGATCCCCGAATTA) was used to design and amplify the upstream primer (F: 5 '- TTCTCCATGCTGC CCAACTT-3') and the downstream primer (R: 5 '- ACCTGATGTTCG CGCA-3') to identify the genotype (heterozygous and homozygous) of the inserted mutant. PCR reaction system includes DNA template 2 μL, 10 x Buffer 2.5 μL. 25 mmol · L-1 $MgCl_2$ 1.5 μL. dNTP 2.0 of 2.5 mmol · L^{-1} μL. 1.0 for upstream primer and 1.0 for downstream primer μL, 5 U· μ Taq enzyme 0.4 of l-1 μL. Supplement sterile water to 20 μL。PCR reaction conditions were 94 ℃ pre-denaturation for 5 min, 94 ℃ denaturation for 30 s, 55~61 ℃ annealing for 30 s, 72 ℃ extension for 30~90 s, 32 cycles of reaction followed by 72 ℃ extension for 10 min, and 25 ℃ storage.

3. Results and Analysis

3.1. Identification of *OsAAP3* Mutant in Rice

T-DNA is a piece of movable DNA located on the Ti plasmid of Agrobacterium tumefaciens or Agrobacterium tumefaciens [26, 27]. The combination PCR technology between specific primers on T-DNA and the upstream and downstream primers of the *OsAAP3* gene can be used to determine whether the *OsAAP3* gene mutant is homozygous, heterozygous, or wild type (Fig. 1). The mutant *OsAAP3* plants were detected by electrophoresis after PCR amplification, and the electrophoresis results were as follows: when the upstream primer of *OsAAP3* gene was combined with T-DNA primer for PCR reaction, it was found that all swimming lanes had target bands (Fig. 2-A); After PCR reaction of upstream and downstream primers, it can be seen that all swimming lanes have no target bands (Fig. 2-B); however, when the downstream primer of *OsAAP3* gene combines with T-DNA primer for PCR reaction, all swimming lanes have no bands (Fig. 2-C), that is, *OsAAP3* plants obtained are homozygous mutants.

Fig-1. Identification diagram of T-DNA insertion mutants in rice

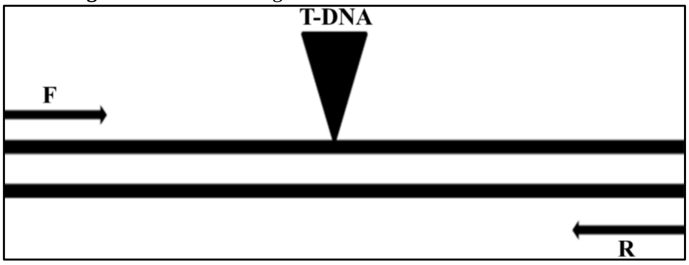


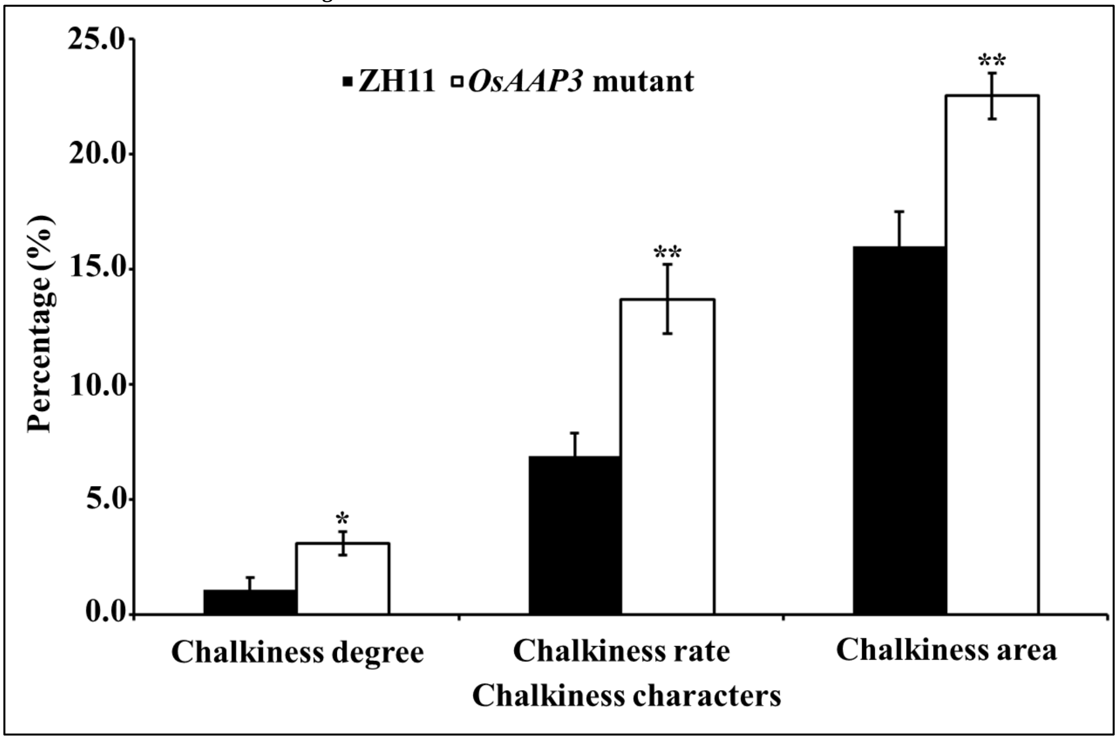
Fig-2. Identification of *OsAAP3* mutant in rice

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

3.2. The Difference and Analysis Results of Chalkiness Characters Between ZH11 and *OsAAP3* Mutant

The chalkiness, chalkiness rate, and chalkiness area of *OsAAP3* mutant in rice are higher than those of ZH11. The chalkiness character with the largest difference is the chalkiness rate, followed by chalkiness. The chalkiness area of the mutant *OsAAP3* is also significantly different from that of ZH11 (Fig. 3). The chalkiness of the mutant *OsAAP3* was 3.1%, while that of the controlling parent ZH11 was only 1.1%. In terms of the chalkiness rate of rice seeds, the mutant *OsAAP3* is 6.8% higher than ZH11, the mutant *OsAAP3* is 13.7%, and ZH11 is 6.9%. The chalkiness area of the mutant *OsAAP3* is 6.5% higher than that of ZH11, the mutant is 22.5%, and the chalkiness area of ZH11 is 16.0%. Compared with ZH11, chalkiness of the mutant *OsAAP3* mainly occurs in the belly of rice seed endosperm, and the rest occurs in the center of rice seed endosperm. Through further analysis, it was found that there was a clear correlation between the chalkiness rate and the chalkiness degree in rice seeds. The chalkiness rate in the seeds of the mutant *OsAAP3* was higher, and its corresponding chalkiness degree was also higher.

Fig-3. Detection of chalkiness in rice *OsAAP3* mutant seeds

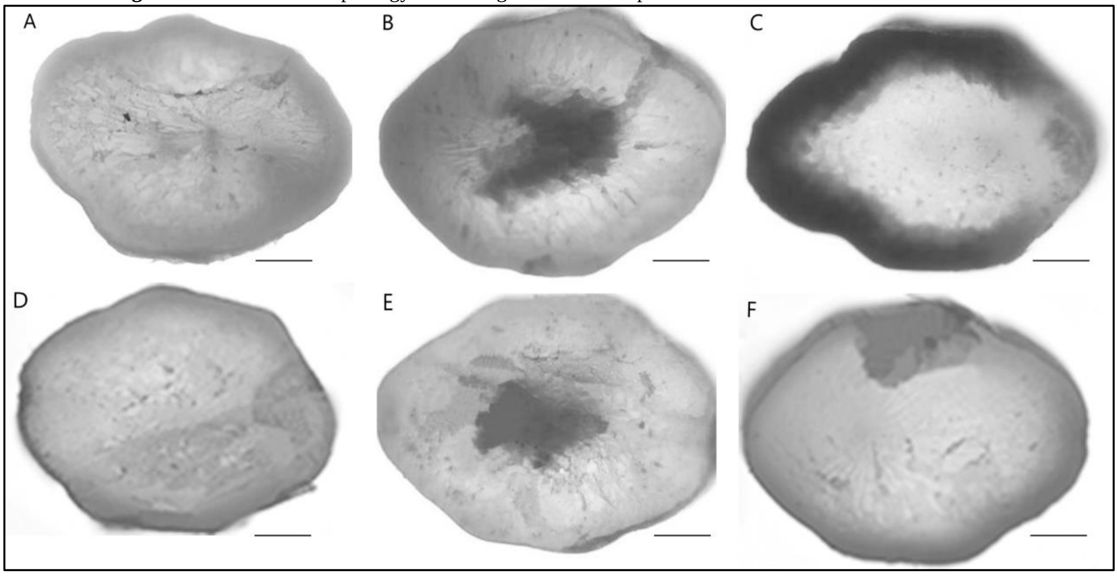


The data are based on three biological replications, error bars, s.e.m. Significant differences **P*<0.05, ***P*<0.01. Significant differences are based on two-tailed *t*-test.

3.3. Endosperm Morphology and Structure of *OsAAP3* Mutant

Chalkiness refers to the opaque part of rice endosperm, which has a very significant negative correlation with transparency. Chalkiness is opaque because its starch grains are loosely arranged, and air inflation between grains causes light refraction. Chalkiness can be divided into white belly, white heart, white back, and other types according to its location [28, 29]. White heart may be the result of incomplete development of endosperm cells in the heart at the early grain-filling stage, while the formation of white belly may mainly occur in the middle and late grain-filling stage, which is the result of insufficient sucrose supply [30]. The typical millet was selected from the mutant *OsAAP3* and its parent ZH11, and the arrangement and morphology of endosperm cells and the distribution of starch grains in the cross-section were observed under an ordinary light microscope. The results showed that the chalky endosperm of the mutant *OsAAP3* was roughly the same as that of ZH11, and the cells in the chalky endosperm were arranged in a non-radiative manner (Fig. 4-A, Fig. 4-D). Both the mutants *OsAAP3* and ZH11 have white heart and white belly. The endosperm cells of white heart show obvious radiation from the middle of the cross-section of the endosperm to the surrounding, and the radiation is longer. There are many columnar cells and elliptical cells around (Fig. 4-B, Fig. 4-E); The white belly is mainly concentrated in the dorsal and ventral direction. The middle of the endosperm cross section is not obvious in radiation, and the endosperm cells arranged in radiation are curved and arranged together. There are irregular cell groups, and the periphery is multi-layer polygonal columnar cells. Compared with ZH11, the white heart area of the mutant *OsAAP3* is larger, and the white belly of the mutant *OsAAP3* is distributed in the edge area of the whole cross-section, while the white belly of ZH11 only exists in a small part of the edge of the cross-section (Fig. 4-C, Fig. 4-F). Therefore, the arrangement and morphology of rice endosperm cells of the mutant *OsAAP3* were significantly different from its corresponding parent material ZH11.

Fig-4. Observation on morphology and arrangement of endosperm cells in *OsAAP3* mutant cross section

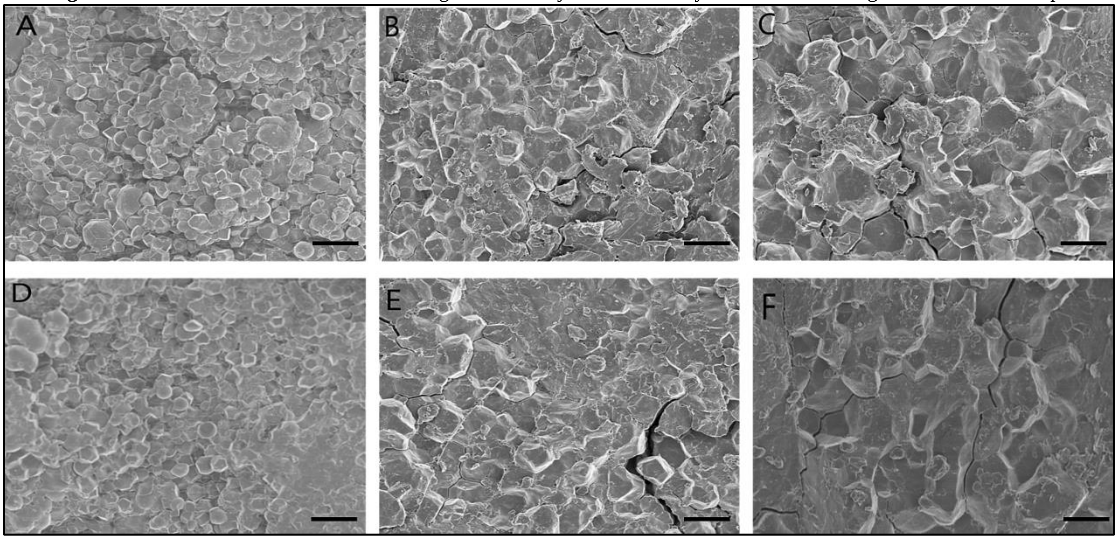


A: *OsAAP3* mutant with non-chalky rice; B: *OsAAP3* mutant with white heart; C: *OsAAP3* mutant with abdominal white; D: ZH11 with non-chalky rice; E: ZH11 with white heart; F: ZH11 with abdominal white.

3.4. Morphological Variation of Starch Grains in Rice Mutant *OsAAP3*

Rice with chalkiness in *OsAAP3* mutant and rice with ZH11 as control were selected to observe the morphology of starch grains in cross-section by scanning electron microscope. The results showed that the starch grains in the chalky part of *OsAAP3* mutant chalky rice differed greatly from those in the transparent part. Most of the starch grains in the chalky part of chalky rice were spherical, small in diameter, loosely arranged, with obvious gaps, and existed in the form of a single starch grain, with poor starch development (Fig. 5-A, Fig. 5-D); Most of the starch grains in the transparent part of chalky rice are in polyhedral shape, with sharp edges and corners, and a few are in irregular shape, closely arranged. Compared with the chalky part of the mutant, the starch development is better, but compared with ZH11, the starch development is worse (Fig. 5-B, Fig. 5-E); Further observation on the starch grains of chalky rice of ZH11 shows that the starch grains of ZH11 are arranged more closely than those of the mutant (Fig. 5-C, Fig. 3-F). The above results showed that the formation of chalkiness of *OsAAP3* mutant rice was closely related to the shape, structure, and arrangement of starch grains, and the arrangement of starch grains of the mutant was loose, the grain type was mostly irregular polyhedron, and the starch development was poor.

Fig-5. Observation of *OsAAP3* mutant starch grain of chalky and non-chalky rice under scanning electronic microscope

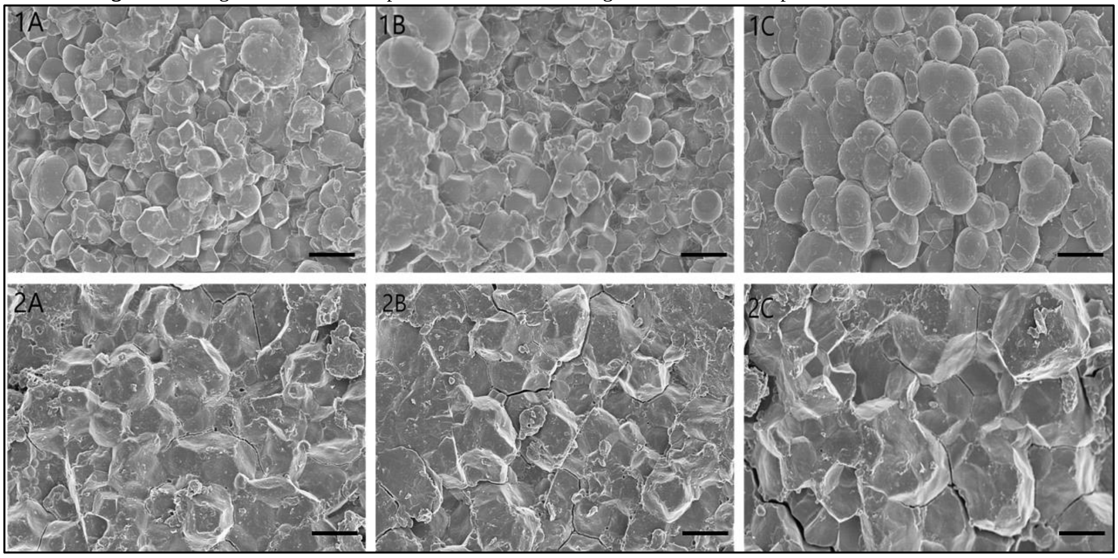


A, D: Starch granules in the chalky rice; B, E: Starch granules in the transparent parts of rice;
C, F: Starch granules in the non-chalky rice; Bar=20 μm.

3.5. Difference of Starch Granule Development in Different Parts of Mutant *OsAAP3*

According to the different positions of chalkiness formation in rice endosperm, chalkiness characters can be divided into three chalkiness subtypes: white belly, white heart, and white back. The abdomen, center, and back of chalky rice grains of *OsAAP3* mutant and ZH11 were observed under the scanning electron microscope. It was found that the endosperm of ZH11 had better starch development in the center, abdomen, and back, while the endosperm of the *OsAAP3* mutant was poorer than that of ZH11. Compared with the endosperm abdomen and back of the *OsAAP3* mutant, the starch in the endosperm center of the *OsAAP3* mutant is well developed, and the starch grains are mostly polyhedral in shape and arranged closely, while the starch in the endosperm abdomen and back of the *OsAAP3* mutant is poorly developed, and most of the starch grains are round or oval, arranged loosely, and have obvious gaps (Fig. 6-1, Fig. 6-2). The above results showed that the formation of chalkiness was closely related to the development of starch grains in different parts of rice, and the starch development in the center, abdomen, and back of *OsAAP3* mutant rice was poor.

Fig-6. Scanning electron microscopic observation of starch granules in different parts of *OsAAP3* mutant rice



1: Mutant *OsAAP3*; 2: ZH11; A: Starch granules in the center of endosperm; B: Starch granules in the belly of endosperm; C: Starch granules on the back of endosperm; Bar=20 μm.

4. Discussion

4.1. Chalkiness and Starch Grain Development of *OsAAP3* Mutant Rice

Chalkiness is an important rice quality character, which not only affects the appearance and processing quality of rice but also affects the cooking and eating quality [31, 32]. Different varieties and qualities of rice have their fixed starch grains. 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 [33-36]. The starch grains of ZH11 are evenly distributed, while the starch grains of *OsAAP3* mutant rice endosperm is obviously unevenly distributed on the cross-section. It can be seen that the varieties with uniform starch grains distribution on the cross-section have low chalkiness grain rate and chalkiness degree, and are not easy to form the chalkiness character; The starch grains on the cross-section are unevenly distributed, with large gaps and loose arrangement. The varieties with poorly developed starch grains have high chalkiness grain rate and chalkiness, which are easy to form chalkiness. Compared with the chalky rice starch grains of ZH11, the chalky rice starch grains of *OsAAP3* mutant mainly exist in a free state and cannot further develop into a starch grain complex with a certain shape: If a single starch grain can be orderly and closely arranged to form a starch grain complex with a certain shape, the chalk character will not be formed in this part; If the spherical or ellipsoidal starch grains are arranged irregularly and deposited in a disordered state, the chalk character will be formed in this part.

4.2. Reasons for the Formation of Chalkiness Character of Mutant *OsAAP3*

The chalkiness of rice endosperm is one of the most important quality traits of rice, but chalkiness is a complex quantitative trait controlled by multiple genes, which are jointly controlled by multiple QTLs or genes [6, 37-40]. It is generally believed that rice chalkiness is caused by genetic factors and environmental conditions [41, 42]. The hardness of chalky rice of the same size of the same variety is smaller than that of non-chalky rice in the back abdomen diameter and transverse diameter. The brown rice rate, milled rice rate, and head rice rate of chalky rice are lower than those of non-chalky rice, so the broken rice rate is higher [43]. The formation of rice chalkiness is the result of multiple metabolic and regulatory pathways such as grain carbon and nitrogen [44]. The scanning electron microscope results showed that the endosperm starch grains in the chalky part of *OsAAP3* mutant rice were mostly spherical, resulting in a loose endosperm structure and opaque appearance. Some studies have also shown that the incomplete development of the protein body leads to the loose combination of the starch body and the protein body, and the gap between them is also an important reason for the formation of chalky [45, 46]. Therefore, based on the results of this study, it is speculated that the occurrence, development, late deposition, and distribution of starch grains in the endosperm of *OsAAP3* mutant rice are critical to the formation of chalkiness. However, the causes of chalkiness formation in *OsAAP3* mutant rice, the early occurrence and development of starch grains, the late sedimentation regulation mode, the genes involved and their roles in the formation of chalkiness traits of the mutant rice need to be further studied.

5. Conclusion

Chalkiness is one of the important quality traits of rice, and it has an important influence on other quality traits. In recent years, the rapid development of rice functional genomics and molecular marker technology has greatly promoted the research on the genetic mechanism of chalkiness traits and beneficial gene mining. However, the mechanism of chalkiness of the mutant and the relationship between the *OsAAP3* gene and chalkiness are still unclear. This study found that the chalkiness of the rice with *OsAAP3* mutant increased significantly, and the chalkiness of the mutant mainly occurred in the abdomen, while the rest occurred in the center of rice endosperm. Moreover, the arrangement of starch grains in *OsAAP3* mutant rice is loose, the grain type is the mostly irregular polyhedrons, and the starch development is poor. It is speculated that the *OsAAP3* gene may affect the anabolism of starch grains in rice, thus leading to chalkiness in *OsAAP3* mutant rice.

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