agronomy

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Physiological Basis of High Nighttime Temperature-Induced Chalkiness Formation during Early Grain-Filling Stage in Rice (*Oryza sativa* L.)

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Abstract: Heat stress during the grain-filling stage seriously affects grain quality in rice. However, very limited information is available regarding the effects of short-term high nighttime temperature (HNT) on grain chalkiness formation in rice. In this paper, the effects of HNT at the early grain-filling stage (7 days after ear emergence) on rice chalkiness formation and the potential causes were investigated by using two rice varieties that differed in susceptibility to high temperature. Although the HNT treatment at night dramatically increased the grain chalkiness in Jiuxiangzhan (JXZ) and Huanghuazhan (HHZ), the increase was greater in JXZ compared to HHZ. The net photosynthetic rate and SPAD value were significantly reduced by HNT treatment in the flag leaves of JXZ, while no significant differences were observed in HHZ. Furthermore, HNT treatment reduced the antioxidant enzyme activity in the flag leaves of JXZ, while the opposite was observed in HHZ, exhibiting increased antioxidant enzyme activity. Moreover, HNT treatment altered the endogenous hormone levels, enhanced the enzymatic activities related to starch biosynthesis, and accelerated the filling rate in grains of JXZ when compared to HHZ. Scanning electron microscopy (SEM) observation exhibited that the starch granules in the endosperm of JXZ were loosely packed together and more starch granules with small pits were produced after HNT treatment. Based on these data, we inferred that HNT stress during the early stage of rice grain filling accelerated the grain-filling rate but shortened the grain-filling duration by changing the endogenous hormone levels and enhancing the enzymatic activities responsible for starch biosynthesis, resulting in significant changes in the morphological structure and arrangement of starch granules and eventually causing the occurrence of grain chalkiness.

Keywords: chalkiness; early grain-filling stage; high nighttime temperature; rice (*Oryza sativa* L.)

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Citation: Du, Y.; Long, C.; Deng, X.; Zhang, Z.; Liu, J.; Xu, Y.; Liu, D.; Zeng, Y. Physiological Basis of High Nighttime Temperature-Induced Chalkiness Formation during Early Grain-Filling Stage in Rice (*Oryza sativa* L.). *Agronomy* **2023**, *13*, 1475. https://doi.org/10.3390/agronomy13061475

Academic Editor: María del Pilar Cordovilla

Received: 19 April 2023
Revised: 24 May 2023
Accepted: 25 May 2023
Published: 26 May 2023

1. Introduction

Temperature is an important environmental cue regulating the growth and development of higher plants. As the global temperature gradually increases, a high temperature limits crop yield and quality improvement. Rice is a major food crop worldwide and plays a pivotal role in ensuring food and nutritional security. High temperature affects different growth stages of rice, starting from germination to maturity. In particular, the grain-filling period is the most affected stage under high-temperature stress, which greatly impacts rice yield and quality [1–3]. Therefore, it is very important to investigate the physiological responses of rice to high-temperature stress during the grain-filling stage.

As high-temperature occurrence during the grain-filling stage seriously limits the yield and quality of rice, it has attracted much research attention in recent years. On the one hand, the high temperature exacerbates the premature senescence of rice leaves. It prevents the production of photosynthetic assimilates resulting in reduced photosynthetic assimilates' transport from the photoautotrophic source leaves to the heterotrophic sink organs [4–6]. On the other hand, although the high temperature accelerates the grain-filling rate, the duration of grain filling is shorter, which has a significant negative effect on both yield and grain quality [7–9]. Further in-depth studies revealed that a high temperature during the night has a greater effect on rice yield than a high temperature during the day [10,11]. For example, when the night temperature increased by 1 °C, rice yield decreased by 10%, while daytime temperature changes had little effect on rice yield [11]. Over the last decades, numerous studies have been conducted to investigate the mechanisms underlying the decrease in rice yield caused by the increased night temperature [4,5,10,12–15]. Among these extensive studies, the multiple causes of rice yield reduction under high nighttime temperature were analyzed, i.e., smaller amounts of assimilates supplied due to decreased photosynthetic capacity in leaves [12,13], poor translocation of assimilates to grains from vegetative organs [5], or low enzymatic activities related to starch biosynthesis in developing grains [14]. In addition to the effects on yield, elevated nighttime temperatures also severely negatively impact rice quality. For example, the high nighttime temperature usually increases grain chalkiness, decreases the head milled rice rates, and reduces the eating quality characteristics of rice [16–19].

Chalkiness degrades the value of rice due to its undesirable appearance [20]. Therefore, investigating the physiological mechanisms of chalkiness formation and reducing its occurrence has become one of the most important research directions for rice quality improvement. Apart from genetic conditions, external environmental cues such as light, temperature, and nutrition can exert an important influence on rice chalkiness formation [20,21]. Among these environmental factors, it is assumed that temperature has the greatest impact [20,22]. Both high and low temperatures during the grain-filling stage affected rice chalkiness, with high temperatures having a much greater effect compared to low temperatures [23]. A high temperature causes an increase in rice chalkiness due to three main reasons. Firstly, a high temperature results in an abnormal structure of starch granules, affecting their arrangement in developing endosperm cells [24,25]. Secondly, a high temperature alters the endosperm starch content and composition [26–29]. Thirdly, a high temperature changes the fine structure of amylopectin, increasing the ratio of long chains to short chains of amylopectin [27]. In addition to the increasing extent of the average temperature, the choice of the starting point of high-temperature treatment also has an important effect on rice chalkiness formation. It was previously reported that the chalkiness significantly increased when the rice plants at 20 days after heading were subjected to high-temperature treatment [30]. Consistent with this, Siddik et al. [23] reported that the effect of high-temperature treatment at different periods on rice chalkiness differed significantly during the grain-filling stage, with the greatest effect on increased chalkiness formation in the second week of post-heading, while gradually diminishing in the later periods. Although it is an indisputable fact that global warming is becoming increasingly severe, a greater increase in nighttime compared with daytime temperatures has been reported [31]. In recent years, the effect of high nighttime temperature (HNT) on rice chalkiness formation has attracted increased research interest worldwide. Compared with an increased daytime temperature, an increased nighttime temperature has been shown to exert similar effects on rice chalkiness formation; however, the mechanisms by which high nighttime and daytime temperatures act may differ [32].

Many studies have focused on rice chalkiness formation in response to extended periods of HNT during the grain-filling stage. However, little information is provided regarding the effects of short periods of HNT on grain chalkiness formation in rice. In the major rice-planting areas across China, short-term high-temperature events frequently occur at the reproductive stage of rice, which has become a severe constraint on rice production. The early stage of rice grain-filling (second week of post-heading) is a critical period for endosperm cell development and grain filling. High temperatures during this period have a serious impact on rice chalkiness formation [23]. In this study, the effects of HNT on rice chalkiness formation during the early grain-filling stage and the potential causes were therefore investigated by using two rice varieties (Jiuxiangzhan and Huanghuazhan) with different susceptibilities to high temperatures that were not previously investigated together. The main objective of this study was to explore the physiological processes governing rice chalkiness formation under HNT conditions during the early grain-filling period.

2. Materials and Methods

2.1. Plant Materials and High-Temperature Treatment

Two *indica* rice varieties (Jiuxiangzhan and Huanghuazhan) with different chalkiness variation in response to high temperature were used [33]. The experiments in our study were performed with potted planting. Specifically, the pregerminated seeds were sown onto wet paddy soils at an experimental site located at Jiangxi Agricultural University. At the third to fourth leaf stage, the rice seedlings were transplanted into plastic pots (27.5 cm in length, 21.0 cm in width, and 32.0 cm in height,) filled with 8.0 kg of soil. The fertilizer management was conducted using a previously reported method [34]. On the 8th day after heading, the rice plants at the same developmental stage were transferred to two environmentally controlled chambers (12 h light/12 h dark photoperiod, 600 μmol m^{-2} s^{-1} light intensity). Specific temperature settings of growth chambers are shown in Table S1. After 7 days of continuous HNT treatment, the rice plants were then transferred to an outside natural environment and allowed to grow until maturity. On the morning (between 9:30 a.m. and 10:30 a.m.) of the 7th day of HNT treatment, all the physiological indexes including photosynthetic characteristics, enzymatic activities, and endogenous hormone levels were determined.

2.2. Evaluation of Grain Chalkiness

The stored grains (about 200 g) were dehulled using a sheller (THU35C, SATAKE, Suzhou, China) and milled using a rice miller (TM05C, SATAKE, Suzhou, China). The machine MRS-9600TFU2L (MICROTEK Inc., Shanghai, China) was used to measure the percentage of chalky grains (chalky grain rate), percentage area of chalky endosperm (chalky area), and percentage of degree of endosperm chalkiness (chalkiness degree).

2.3. Measurement of Photosynthetic Parameters

At HNT treatment stage, about 15 rice plants were selected to conduct the photosynthetic determinations. The net photosynthetic rate and SPAD value were measured on flag leaves by CI-340 portable photosynthesis system (CI-340, CID Inc., WA, USA) and SPAD-502Plus portable chlorophyll meter (SPAD-502Plus, Konica Minolta, Osaka, Japan), respectively.

2.4. Ultrastructural Observation of Starch Granules

Representative samples of rice grains collected from each variety and treatment were analyzed for starch granule ultrastructure. The grains were fixed in 2.5% glutaraldehyde, rinsed three times with PBS buffer, fixed in 1% osmium acid, and rinsed with double-distilled water. Afterward, samples were dehydrated with an ethanol gradient, dried using liquid CO_2 (K850, Quorum, East Sussex, UK), and coated with gold (108Auto, Cresstington, Watford, UK). The SEM instrument (GeminiSEM300, Zeiss, Oberkochen, GER) was used to observe the ultrastructure of starch granules.

2.5. Measurement of Activities of Antioxidant Enzymes and Contents of Malondialdehyde

The activities of antioxidant enzymes and the contents of malondialdehyde were determined in the flag leaves of rice plants by a colorimetric method using a 96-well microplate assay. Briefly, approximately 0.1 g of each sample was ground into powder and the ground samples were then transferred into a centrifuge tube containing 1.0 mL of extraction solution. Next, the sample was centrifuged and the supernatant was used as a test solution for further analysis. The activities of superoxide dismutase (SOD, M0102A), peroxidase (POD, M0105A), and catalase (CAT, M0103A) and the contents of malondialdehyde (MDA, M0106A) were determined using the commercial kits (Suzhou Michy Biomedical Technology Co., Ltd., Suzhou, China).

2.6. Measurement of the Activities of Enzymes Involved in Starch Biosynthesis

The enzymatic activities related to starch biosynthesis were determined in all the grain paddy rice samples by a colorimetric method using a 96-well microplate assay. Approximately 0.1 g of each sample was ground into powder, and the ground samples were then transferred into a centrifuge tube containing 1.0 mL of extraction solution. After centrifugation, the supernatant was used as a test solution for further analysis. The activities of sucrose synthase (SS-II, M1204A), adenosine diphosphoglucose pyrophosphorylase (AGPase, M1107A), soluble starch synthase (SSS, M1108A), starch branching enzyme (SBE, M1110A), and debranching enzyme (DBE, M1111A) were measured using the commercial kits (Suzhou Michy Biomedical Technology Co., Ltd., Suzhou, China).

2.7. Measurement of the Contents of Endogenous Hormones

The levels of endogenous hormones IAA (indole-3-acetic acid), GAs (gibberellins), CTKs (cytokinins), ABA (abscisic acid), and ACC (1-aminocylopropane-1- carboxylicacid) were measured by UHPLC-MS/MS in the whole grain paddy rice samples. Briefly, the frozen grain tissue (0.1 g) was extracted in 1 mL of 50% aqueous acetonitrile. After ultrasonication, the samples were extracted using a Stuart SB3 benchtop laboratory rotator. Afterwards, the sample was centrifugated and the supernatant was purified and then determined using a UPLC Orbitrap-MS system.

2.8. Rate and Duration of Grain Filling

The panicles were sampled at 10th, 12th, 14th, 21st, 28th, and 35th day after heading. Freshly harvested rice grain samples were inactivated at 105 °C for 30 min and then dried at 75 °C for 3 days. Calculations for the rates and duration of grain filling were performed according to the method described previously [5].

2.9. Statistical Analysis

All experiments were repeated three times independently. Data represent the mean values with the standard errors. The values with different letters are significantly different ($p < 0.05$) according to Student's *t*-test. Two-way analysis of variance (ANOVA) was used to analyze the data with SPSS version 26.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Chalky Grain Rate, Chalkiness Area, and Chalkiness Degree

In the previous studies, we found that there was a great variation in grain chalkiness among rice varieties in response to temperature changes. Jiuxiangzhan (JXZ) was sensitive to high temperature with high chalkiness occurrence, while Huanghuazhan (HHZ) was tolerant to high temperature with low chalkiness occurrence [33]. There is little difference in heading date between the two rice cultivars when grown under local environmental conditions (about 5 days). In order to investigate the effects of HNT treatment during the early grain-filling stage on rice grain chalkiness, the rice plants (JXZ and HHZ) were treated at nighttime with high temperature for 7 days. The short-term HNT stress dramatically increased the percentage of chalky grains and the percentage of degree of endosperm chalkiness of both rice varieties, with JXZ exhibiting a greater increase than HHZ (Figure 1a–c). Specifically, the rate of chalky grains and the degree of endosperm chalkiness in JXZ, respectively, increased by 1.41-fold and 1.53-fold, while those of HHZ, respectively, increased by only 1.21-fold and 1.14-fold. Moreover, compared to normal temperature, the HNT treatment dramatically increased the percentage area of chalky endosperm by 1.13-fold for JXZ, whereas no significant changes were observed in HHZ (Figure 1d). These findings indicate that although the grain chalkiness was significantly induced in both rice varieties by HNT stress, the extent of induction was greater in JXZ that in HHZ.

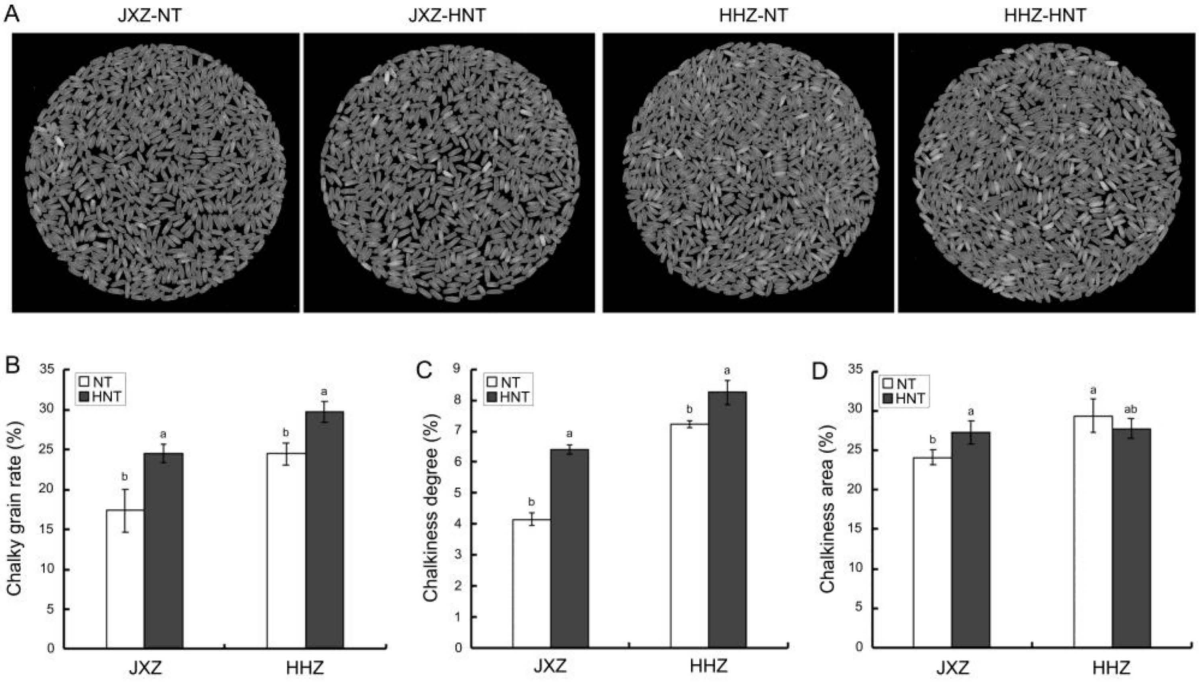

Figure 1. JXZ was more sensitive to HNT-induced chalkiness formation than HHZ. (**A**) Appearances of milled rice of the JXZ and HHZ grown under NT and HNT conditions. (**B**–**D**) The chalky grain rate (percentage of chalky grains), chalkiness degree (percentage of degree of endosperm chalkiness), and chalkiness area (percentage area of chalky endosperm) of milled grains of JXZ and HHZ. Different letters represent significant differences.

3.2. Amyloplast Development in Endosperm

Since the morphology and arrangement of starch granules affect the chalkiness formation of rice grains, we, therefore, investigated the effects of HNT treatment on the morphology and arrangement of starch granules in the grains of the two varieties (JXZ and HHZ). SEM observation of the endosperm cross-section exhibited that the elevated nighttime temperature has an obvious influence on the morphology and arrangement of starch granules in the endosperm of JXZ (Figure 2a). Under normal conditions, the amyloplasts in the endosperm were compounded and highly densely packed with similar sized polyhedral starch granules. However, the starch granules in the endosperm exposed to HNT were arranged loosely and most existed in the form of a single starch body. Moreover, HNT treatment resulted in numerous small pits appearing on the surface of single starch granules (Figure 2a). Unlike JXZ, the SEM photomicrographs showed that most of the starch granules in the endosperm of HHZ were largely unchanged under the conditions of HNT (Figure 2b). All these results suggest that the extent of the effects of HNT on the morphological structure and arrangement of the starch granule in JXZ was higher than that in HHZ.

3.3. Photosynthetic Capacity and Activities of Antioxidant Enzymes

The photosynthetic capacity of leaves is an important factor reflecting the strength of photosynthesis, which directly determines the productivity potential of crop plants. To investigate the effects of HNT treatment on the photosynthetic capacity of the two rice varieties (JXZ and HHZ), the net photosynthetic rate and SPAD value of the flag leaves were examined. As shown in Figure 3a,b, the two rice varieties exhibited different responses to the elevated nighttime temperature: HNT treatment dramatically decreased the net photosynthetic rate and SPAD value in JXZ as compared with the control plants (decreased by 16.4% and 9.0%, respectively), while no significant changes were found in HHZ. The results suggest that the decrease in the net photosynthetic rate under HNT conditions may be due to the decrease in the chlorophyll contents in the flag leaves of JXZ.

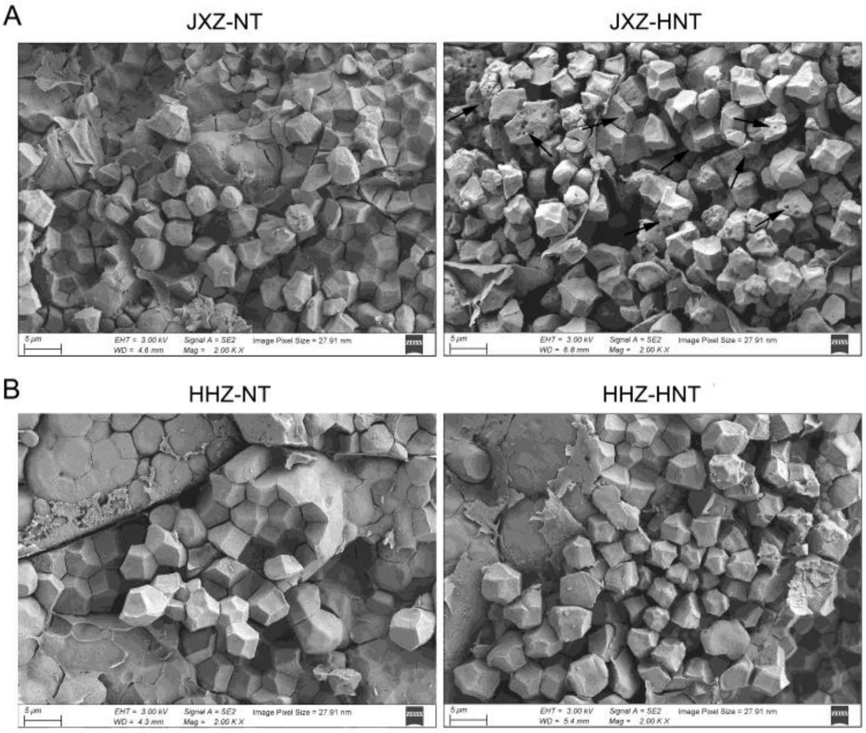

Figure 2. The extent of effects of HNT on morphological structure and arrangement of starch granules in JXZ was higher than that in HHZ. (**A**,**B**) SEM observation of transverse sections of grains of JXZ and HHZ grown under NT and HNT conditions. The arrows show the small pits on the surface of starch granules.

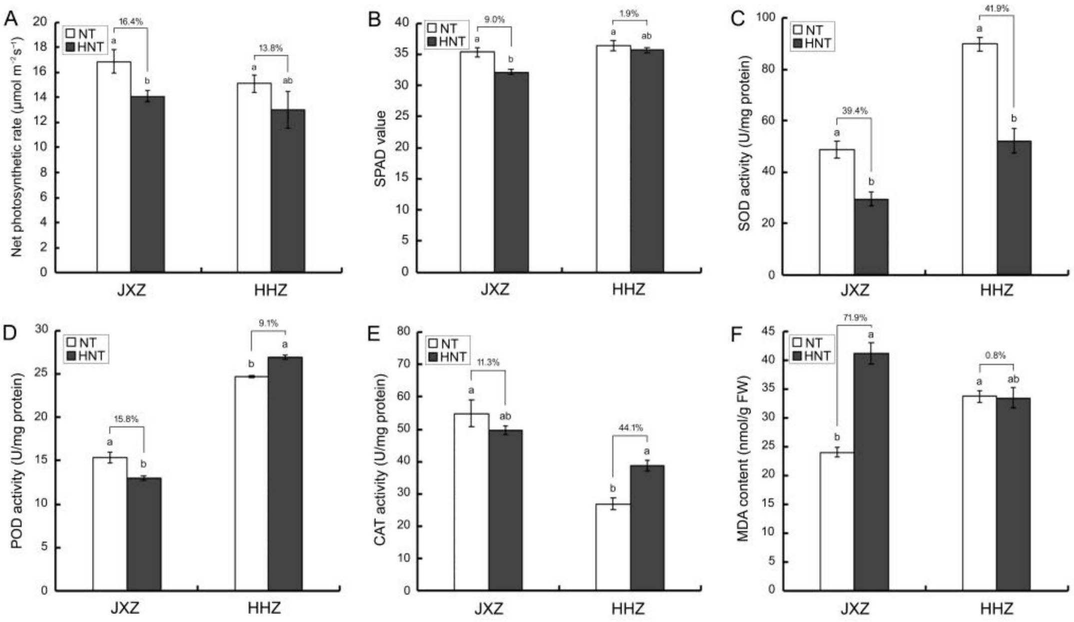

Figure 3. The leaves of JXZ were more sensitive to HNT stress than those of HHZ. (**A**,**B**) The net photosynthetic rate and SPAD value of flag leaves of JXZ and HHZ grown under normal (NT) and high nighttime temperature (HNT) conditions. (**C**–**F**) Comparative analysis of the antioxidant enzyme activities and MDA contents in flag leaves of the two rice varieties (JXZ and HHZ) in response to HNT. €SOD; (**D**) PO€(**E**) CAT; (**F**) MDA. Different letters represent significant differences.

Abiotic stresses such as heat, cold, and drought affect plant growth and yield production. Plants respond to these stresses by increasing the activities of antioxidant enzymes in leaves to alleviate the damage of reactive oxygen species (ROS) to photosynthetic machinery [35]. Since the two rice varieties showed a different response to the HNT stress (photosynthetic capacity), we therefore explored the stress-induced changes in antioxidant enzyme activities in the flag leaves. Compared to the normal temperature, HNT treatment dramatically reduced the activity of superoxide dismutase (SOD) by 39.4% for JXZ and 41.9% for HHZ (the extent of the reduction was similar; Figure 3c). Under HNT conditions, the peroxidase (POD) activity of JXZ decreased by 15.8%, while that of HHZ increased by 9.1% compared to the normal temperature (Figure 3d). The catalase (CAT) activity of HHZ increased by 44.1% under HNT conditions, whereas there were no significant changes in JXZ (Figure 3e). Malondialdehyde (MDA) levels are important indicators for assessing the integrity of the membrane structure [36]. We further analyzed the effects of HNT on the MDA content in the flag leaves of the two rice varieties. As shown in Figure 3f, HNT treatment significantly increased the MDA content in JXZ but did not alter it significantly in HHZ. These results suggest that the leaves of JXZ were more sensitive to HNT stress than those of HHZ. The limited impact of HNT stress on the photosynthetic capacity in HHZ is probably attributed to the increase in the activities of antioxidant enzymes and the constant MDA content in the flag leaves.

3.4. Rate and Duration of Grain Filling

It has been assumed that both the rate and duration of grain filling have a great influence on rice chalkiness formation [37]. Therefore, we examined the influences of HNT treatment on the grain-filling characteristics in the two rice varieties. Compared with normal temperature, HNT stress significantly increased the GR_{max} (maximum grain-filling rate) and GR_{mean} (mean grain-filling rate) of JXZ, while there was only a slight impact on GR_0 (initial grain-filling rate) of HHZ. In JXZ, the T_{max} (time taken to reach the maximum grain-filling rate) in JXZ was decreased by 25.76% (shortened by 2.71 days) under HNT conditions. Although HHZ exhibited a similar response, the extent of this shortening effect was smaller than that in JXZ (decreased by 18.46%, 1.96 days; Table 1). Moreover, the D (active grain-filling duration) was shortened by 4.60 days with HNT treatment; however, in HHZ, it was only shortened by 2.73 days (Table 1). These results indicate that the impact of HNT on the grain filling in JXZ was greater than that in HHZ.

Table 1. The impact of HNT on the grain filling in JXZ was greater than that in HHZ. Grain-filling parameters of the two rice varieties (JXZ and HHZ) under normal (NT) and high nighttime temperature (HNT) conditions.

Variety	Treatment	W	GR_0	GR_{max}	GR_{mean}	T_{max}	D	R^2
JXZ	NT	12.39a	0.28a	1.21b	0.64b	10.52a	17.31a	0.99
	HNT	13.89a	0.29a	1.99a	0.97a	7.81b	12.71b	0.97
HHZ	NT	17.39a	0.23b	2.78a	1.23a	10.62a	14.36a	0.99
	HNT	15.49b	0.25a	2.32a	1.08a	8.66b	11.63b	0.99

W, grain weight (mg grain^{-1}), GR_0, initial grain-filling rate (mg per grain d^{-1}), GR_{max}, maximum grain-filling rate (mg per grain d^{-1}), GR_{mean}, mean grain-filling rate (mg per grain d^{-1}), T_{max}, time taken to reach maximum grain-filling rate (d), D, active grain-filling duration (d). The values with different letters are significantly different ($p < 0.05$) according to Student's *t*-test.

3.5. Enzymatic Activities Related to Starch Biosynthesis

To explore the effects of HNT treatment on the starch biosynthesis in grains of the two rice varieties, the enzymatic activities related to starch biosynthesis, including SS-II, AGPase, SSS, SBE, and DBE, were determined. The results showed that the change trends of these enzyme activities responding to HNT stress differed largely between JXZ and HHZ (Figure 4). Compared to the control, the HNT treatment dramatically increased the activities of all five enzymes in the grains of JXZ. However, the activities of most enzymes

(SS-II, SSS, and DBE) were decreased significantly in HHZ as compared to the control, except for AGPase and SBE, which remained unchanged (Figure 4).

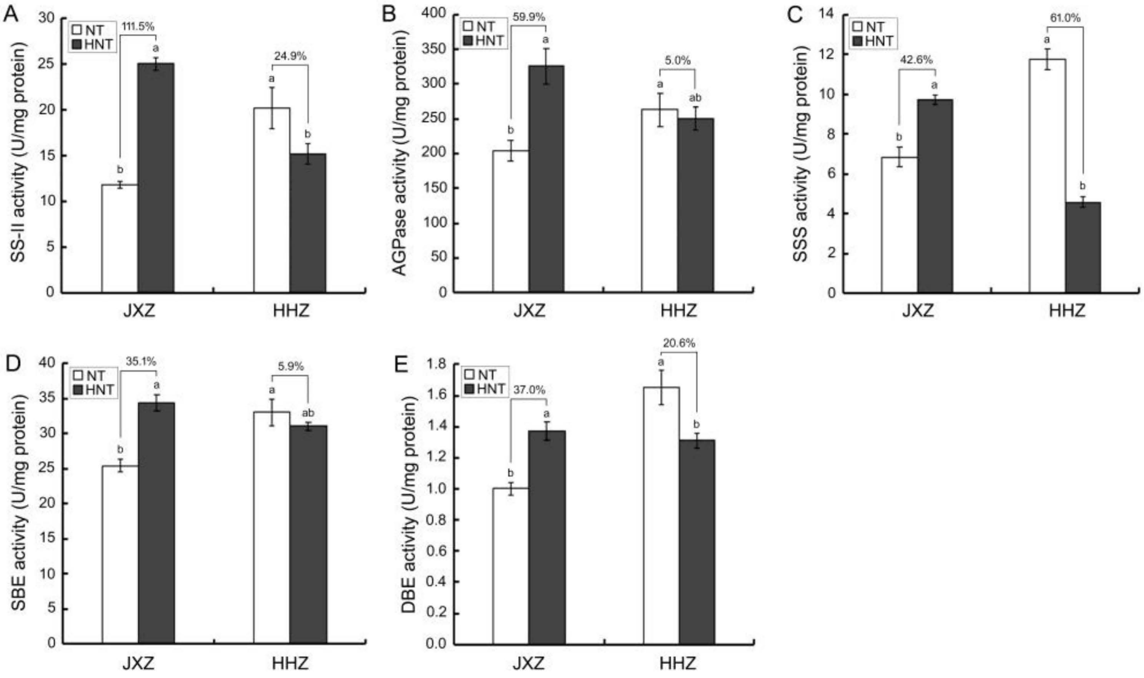

Figure 4. HNT treatment increased the enzymatic activities related to starch biosynthesis in grains of JXZ, while decreased or remained unchanged in grains of HHZ. (**A**) SS-II; (**B**) AGPase; (**C**) SSS; (**D**) €E; (**E**) DBE. "NT" and "HNT" represent normal and high nighttime temperature conditions, respectively. Different letters represent significant differences.

3.6. Levels of Endogenous Hormones

Plant hormones play a crucial role in modulating starch biosynthesis. Considering that the two rice varieties showed different responses in the activities of enzymes involved in starch biosynthesis to the HNT stress, we further examined the effect of HNT treatment on the levels of endogenous hormones in the grains of the two varieties. As shown in Figure 5, the change trends of the contents of IAA, Gas, and ABA responding to HNT stress were similar between JXZ and HHZ. HNT treatment caused the elevation of IAA contents and a reduction in the contents of GAs and ABA in both rice varieties (Figure 5a,b,d). It should be noted that the extent of change in the contents of IAA and ABA differed between the two rice varieties (Figure 5a,d). Specifically, compared to normal temperature, HNT stress dramatically increased the IAA content by 50.48% for JXZ and only 4.56% for HHZ (Figure 5a). HNT treatment reduced the ABA content dramatically for HHZ, by approximately 52.06%, compared to the control. However, the content of ABA was only reduced by approximately 7.86% for JXZ (Figure 5d). Moreover, the change trends of the contents of CTKs and ACC responding to HNT differed between the two rice varieties (Figure 5c,e). HNT treatment significantly decreased the contents of CTK and ACC in JXZ, while these significantly increased in HHZ (Figure 5c,e).

3.7. Relationship between Chalkiness and Physiological Indexes

To further understand the physiological mechanisms of HNT-induced chalkiness formation during the early grain-filling stage in rice, the relationship between chalkiness and physiological indexes measured in this study was analyzed by two-way analysis of variance. The results showed that rice chalkiness was closely related to these physiological indexes. Both the chalky grain rate and chalkiness degree were significantly and negatively correlated to the net photosynthetic rate, POD, GAs, ACC, and active grain-filling duration, but were significantly and positively correlated to the MDA, SBE, IAA, GR_{max}, and

GR_{mean}. The chalkiness degree was negatively correlated to CAT and CTKs, whereas it was positively correlated with DBE (Table 2).

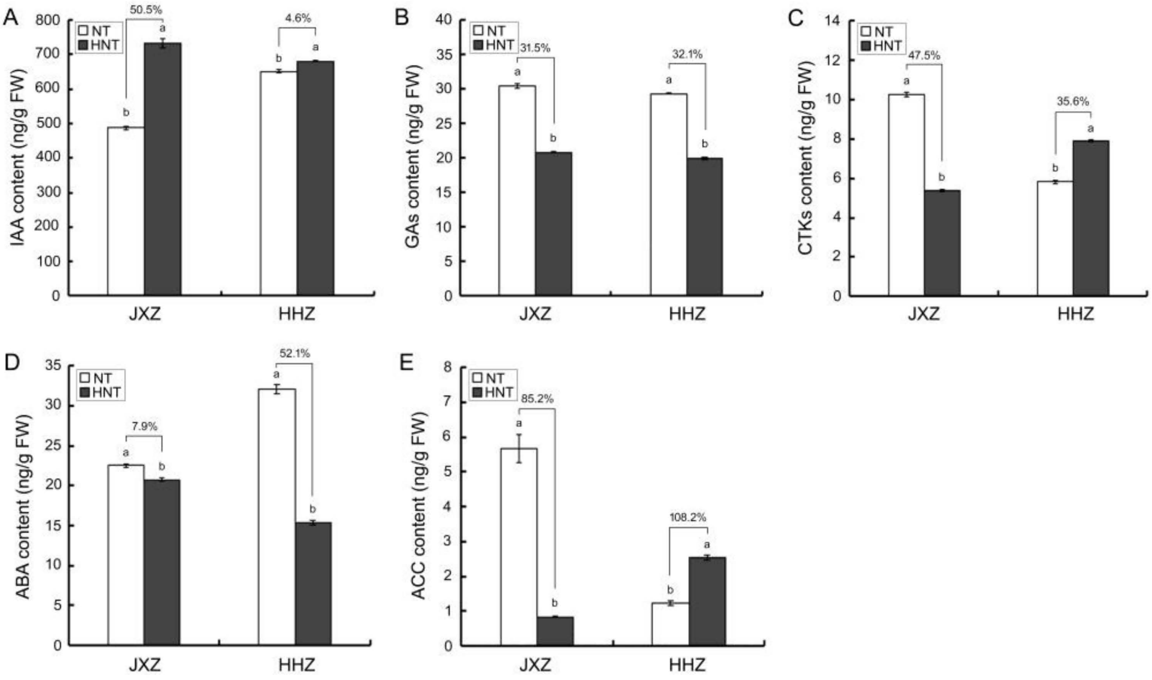

Figure 5. The two rice varieties (JXZ and HHZ) exhibited different changes in endogenous hormone levels under HNT conditions. (**A**) IAA; (**B**) GAs; (**C**) CTKs; € ABA; (**E**) ACC. "NT" and "HNT" represent normal and high nighttime temperature conditions, respectively. Different letters represent significant differences.

Table 2. Correlation analysis between chalkiness and other physiological indexes.

Index	Chalky Grain Rate	Chalkiness Degree
Net photosynthetic rate	−0.82 **	−0.81 **
SPAD	0.08	0.17
Superoxide dismutase (SOD)	0.07	0.28
Peroxidase (POD)	−0.64 *	−0.75 **
Catalase (CAT)	−0.53	−0.73 **
Malondialdehyde (MDA)	0.59 *	0.58 *
Sucrose synthase (SS-II)	0.31	0.33
Adenosine diphosphoglucose pyrophosphorylase (AGP)	0.41	0.39
Soluble starch synthase (SSS)	−0.17	−0.04
Starch branching enzyme (SBE)	0.64 *	0.66 *
Debranching enzyme (DBE)	0.54	0.67 *
Indole-3-acetic acid (IAA)	0.77 **	0.77 **
Gibberellins (GAs)	−0.74 **	−0.62 *
Cytokinins (CTKs)	−0.50	−0.58 *
Abscisic acid (ABA)	−0.34	−0.15
1-aminocylopropane-1- carboxylicacid (ACC)	−0.61 *	−0.69 *
Initial grain-filling rate (GR_0)	−0.08	−0.19
Maximum grain-filling rate (GR_{max})	0.61 *	0.75 **
Mean grain-filling rate (GR_{mean})	0.62 *	0.75 **
Time taken to reach maximum grain-filling rate (T_{max})	−0.47	−0.36
Active grain-filling duration (D)	−0.76 **	−0.79 **

* and ** indicate the significance at $p < 0.05$ and $p < 0.01$, respectively.

4. Discussion

High temperatures during the grain-filling stage induced chalkiness formation, severely affecting the appearance quality of rice [2,20]. Under the trend of global warming, it has been reported that the rate of temperature increase at night is faster than that in the daytime [31]. Therefore, it is very important to investigate the impact of HNT on rice grain quality. In our previous work, it was found that there was a significant variation in grain chalkiness among rice varieties in response to temperature changes. JXZ was sensitive to high temperatures with high chalkiness occurrence, while HHZ was tolerant to high temperature with low chalkiness occurrence [33]. Since the early stage of rice grain-filling is a critical period for the endosperm cell development and grain filling, changes in the ambient temperature during this period have a great impact on rice quality [23]. In this study, the effects of HNT during the early grain-filling period on rice chalkiness formation and the potential causes were investigated by using the two rice varieties (JXZ and HHZ). By comparing their physiological responses to HNT stress, we elucidated the potential mechanisms underlying rice chalkiness formation caused by elevated nighttime temperatures during the early grain-filling stage.

The flag leaf is especially important since it is the primary photosynthetic organ at the grain-filling stage of rice. Therefore, any injury to the flag leaf caused by abiotic stress could lead to yield loss and quality deterioration. Considering the two rice varieties (JXZ and HHZ) showed different responses to HNT stress, we evaluated the changes in the net photosynthetic rate and SPAD value of their flag leaves after treatment with HNT. High temperatures at night significantly reduced the net photosynthetic rate and SPAD value in the flag leaves of JXZ but had no significant effect on HHZ (Figure 3a,b). High temperature is one of the abiotic stresses that induces a cascade of excessive ROS production. Excessive ROS leads to lipid peroxidation and cell apoptosis that can be an important mediator of damage to nucleic acids and proteins [38]. The ROS generated by oxidative stress may also damage the photosynthetic machinery and destroy the structure and function of the thylakoid membrane, eventually leading to the inhibition of photosynthesis [39]. In order to reduce the harm of high temperatures, plants have developed a highly efficient antioxidant enzyme system to scavenge the excessive ROS [38]. To further investigate the activities of antioxidant enzymes in the flag leaves of the two rice varieties, the changes in the activities of SOD, POD, and CAT were examined. The change trends of these enzymatic activities responding to HNT stress differed between the two rice varieties except for SOD (exhibited a similar change trend; Figure 3c–e). HNT treatment dramatically decreased the activities of POD in JXZ, while these dramatically increased in HHZ (Figure 3d). Although the activities of CAT in HHZ were greatly induced under HNT conditions, no obvious changes were observed in JXZ (Figure 3e). Consistent with these results, HNT treatment significantly increased the MDA content in JXZ but this did not alter significantly in HHZ (Figure 3f). Based on these results, we draw the following conclusions: (i) The flag leaves of JXZ were more sensitive to HNT stress, resulting in greater suppression of photosynthetic capacity than that of HHZ. (ii) The photosynthetic capacity of the flag leaves in HHZ remained relatively stable under the conditions of HNT, mainly due to the enhancement of enzymatic activities related to the antioxidant system (including POD and CAT). The results of the correlation analysis showed that the chalkiness degree was negatively correlated to the net photosynthetic rate, POD, and CAT, whereas it was positively correlated with MDA. It is widely believed that the enhancement of antioxidant enzyme activity, by lowering the levels of ROS and MDA, contributes to maintaining higher leaf photosynthesis under various abiotic stress conditions. This indicates that the suppression of photosynthetic production in the leaf lamina is an important factor leading to the formation of chalky grains. In the current study, we focused only on the impact of the photosynthetic capacity of the flag leaves on assimilates' supply; however, the influence of carbohydrates stored in the stems and leaf sheaths was not explored.

The decrease in the photosynthetic rate of leaves caused by high temperature stresses is associated with a reduction in grain yield [4]. Furthermore, the decrease in grain weight is partially attributed to the decline of the photosynthetic rate in leaves, which results in a reduction in grain yield [40,41]. High temperatures reduced the grain weight by affecting the rate and duration of grain filling [42]. In our research, the decline of grain weight caused by HNT stress at the early grain-filling stage was not related to the decrease in the photosynthetic capacity of the flag leaves, but was related to the changes in the rate and duration of grain filling. This hypothesis can be validated by the following evidence. Firstly, although the HNT stress significantly reduced the net photosynthetic rate of the flag leaves of JXZ, it did not affect the grain weight of JXZ (Figure 3a; Table 1). Under HNT conditions, the net photosynthetic rate of the flag leaves of HHZ was not affected, while the grain weight of HHZ was dramatically reduced compared to the control (Figure 3a; Table 1). Secondly, compared to the normal temperature, the active grain-filling duration was dramatically shortened in both rice varieties under HNT conditions (Table 1). Generally speaking, high temperature led to a decreased grain weight by shortening the duration of grain filling. However, an increased grain-filling rate in JXZ under HNT stress could compensate for the reduction in the grain-filling duration, eventually leading to the grain weight not being affected (Table 1). Unlike JXZ, HHZ recorded a shorter grain-filling duration, while the mean grain-filling rate remained unchanged, therefore decreasing the grain weight (Table 1).

It has been documented that the duration of grain filling affects rice chalkiness formation [37]. High temperature exposure post-flowering resulted in an increase in the grain-filling rate and a shortened grain-filling duration in rice [43,44]. In our research, we found that the impact of HNT on the grain filling in JXZ was greater than that in HHZ. In particular, the T_{max} for JXZ and HHZ was shortened by 2.71 days and 1.96 days, respectively. Moreover, the D for JXZ was shortened by 4.60 days, whereas it was shortened only by 2.73 days for HHZ (Table 1). Further correlation analysis revealed that both the chalky grain rate and chalkiness degree were significantly positively correlated with $\mathrm{GR}_{\mathrm{max}}$ and $\mathrm{GR}_{\mathrm{mean}}$ but negatively correlated with the D. Combined with the results of the different responses of chalkiness formation of the two rice varieties to HNT (Figure 1), we speculated that the shortened duration of grain filling caused by the accelerated grain-filling rate under HNT conditions may be responsible for the rice chalkiness formation. Considering that the grain-filling rate under HNT conditions increased to a much greater extent in JXZ than in HHZ (Table 1), it is unsurprising that JXZ was more sensitive to HNT-induced chalkiness formation than HHZ. For JXZ, although an increased grain-filling rate could compensate for the reduction in grain weight caused by HNT stress, the effects of HNT on the induction of chalkiness have not yet been eliminated (Figure 1 and Table 1). These findings suggest that the chalkiness formation is not directly associated with the reduction in grain weight caused by HNT.

During the grain-filling stage, the levels of endogenous hormones and the enzymatic activities related to starch biosynthesis in grains are tightly associated with the grain-filling rate [45,46]. Since the two rice varieties showed different responses in the grain-filling rate to the HNT stress, we explored the effects of HNT on the levels of endogenous hormones and the starch biosynthesis-related activities in grains of the two varieties. HNT caused the elevation of IAA contents and a reduction in ABA contents in both rice varieties. The change in IAA contents in JXZ was greater than that in HHZ, while the change in ABA contents in HHZ was greater than that in JXZ (Figure 5a,d). It was reported that IAA had a positive effect on grain filling by regulating the division of endosperm cells and enhancing sink strength [41]. ABA could also promote grain filling by modulating the enzymatic activities related to starch biosynthesis in cereal sink organs [47]. Therefore, changes in the IAA and ABA contents responding to HNT in JXZ may result in increased cell division and starch biosynthesis in the endosperm, eventually leading to fast grain filling. It is generally assumed that high temperature stress reduces CTKs content in the grains of rice. Under heat stress conditions, the heat-sensitive rice variety exhibited a significant reduction in CTKs content, while the heat-tolerant variety exhibited stable levels of CTKs [48]. In this study, we found that HNT decreased the content of CTKs in JXZ but not in HHZ (Figure 5c), suggesting that the grains of JXZ are more sensitive to HNT stress than those of HHZ. Accumulating studies have revealed that ethylene has a negative impact on grain filling in cereal crops [49–51]. For example, Sekhar et al. [49] pointed out that ethylene inhibited the starch biosynthesis and therefore caused the contents of soluble sugars to increase in developing rice grains. In our study, HNT treatment significantly decreased the contents of ACC (precursor for ethylene biosynthesis) in the grains of JXZ, while significantly increasing them in HHZ (Figure 5e). The decrease in ACC contents in the grains of JXZ may contribute to the acceleration of grain filling under HNT stress. Consistent with these results, we found that HNT dramatically increased the enzymatic activities related to starch biosynthesis (Figure 4). Overall, our results suggest that HNT treatment during the early stage of rice grain filling accelerated the grain-filling rate by changing the levels of endogenous hormones and enhancing the enzymatic activities related to starch biosynthesis. To better understand the relationship between rice chalkiness formation and the changes in the physiological characteristics of sink tissues to HNT stress, we performed two-way analysis variance. The results showed that the chalkiness degrees were positively correlated to SBE, DBE, and IAA, whereas they were negatively correlated with GAs, CTKs, and ACC. It was previously reported that the formation of chalky grains under high temperatures is mainly attributable to the inhibition of starch biosynthesis [52]. Liu et al. [53] demonstrated that the near-isogenic line CSSL50-1 (with high chalkiness) exhibited higher expression levels of genes encoding enzymes involved in starch biosynthesis in the developing grains. This is consistent with our findings that the activities of all five enzymes (SS-II, AGPase, SSS, SBE, and DBE) were dramatically increased in the grains of JXZ under HNT conditions. Considering that changes in endogenous hormone levels ultimately affect starch biosynthesis in developing grains, it is therefore important to control the HNT-induced chalkiness formation by regulating the activities of enzymes involved in starch biosynthesis.

Starch is the most abundant substance in rice endosperm, and the morphology and arrangement of starch granules are associated with the occurrence of grain chalkiness [54,55]. Chalkiness is the opaque portion of rice endosperm caused by loose packing of the starch granules. High temperatures during the grain-filling period affect the development of amyloplast in the endosperm, which leads to irregular packing of starch granules and causes an overall increase in chalkiness [20]. It has also been reported that in addition to the arrangement of starch granules, the morphological characteristics, such as the size and shape of starch granules, also displayed significant changes under high-temperature conditions [32,41]. In this study, we found that the effects of HNT on the morphology and arrangement of starch granules in JXZ were greater than those in HHZ (Figure 2). Under HNT conditions, starch granules in the endosperm of JXZ were poorly developed and single, and large air spaces were observed among the individual starch granules (Figure 2a). It should be specifically pointed out that HNT stress resulted in many small pits that were produced on the surface of single starch granules of JXZ (Figure 2a). At present, most studies have focused on the impact of the size, shape, and arrangement of starch granules on the rice chalkiness formation, while there are few reports on the correlation between the formation of small pits and the occurrence of grain chalkiness. Interestingly, it was reported that the starch granules with a similar structure (small pits) were also observed in the germinated rice grains [56,57], suggesting that the appearance of small pits on the surface of starch granules may be attributed to the partial degradation of endosperm starch. α-Amylase is the major hydrolytic enzyme that causes the degradation of starch in the endosperm of germinated rice seeds. It has been reported that high-temperature stress at the grain-filling stage significantly induced the expression of genes encoding α-amylase in developing rice grains [58]. The site-specific elevation of α-amylase genes in developing endosperm resulted in an increase in rice grain chalkiness. Furthermore, the SEM results demonstrated that the chalky endosperm was composed of loosely arranged starch granules that had many small pits on their surface [59]. Therefore, the degradation of starch granules caused by HNT might be one of the important reasons for the chalkiness formation in the grains of JXZ. Considering that the partially degraded starch granules were not observed in the grains of HHZ (Figure 2b), the sensitivity of the α-amylase to HNT stress should be further investigated in the two rice varieties (including the abundance and activity of α-amylase).

5. Conclusions

In conclusion, the effects of HNT at the early grain-filling stage on rice chalkiness formation were investigated by using two rice varieties that differed in susceptibility to high temperature. Our findings provided an insight into the physiological changes that cause the formation of chalky grains under HNT conditions during the early grain-filling period. The multiple reasons for the increase in rice chalkiness were analyzed, i.e., smaller amounts of carbohydrates supplied owing to decreased photosynthetic capacity in flag leaves, a shorter grain-filling duration resulting from an increased grain-filling rate, and higher activity of enzymes involved in starch biosynthesis due to imbalanced hormone levels in developing grains. The findings addressed in this study will provide a useful guideline for producing high-quality rice with low chalkiness to meet growing market demands.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13061475/s1, Table S1: Temperature setting of the different treatments in the climate chambers.

Author Contributions: Conceptualization, Y.D. and C.L.; methodology, Y.D. and X.D.; software, Y.D.; validation, Y.D., C.L. and Z.Z.; investigation, Y.D., X.D., J.L. and Y.X.; data curation, Y.D. and C.L.; writing—original draft preparation, Y.D. and D.L.; writing—review and editing, Y.D., D.L. and Y.Z.; project administration, D.L. and Y.Z.; funding acquisition, Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National High-Level Personnel of Special Support Program (“Ten Thousand Talents Program”), the National Natural Science Foundation of China (31971847) and the Jiangxi Province Rice Industry Technology System Special Project (JXARS-02-03).

Data Availability Statement: Data related to the research are reported in the manuscript. Any additional data may be acquired from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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