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Growth characteristics and endosperm structure of superior and inferior spikelets of *indica* rice under high-temperature stress

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Abstract

Heat stress severely reduces rice yield and quality; however, differences between the superior, early-flowering and inferior, later-flowering spikelets of *indica* rice in response to high-temperature stress during grain filling remain unclear. This study investigated the effects of high temperature (HT, 33.6/20.7 °C day/night) on growth, endosperm structure, and hormone and polyamine content of superior and inferior spikelets of heat-sensitive (SG-1) and heat-tolerant (HHZ) *indica* cultivars. The HT decreased fertilization rate, caused earlier grain filling, and reduced duration of grain filling, thus resulting in decreased grain mass and a poor endosperm structure. In addition, soluble sugar and sucrose content increased, and starch synthesis decreased by HT at the early stage of grain filling. The HT increased polyamine [spermidine (Spd) and spermine (Spm)] and abscisic acid (ABA) content, but reduced zeatin (Z) + zeatin riboside (ZR) and indole-3-acetic acid (IAA) content in the grains. Such effects were more apparent in the inferior than superior spikelets; however, the inferior spikelets of SG-1 were more affected than those of HHZ. At the middle grain filling stage, HT produced little difference between the two cultivars. Our results suggest that the poor development of inferior spikelets of SG-1 under the HT could be attributed, at least in part, to the changed content and ratios of free polyamines [putrescine (Put), Spd, and Spm] and phytohormones (Z+ZR, IAA, and ABA) and the conversion efficiency of sucrose into starch.

Additional key words: abscisic acid, auxin, cytokinin, *Oryza sativa,* polyamines, starch, sugars.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops as it serves as the main food for more than a half of the world's population. High temperature (HT) is becoming a major environmental stress factor limiting crop growth, development, productivity, and quality (Crowley 2000, Hakata *et al.* 2012). Previously, some researchers proposed that a high night temperature is more harmful than a high daytime temperature (Peng *et al.* 2004, Mohammed and Tarpley 2011, Coast *et al.* 2015). However, HT in the daytime is also harmful to rice production. At the heading and grain filling stages, rice is very sensitive to daytime HT (Kobata and Uemuki 2004, Morita *et al.* 2005). During these reproductive stages, HT impairs pollen development leading to yield losses (Tashiro and Wardlaw 1991), increased chalking rice (Zakaria *et al.* 2002); and shortening assimilate

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Abbreviations: ABA - abscisic acid; G - grain filling rate; HT - high temperature; IAA - indole-3-acetic acid; PAs - polyamines; Put - putrescine; Spd - spermidine; Spm - spermine; Z - zeatin; ZR - zeatin riboside.

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supply time by triggering early senescence (Fitzgerald and Resurreccion 2009). Grain filling is an important physiological process in rice development and starch is a major component of rice grain; therefore, a decrease in starch formation is considered a primary factor in the decrease of grain mass under HT (Yamakawa and Hakata 2010).

Polyamines (PAs) are group of low molecular nitrogen-containing compounds ubiquitous in all life forms. In plants, their major forms are diamine putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm). PAs are involved in various physiological events, such as growth, development, and response to stresses including HT (Alcazar *et al.* 2006). Reports indicated that HT could enhance content of PAs, thus increasing stress tolerance (Cvikrova *et al.* 2012). Plant hormones play important roles in regulation of pollen and grain development. Some researchers demonstrated that indole-3-acetic acid (IAA) deficiency and excessive abscisic acid (ABA) could trigger pollen abortion (Chakrabarti *et al.* 2010). High temperature decreases IAA content and increases ABA content in rice anthers and grains leading to floret sterility and poor grain filling (Wang *et al.* 2011).

In rice, the classification of superior and inferior spikelets is performed according to their flowering date and location within a panicle. Superior spikelets are usually located on apical primary branches. They flower earlier, fill faster, and have larger and heavier grains. In contrast, inferior spikelets are located on proximal secondary branches, flower later, fill slowly, and have smaller and lighter grains (Yang *et al.* 2008). The effect of HT on hormone changes of the superior and inferior grains has been reported in maize (Zhao *et al.* 2014). However, its effect on the superior and inferior spikelets of rice during grain filling remains unclear.

In the present study, two widely grown rice cultivars, heat-sensitive Shuangui No. 1 (SG-1) and heat-tolerant Huanghuazhan (HHZ), were selected (Cao *et al.* 2008, 2009). We treated these two cultivars with a high daytime temperature during grain filling and measured following data: spikelet sterility, yield and yield components, grain filling characteristics, and content of starch, sucrose, soluble sugars, PAs (Put, Spd, and Spm), and hormones [zeatin (Z) + zeatin riboside (ZR), IAA, and ABA] in the superior and inferior spikelets. The objective was to determine the effect of the high daytime temperature during grain filling on the superior and inferior spikelets and the role of PAs and hormones in regulation of rice grain filling.

Materials and methods

Plants and growing conditions: The experiment was conducted with two mid-maturity *indica* cultivars with different heat tolerance, Huanghuazhan (HHZ, heat-tolerant) and Shuanggui 1 (SG-1, heat-sensitive), at a farm of the Yangzhou University, Jiangsu Province, China (32° 30′ N, 119° 25′ E) during the growing season (May - October) of 2010 and repeated in 2011. Heat tolerance of the two cultivars was analyzed in our previous studies (Cao *et al.* 2008, 2009). Seeds of both genotypes were sown on 12 to 14 May and the seedlings transplanted to plastic pots on 4 to 6 June. Each pot contained three hills with two seedlings per hill. Soil nutrient conditions were as follows: an organic matter of 19.2 g kg^{-1}, available nitrogen of 97.5 mg kg^{-1}, available phosphorus of 25.3 mg kg^{-1}, and available potassium of 80.4 mg kg^{-1}. The time and amount of fertilizer applied during planting was according to Cao *et al.* (2009). Each genotype was planted in 90 pots. All the pots maintained a water layer of 1 - 2 cm in depth during the whole growth period except for drainage at the end of tillering. Other crop managements were conducted following the same procedure as the conventional high-yielding cultivation.

Heat stress treatment: Plants approaching the heading stage (2 - 7 August) were moved to a greenhouse. The period of heat treatment was from initial heading to 10 d after heading [T1: the early grain filling stage, August 2 - 11 (2010) or 4 - 13 (2011) for SG-1; and August 4 - 13 (2010) or 7 - 16 (2011) for HHZ)] and from 11 to 20 d after heading [T2: the middle grain filling stage, August 12 - 21 (2010) or 14 - 23 (2011) for SG-1 and August 14 - 23 (2010) or 17 - 26 (2011) for HHZ]. Thereafter, the plants resumed their growth under normal conditions outside the greenhouse until maturity. Each treatment comprised 30 pots as replicates. Average day/night temperatures in the greenhouse were 33.6/20.7 °C and control temperatures in the normal outdoor conditions were 29.8/20.7 °C (for details see Cao *et al.* 2008, 2009). During the period of the heat stress treatment, temperatures in the T1 and T2 treatments were consistently higher than those in the control, and the relative humidity in T1 and T2 was consistent with that in the control (Fig. 1 Suppl.).

Sampling methods: A total of 300 to 400 panicles that headed on the same day were chosen and tagged for each treatment. The flowering date and position of each spikelet on some tagged panicles were recorded. A total of 40 to 50 tagged panicles from each treatment were sampled every 5 d from anthesis to maturity. The sampled panicles were divided into two parts (the superior and inferior spikelets) as described in Yang *et al.* (2008). Approximately 80 to 100 sampled grains were dried at 70 °C for 72 h, and the constant masses were recorded. The other grains were frozen in liquid nitrogen and then stored at -80 °C to measure content of phytohormones and PAs.

Grain filling process fitted the Richards (1959) growth equation, and grain filling rate was calculated as described by Yang *et al.* (2003). Five pots of plants from each treatment were harvested and data, including grain yield, fertilization rate, 1000-grain mass, seed-setting rate, and plump grain percentage were determined for the superior and inferior spikelets, respectively, according to Yang *et al.* (2007).

Observation of endosperm structure: For scanning electron microscopy analysis, mature 10-hulled superior and inferior grains from each treatment were divided into halves, by hitting and not by cutting, with the edge of a scalpel blade to produce natural fracture surfaces at the largest diameters. The grain halves were fixed on a copper sample table using a double-sided tape, sputter-coated with gold in an ion sputter, and then observed with a scanning electron microscope (*XL-30, Philips*, The Netherlands).

Determination of soluble sugars, sucrose, starch, PAs, and phytohormones: Soluble sugars and sucrose content were analyzed by anthrone colorimetry (Yemm and Willis 1954). Starch content was determined by dual wavelength colorimetry (Williams *et al.* 1958).

Polyamines (free Spd, Spm, and Put) were extracted and measured according to Yang *et al.* (2008). Hormones (Z+ZR, IAA, and ABA) were extracted and purified according to previous studies (Yang *et al.* 2003) and measured by high-performance liquid chromatography (*P680 Pump/UVD170U UV-VIS Detector*, Waters, USA). Exactly 0.01 cm^3 of a sample was injected and loaded onto a 4.6 mm × 250 mm, 5-mm particle size, reverse-phase (C18) column. A column temperature was 30 °C, a flow rate of 1.0 cm^3 min^{-1}, and an absorbance was read at 254 nm.

Statistical analysis: The results were analyzed using the *SAS/STAT* statistical package (*v. 6.12*; SAS Institute, Cary, NC, USA). Where there was no significant difference between the data from the two years, then the values from all the experiments for that parameter were used to obtain the mean and standard deviation. The means were separated using Duncan's test at $\alpha = 0.05$ or 0.01.

Results

The high-temperature treatment during early grain filling (T1) significantly decreased spikelet fertility of the cv. SG-1. The percentage of fertilized spikelets decreased by 16.3 and 23.2 % for the superior and inferior spikelets, respectively. However, the HT treatment only slightly affected the superior spikelets of the heat-tolerant cv. HHZ. By contrast, the inferior spikelets decreased by 11.3 % showing a significant difference from the control (Fig. 1). These observations indicate that the inferior spikelets exhibited a lower fertilization rate than the superior ones, and that SG-1 showed an overall lower fertilization rate than HHZ. The effects of the HT at the middle grain filling stage (T2) were not significantly different between the two cultivars.

Similarly to spikelet fertilization, seed-setting rate and plump grains percentage of the two cultivars decreased under the HT; the percentage decrease was larger in SG-1 compared with HHZ, in the later-flowering inferior spikelets compared with the earlier-flowering superior spikelets, and at the T1 than T2 treatment (Table 1).

The HT had no obvious effect on 1 000 fertilized grain mass in the cv. HHZ, but in SG-1, the T1 treatment

Table 1. Effects of high temperatures (33.6/20.7 °C day/night) during heading and grain filling on filled-grain percentage and 1000 fertilized grain mass of superior and inferior grains of rice (HHZ - Huanghuazhan, SG-1 - Shuanggui 1; S - superior spikelets, I - inferior spikelets, T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading). Means ± SDs were calculated from the data of five pots. Values followed by different letters within the same column and cultivar are significantly different at $P < 0.05$ between the treatment and control.

Cultivar	Treatment	Yield [g m^{-2}]	Seed-setting rate [%]		Plump grains percentage [%]		1000 grains mass [g]	
			S	I	S	I	S	I
HHZ	CK	803 ± 40 a	91.9 ± 1.4 a	91.6 ± 2.5 a	92.9 ± 1.3 a	92.6 ± 2.5 a	20.5 ± 0.7 a	16.4 ± 0.8 a
	T1	753 ± 38 a	88.4 ± 5.2 a	80.2 ± 4.1 b	90.2 ± 3.8 a	82.4 ± 4.9 b	19.9 ± 0.6 a	14.7 ± 0.9 b
	T2	765 ± 28 a	89.0 ± 4.8 a	83.3 ± 2.7 ab	92.0 ± 2.4 a	86.8 ± 1.4 ab	20.0 ± 0.5 a	15.0 ± 0.2 ab
SG-1	CK	798 ± 32 a	84.1 ± 3.5 a	76.6 ± 6.4 a	85.2 ± 2.8 a	78.7 ± 5.5a	18.1 ± 0.5 a	14.8 ± 0.8 a
	T1	592 ± 19 c	70.6 ± 8.9 b	59.3 ± 5.7 b	65.5 ± 3.4 b	60.6 ± 3.8 b	14.0 ± 0.9 b	11.6 ± 0.5 c
	T2	718 ± 29 b	83.9 ± 2.6 a	68.3 ± 7.0 ab	85.1 ± 1.5 a	71.1 ± 2.7 a	17.9 ± 0.4 a	13.1 ± 0.5 b

decreased 1 000 fertilized grains mass of the superior and inferior spikelets remarkably; the inferior grains were reduced significantly also under the T2 treatment.

The decrease of seed-setting rate caused by spikelets fertilization, plump grains percentage and 1000 fertilized grains mass under the HT led to a yield reduction. The yield reduction of the cv. SG-1 was higher than that of the cv. HHZ. Moreover, the T1 treatment had larger effects on yield than the T2 treatment. The yield of SG-1 decreased by 25.8 % during the T1 treatment and by 10.0 % during the T2 treatment compared with the control. By contrast, the yield of HHZ decreased by 6.2 % in the T1 and by 4.7 % in the T2 (Table 1).

Under the normal growth conditions, the superior and inferior spikelets reached their maximum grain mass at 15 to 20 d and 25 to 30 d after anthesis, respectively.

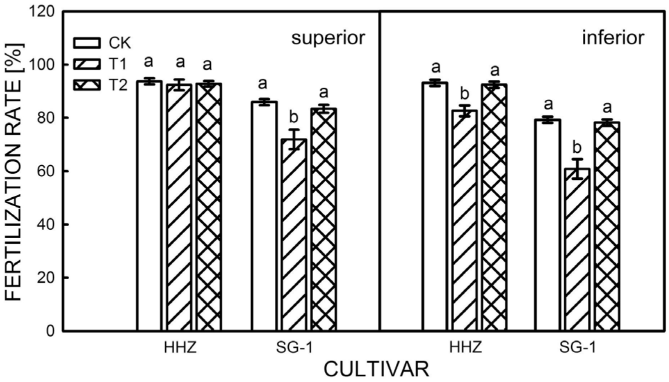

Fig. 1. Effects of high temperatures of 33.6/20.7 °C (day/night) for 0 - 10 d after heading (T1) or after anthesis (11 - 20 d after heading, T2) on spikelet-fertilized rate of superior and inferior spikelets in rice. The fertilization rate was defined as the ratio of the fertilized grains (including both completely and partially filled grains) to the total number of grains. HHZ - Huanghuazhan, SG-1 - Shuanggui 1, CK - normal conditions. Means ± SDs, *n* = 30; different letters for the same cultivar indicate significant differences induced by the treatments at $P < 0.05$.

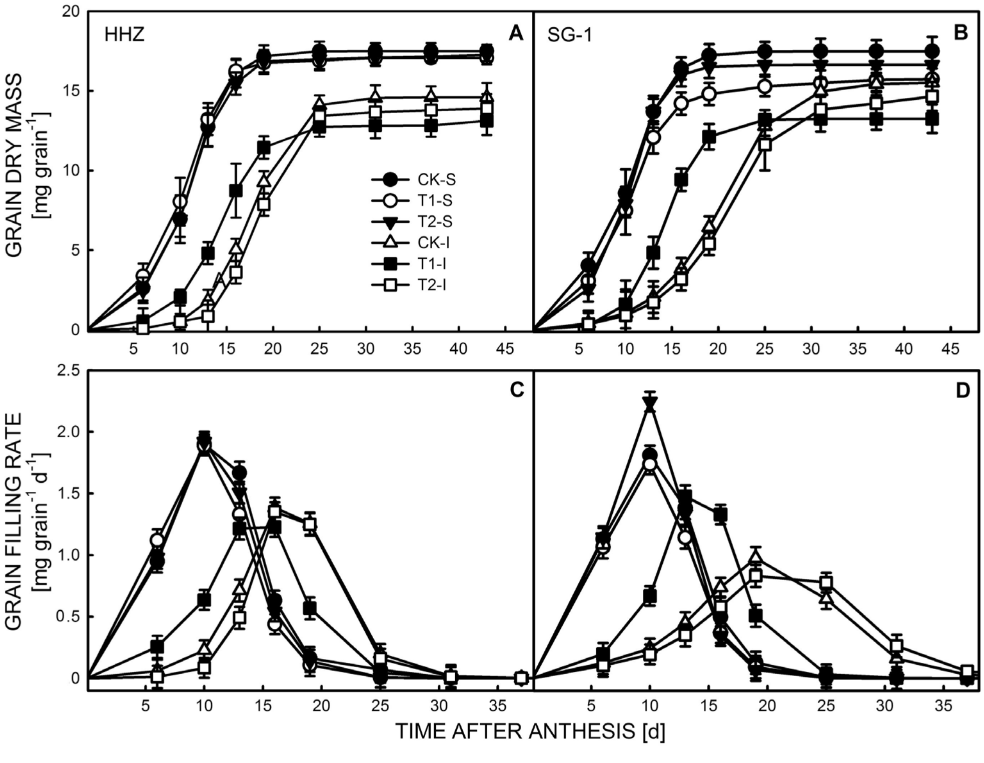

Fig. 2. Changes in superior and inferior grain dry masses (*A*,*B*) and grain filling rates (*C*,*D*) in rice cultivars HHZ and SG-1 under high tempereatures (HT) of 33.6/20.7 °C (day/night). Superior (S) and inferior (I) grain dry masses were calculated using grains harvested from 20 spikelets as one repeat, and each data point represents a mean from three repeats. Grain filling rate was calculated according to the Richards (1959) equation. CK - normal conditions, T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading. Means ± SDs.

Under the HT, maximum dry mass per grain decreased in both the cultivars. The T1 treatment significantly decreased maximum dry mass of the superior (9.7 %) and inferior (14.2 %) spikelets in SG-1, and the inferior spikelets (10.3 %) in HHZ (Fig. 2*A*, *B*). However, maximum dry masses of the superior spikelets in HHZ under the T1 decreased by only 2.3 %, which was not significant (Fig. 2*A*). The T2 treatment had no significant effect on maximum dry mass of the superior (1.1 and 4.8 %) and inferior (4.6 and 5.8 %) grains in HHZ and SG-1, respectively (Fig. 2*A*,*B*).

The grain filling rate curves of the superior and inferior spikelets displayed an obvious asynchronous grain filling shape (Fig. 2*C*,*D*). Under the normal growth conditions, the time to reach the maximum grain filling rate (G_{max}) of the superior spikelets (about 11 d) was shorter than that for the inferior ones (17 to 20 d) in both genotypes (Table 2 Suppl.). Under the HT for the inferior spikelets, the T1 treatment significantly decreased the time to reach G_{max} in both cultivars, and a peak of grain filling in SG-1 was earlier than in HHZ. The T1 prolonged the active grain filling period and reduced G_{max} and G_{mean} in HHZ, whereas in SG-1, the change was opposite for these three parameters. The loss caused by the decreased grain filling rate in HHZ was less than that caused by the short grain filling period in SG-1. Thus, grain mass of the inferior spikelets in HHZ showed a smaller decrease than that of SG-1. For the superior spikelets, the T1 decreased the active grain filling period and grain filling rate resulting in a lower grain mass in SG-1. There was no obvious change in HHZ (Table 2 Suppl.). The T2 treatment significantly increased G_{max} and decreased the active grain filling period resulting in a lower grain mass in the superior spikelets in SG-1. The T2 treatment had no obvious effect on the inferior spikelets in SG-1 or on the superior and inferior spikelets

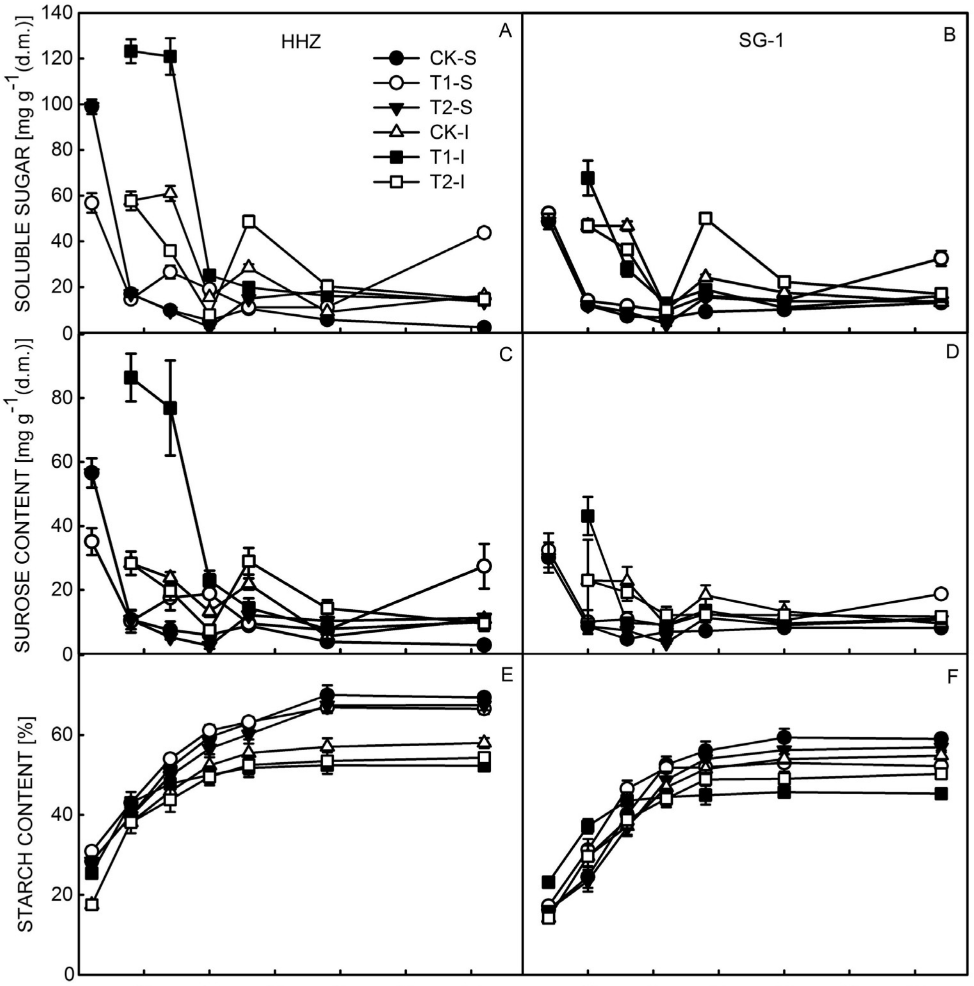

Fig. 3. Changes in content of soluble sugars (*A*,*B*), sucrose (*C*,*D*), and starch (*E*,*F*) in superior (S) and inferior (I) spikelets under high tempereatures (HT) of 33.6/20.7 °C (day/night). HHZ - Huanghuazhan, SG-1 - Shuanggui 1, CK - normal conditions, T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading. Means ± SDs, *n* = 3.

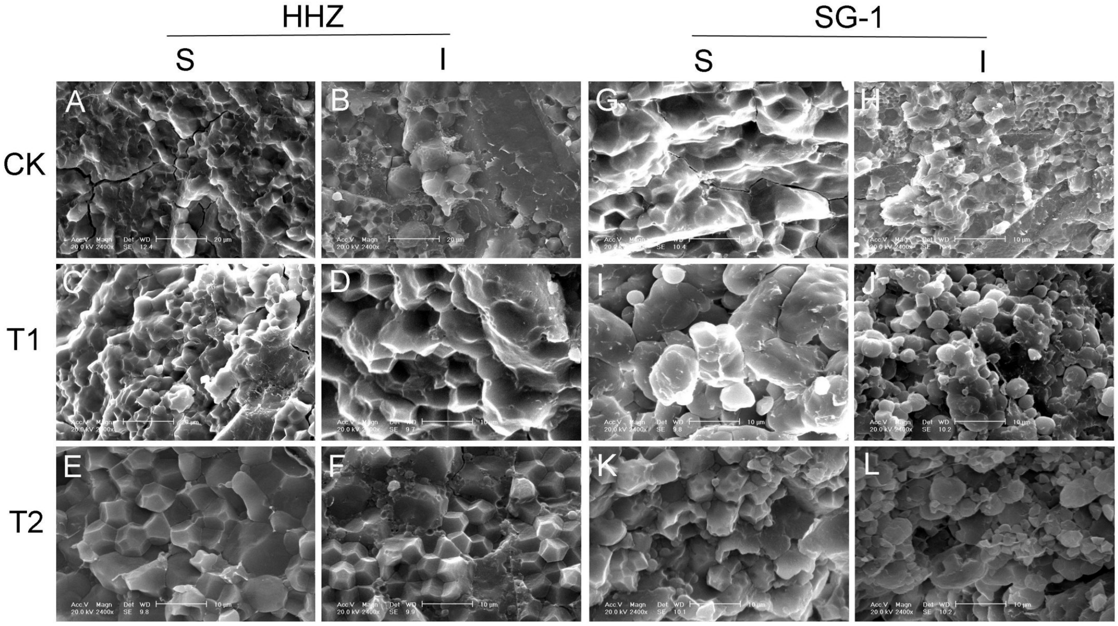

Fig. 4. Effects of high temperatures of 33.6/20.7 °C (day/night) on rice endosperm structure of dorsal parts of superior (S) and inferior (I) spikelets in cv. HHZ (*A-F*) and cv. SG-1 (*G-L*) at maturity. CK - normal temperature (29.8/20.7 °C, day/night), T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading. The *bars* in *A,B,C* 20 µm and in *D,E,F,G,H,I,J,K,L* 10 µm.

in HHZ (Table 2 Suppl.).

In the normal conditions, soluble sugar content declined throughout the grain filling period and varied with temperature (Fig. 3*A*,*B*). Under the T1 treatment, soluble sugar content of the superior spikelets was higher than under the normal temperature in the whole growth period in both cultivars, except that HHZ was lower than the control. The T1 treatment increased soluble sugar content of the inferior spikelets in both cultivars. After the HT treatment, soluble sugar content in SG-1 decreased quickly, but in HHZ remained high for a long time and then decreased. Soluble sugar content during the T2 treatment was equal to (the superior grains) or lower than (the inferior ones) that at the normal conditions. The changes of sucrose content under the HT were consistent with those of soluble sugars (Fig. 3*C*,*D*).

Starch content increased throughout the grain filling period under the normal conditions and varied with temperature (Fig. 3*E*,*F*). The T1 treatment enhanced starch accumulation, but there was no significant difference between the treatment and the control in the T2. Finally, at maturity, starch content of the superior and inferior grains in both cultivars under the T1 and T2 were lower than under the normal temperature: the superior grains had a higher starch content than the inferior ones, and the change in HHZ was lesser than that in SG-1 (Fig. 3*E*,*F*).

To understand whether the HT could affect development of the superior and inferior spikelets, dorsal endosperm structures were observed at maturity under a scanning electron microscope (Fig. 4). In the normal conditions, the superior and inferior spikelets of HHZ and the superior spikelets of SG-1 had similar endosperm structures. Amyloplasts of the endosperm were arranged regularly and tightly with small or no gaps among polygonal complex starch granules, whereas the inferior spikelets of SG-1 had some starch complex granules and single granules.

The T1 treatment had no significant effect on endosperm structure of the superior and inferior spikelets of HHZ (Fig. 4*C*,*D*); however, the T1 treatment significantly affected the cv. SG-1, the superior spikelets of which had heterogeneous granules including many large elliptical starch complex granules and a small amount of spherical starch simple granules with large gaps and irregular arrangements (Fig. 4*I*). The inferior spikelets had a large number of single starch granules with large gaps and few starch complex granules (Fig. 4*J*). The T2 treatment had no obvious effect on the superior spikelets of either cultivar. The edge of some partial starch compound granules was broken in the inferior grains of HHZ (Fig. 4*F*). Clusters of small starch simple grains were intertwined with large starch complex grains in the inferior grains of SG-1 (Fig. 4*L*).

Under the normal temperature, content of Put, Spd, and Spm increased at the early grain filling stage. The superior spikelets had the largest content at 5 to 10 d after anthesis, whereas the inferior ones showed a peak at 15 to 20 d, and then PAs content decreased rapidly (Fig. 5). Polyamines content in the two cultivars showed a similar trend, however, the peak value of the cv. HHZ was higher than that of the cv. SG-1. Free PAs content during the early grain filling (3 to 10 d after anthesis) in the superior spikelets was higher than in the inferior ones, however, after reaching a maximum, free PAs content in the superior spikelets was lower than in the inferior ones.

The T1 treatment increased free PAs content in the superior and inferior spikelets in early grain filling both cultivars, especially in terms of peak content. The time taken by the inferior spikelets to reach a maximum value was shorter than in the control plants. After the maximum, Spd and Spm content was lower under the HT than in the control (Fig. 5*C*,*D*,*E*,*F*), whereas free Put content was higher or not significantly different compared with the control (Fig. 5*A*,*B*). The maximum values of free PAs in the inferior spikelets were larger than in the superior ones, and they were higher in SG-1 than in HHZ. The T2 treatment decreased free PAs content at the early stage, enhanced free Spd content at the middle and later stages, and delayed the time taken by the inferior spikelets to reach peak PA values (Fig. 5).

Content of Z+ZR and IAA was different in the superior and inferior spikelets. Content of Z+ZR and IAA in the superior spikelets transiently increased at the early grain filling stage, reached a maximum at 10 d after anthesis, and then decreased. Content of these hormones in the inferior spikelets slowly increased during the early grain filling stage, reaching a maximum at 15 to 20 d after anthesis, and then decreased slowly. Content of Z+ZR and IAA in the superior spikelets during the early grain filling stage (5 to 15 d after anthesis) was higher than in the inferior ones, but it was lower than that of the inferior ones at 15 to 20 d after anthesis. The HT treatment decreased content of Z+ZR and IAA in the grains. This tendency was more obvious in the cv. SG-1 compared with the cv. HHZ, in the T1 than in the T2, and in the inferior than superior spikelets (Fig. 6*A*,*B*,*C*,*D*).

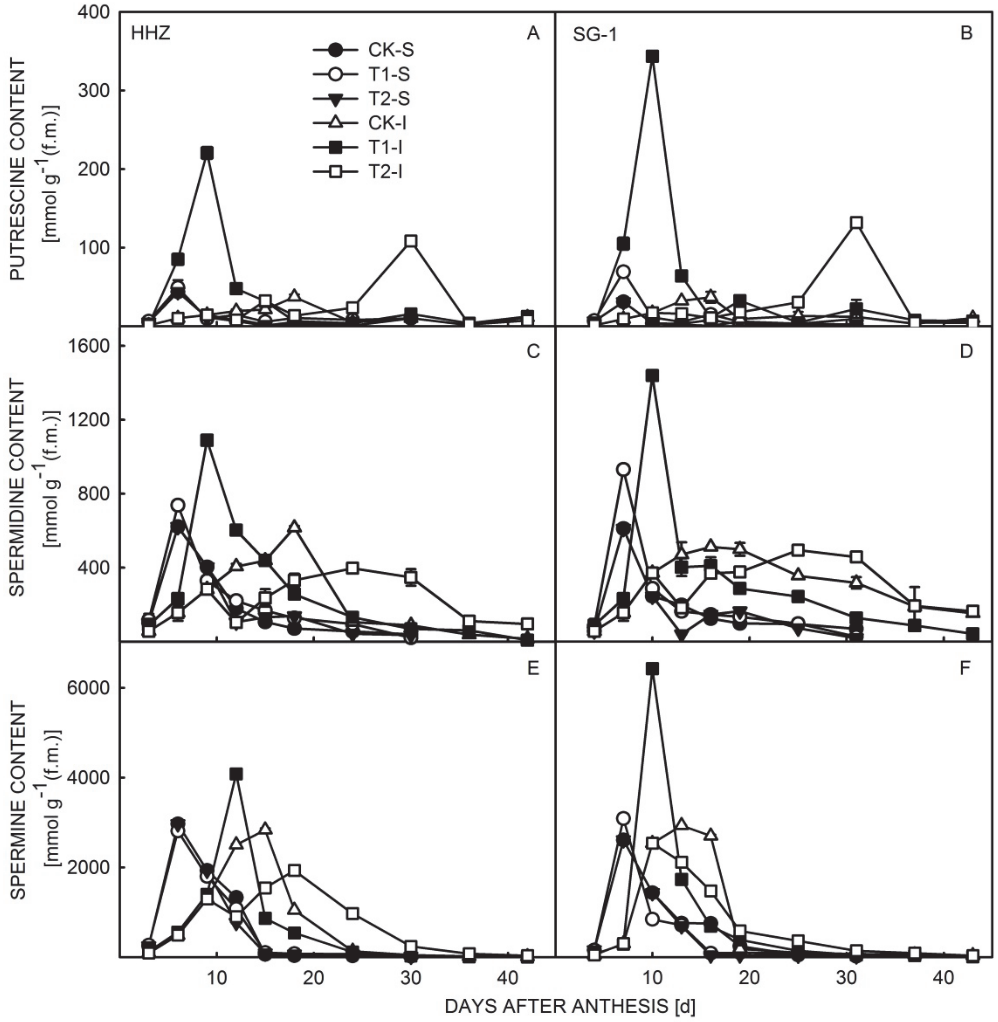

Fig. 5. Changes in content of putrescine (*A*,*B*), spermidine (*C*,*D*), and spermine (*E*,*F*) in superior (S) and inferior (I) spikelets under high tempereatures (HT) of 33.6/20.7 °C (day/night). HHZ - Huanghuazhan, SG-1 - Shuanggui 1, CK - normal conditions, T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading. Means ± SDs, *n* = 3.

Similarly to Z+ZR and IAA content, ABA content under the normal conditions transiently increased at the early grain filling stage and then decreased (Fig. 6*E*,*F*).

The HT affected ABA content of the inferior but not superior spikelets. The T1 treatment increased ABA content of the inferior spikelets significantly. In addition, the time taken by the inferior spikelets to reach a peak ABA content was shorter by 5 d. Then, ABA content of the inferior spikelets decreased during later grain filling. This trend was more obvious in the cv. SG-1 compared with the cv. HHZ. Content of ABA decreased at the early stage of the T2 treatment, and in the middle and later stages, ABA content was enhanced. Content of Z+ZR, IAA, and ABA in the heat-tolerant cultivar was higher than in the heat-sensitive cultivar under the HT.

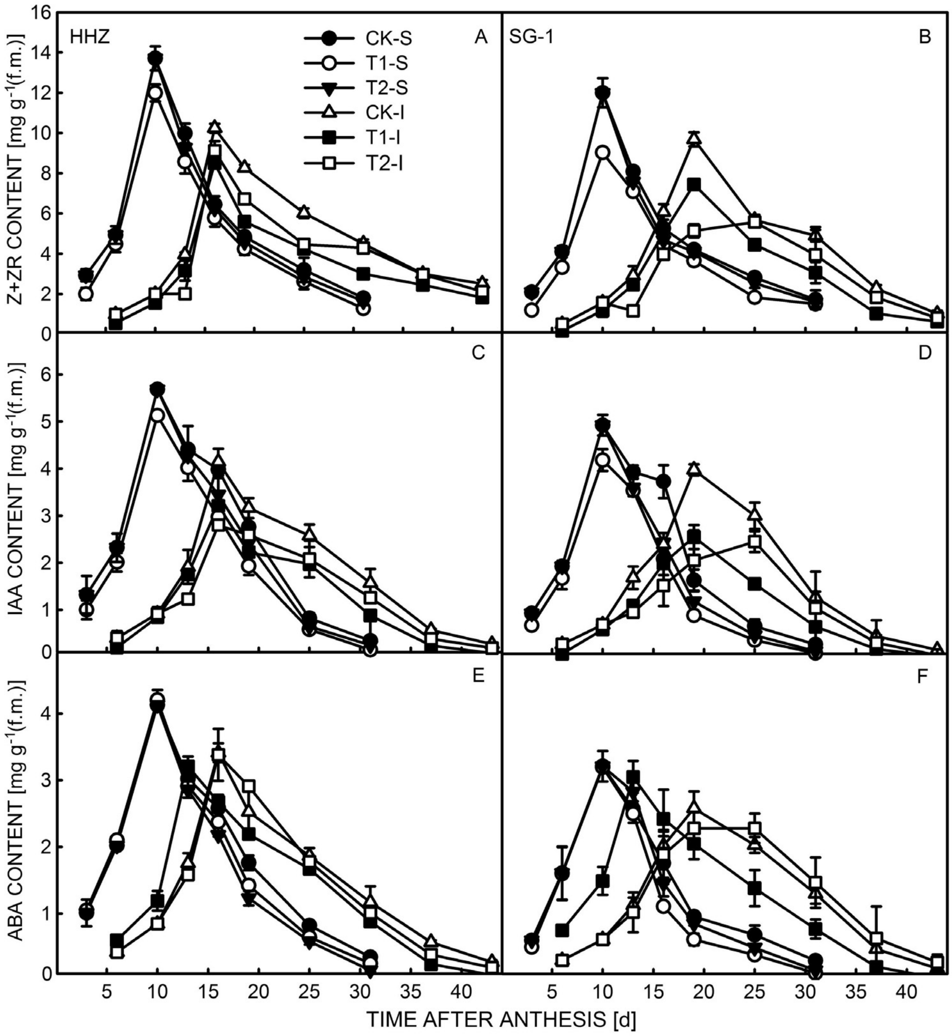

Fig. 6. Changes in content of zeatin+zeatin riboside (Z+ZR; *A,B*), indole-3-acetic acid (IAA; *C,D*), and abscisic acid (ABA; *E F*) in superior (S) and inferior (I) spikelets under high tempereatures (HT) of 33.6/20.7 °C (day/night). HHZ - Huanghuazhan, SG-1 - Shuanggui 1, CK - normal conditions, T1 - HT during 0 - 10 d after heading, T2 - HT during 11 - 20 d after heading. Means ± SDs, *n* = 3.

Discussion

High-temperature stress affects rice grain fertility significantly, leading to a decrease in seed set. Temperatures over 36 °C or about 1 h exposure to a temperature greater than 33.7 °C at anthesis causes pollen sterility (Chakrabarti *et al.* 2010), which is consistent with our results. A high mean daytime temperature over 33.6 °C (Fig. 1 Suppl.) lasting for about 10 d after anthesis leads to spikelet sterility, and a decrease in seed set results in a yield loss (Fig. 1). However, we also found that the grain positions were significantly different under the HT. The superior spikelets had a lower grain sterility (7.6 - 28.1 %) than the inferior ones (11.6 - 39.2 %) under the T1 treatment (Fig. 1). Based on previous studies, a decrease in spikelet fertility or an increase in spikelet sterility under the HT is related to a number of factors, including poor anther dehiscence, reduced numbers of pollen grains, poor pollen germination, and early peak anthesis (Prasad *et al.* 2006). Our study indicates that the effect of the HT on the cultivars demonstrated differential responses in spikelet fertility. Interactions between temperature and duration of exposure to HT are the causes of different spikelet sterility among genotypes (Jagadish *et al.* 2007).

In addition to grain fertility, the HT significantly affected rice grain filling (Fig. 2, Table 2 Suppl.). Similar results have been reported under night-time HT (Morita *et al.* 2005). However, our results reveal that the changes were more significant in the inferior spikelets. The high daytime temperature significantly increased early grain filling rate and decreased later grain filling rate of the inferior spikelets (Fig. 2). Duration of grain filling in the inferior spikelets was shortened, and the increase in grain filling rate did not compensate for the loss of the grain filling duration, leading to a loss of inferior grain mass. A previous study suggested that HT at night could increase grain filling rate, which has a greater effect than daytime HT. However, if the temperature exceeds a threshold, grain filling rate decreases (Morita *et al.* 2005). In addition, we found that the maximum grain filling rate in the cv. SG-1 under the T1 treatment was more affected compared with the control plants, which suggests that the HT might severely affect inferior spikelet growth, leading to obvious grain mass decreases (Fig. 2 and Table 2 Suppl.).

Grain filling is accompanied by starch accumulation and soluble sugar transformation. A previous study indicated that HT could promote starch accumulation at the early grain filling stage and inhibit starch accumulation at the middle and late grain filling stages compared with the control (Phan *et al.* 2013). Our experiment shows the same result under the T1 treatment, but the T2 treatment slightly decreased starch content during the whole filling stage (Fig. 3). The change of grain mass correlated with that of starch content under the HT. Starch is formed from sucrose by a series of reactions, and HT enhances sucrose content (Phan *et al.* 2013). In the present study, the T1 treatment decreased sucrose content of the superior spikelets, while it increased sucrose content of the inferior ones (Fig. 3*C*,*D*). The change in sucrose content of the inferior spikelets may have resulted from accelerating proliferation of endosperm cells during grain filling under the HT (Morita *et al.* 2005). However, their proliferation depends on an increased nutrition, which might then trigger rapid recycling stored carbon from a source to a sink, leading to the inferior spikelets having high sucrose and starch content under the T1 treatment (Fig. 3*C*,*D*,*E*,*F*). These results suggest that assimilate supply (sucrose, soluble sugars) may not be the main cause of poor filling of the inferior spikelets (Wakasa *et al.* 2011). Low activities of starch synthesis-related enzymes, downregulation of respective genes and sucrose transporter genes, and upregulation of starch degradation-related genes lead to a low starch content (Zhao *et al.* 2014).

The structure of the rice endosperm is an important factor in determining rice quality. Previous studies focused on differences in endosperm structure among cultivars (Cheng *et al.* 1999). High temperature accelerates proliferation of endosperm cells and reduces cell size (Morita *et al.* 2005). With an increase of a mean daytime temperature (from 21 to 30 °C) during the grain filling stage, starch granules in the endosperm are regularly shaped and orderly arranged. However, HT changes them into various shapes and causes them to be chaotically arranged and accompanied by obvious natural gaps between starch granules (Xu *et al.* 2012). In our study, similar results were observed in the grains of the heat-tolerant cv. HHZ and for the superior spikelets of the heat-sensitive cv. SG-1. They had regularly shaped and orderly arranged starch granules except at the HT during the early filling stage (Fig. 4). However, we also found that the superior and inferior spikelets in the cv. SG-1 treated with the HT at the T1 contained starch granules of different sizes with obvious gaps that were arranged chaotically (Fig. 4*I*,*J*), resulting in mature grains with a chalky appearance. This confirmed that photosynthetic product conversion could not satisfy the need of grain filling because the grain filling rate was too fast at the early filling stage in inferior spikelets.

Some studies have reported that abiotic stresses affect grain growth and development by regulating content of PAs; a higher PAs content is beneficial for grain growth (Yang *et al.* 2008). In the present study, the T1 treatment increased PAs content at the early filling stage and decreased it at the later stage, ultimately leading to grain yield reduction. This is different to a result obtained under drought stress (Chen *et al.* 2013). The changes in free Spd and Spm content and the ratio of (Spd+Spm) to Put are positively associated with the grain filling rate (Table 3 Suppl.). Application of Spd or Spm to rice panicles increases activities of sucrose synthase, ADP glucose pyrophosphorylase, and soluble starch synthase in grains significantly, whereas polyamine biosynthesis inhibition decreases their activities (Wang *et al.* 2011). In our study, Put content in the grains showed no significant correlation with grain filling (Table 3 Suppl.). The result suggests that free Put in the grains could respond to the HT, but Put played only a minor role in grain development. Previous research reported that a high content of PAs correlates with plant tolerance to a wide array of environmental stresses. Compared with stress-intolerant plants, stress-tolerant plants have generally a larger capacity to enhance PA biosynthesis in response to abiotic stresses (Alcazar *et al.* 2006). In our study, the low PA content in the cv. SG-1 accounted for its higher sensitivity to the HT compared with the cv. HHZ, which had a high PAs content (Fig. 5). The heat-tolerant cultivar or superior spikelets had a relatively stable PAs content under the HT. Grain growth may not depend only on PAs content but also on the ratio of free (Spd+Spm) to Put.

Plant hormones play an important role in regulation of grain growth and development and HT affects the endogenous hormone content in rice. Wang *et al*. (2005) have reported that IAA and ZR content under HT is significantly lower than in control plants during early filling, whereas ABA content is higher than in the control during the whole filling process. Zhao *et al.* (2014) demonstrated that HT decreases content of IAA and ZR of superior and inferior kernels in maize. In our study, Z+ZR, IAA, and ABA content, and the ratio of ABA to (Z+ZR+IAA) were positively associated with grain filling (Table 4 Suppl.). A lower Z+ZR and IAA content, and a higher ABA content and a lower ratio of ABA to (Z+ZR+IAA) might be the main cause of abnormal grain filling and a lower grain mass in the inferior spikelets. The results suggest that grain growth and development depends not only on hormone type and content, but also on balance between the hormones. We speculated that the role of ABA is larger than that of Z+ZR and IAA: a higher hormone content and a higher ratio of ABA to (Z+ZR+IAA+ABA) are beneficial for grain filling.

In conclusion, the HT at the heading and early grain filling stages decreased grain fertility and yield, increased grain filling rate and starch content at the early grain filling stage, decreased them at the middle and later stages, reduced duration of grain filling, and promoted soluble sugars and sucrose accumulation in the inferior spikelets. Under the HT, endosperm structure of the inferior spikelets was abnormal; differently sized starch granules were arranged chaotically and had obvious gaps. Inferior spikelet abnormal grain filling and a small final grain mass were closely associated with a low starch accumulation and content of Spd, Spm, Z+ZR, IAA, and ABA. The ratios of (Spd+Spm) to Put, and ABA to (Z+ZR+IAA) also affected inferior spikelet abnormal grain filling. The effect of the HT on the superior spikelets was lower than that on the inferior ones. The high temperature had also a lower effect on the heat-tolerant cv. HHZ compared with the heat-sensitive cv. SG-1. The earlier the period of the HT, the more effects were observed. Further investigation is needed to reveal the molecular mechanism of the differences in growth of the superior and inferior spikelets between the two cultivars under HT.

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