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Relationships among the chalkiness, kernel size and endosperm cell morphology of rice kernels at different spikelet positions within a panicle

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ABSTRACT

The occurrence of chalky kernels in rice is causally related to kernel size and endosperm morphology. This study aimed to investigate the occurrence of chalky kernels by analyzing kernel size, and the number and area of endosperm cells. Spikelets were sampled from upper and middle primary branches and lower secondary branches in a panicle, and divided into four categories: upper, middle, and lower perfect (PF) kernels and lower milky-white (MW) kernels. On the lower secondary branches, there was a higher percentage of chalky kernels, with smaller kernel lengths, widths and thicknesses, than the kernels on the upper and middle primary branches. MW kernels were smaller in size than PF kernels even on the same lower secondary branches. Regardless of grain appearance quality traits, the total areas of endosperm cross sections in lower kernels were significantly smaller than in upper kernels owing to the decreased cell area, and there was a significant negative correlation between the number of cells and average cell area. When the numbers and the areas of cells were analyzed using angular 30° intervals from the line connecting the center point and the dorsal vascular bundle, the MW kernels had significantly less cells than PF kernels near the ventral side at 120–180°. Thus, the decrease in the number of cells near the ventral side was a main causal factor in the decrease in MW kernel widths compared with PF kernel widths, and this suggested that cell division in MW kernels was inhibited at the early grain-filling stage.

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Chalky kernel; cross section; endosperm cell; grain appearance quality traits; kernel size; rice

Introduction

The percentage of first-grade rice kernels in Japan has decreased recently, reducing rice farmer incomes (MAFF, 2011; Morita, Wada, & Matsue, 2016). A major factor of this decrease is the occurrence of chalky kernels (Kawatsu, Homma, Horie, & Shiraiwa, 2007; Terashima et al., 2001). Chalky kernels result from a decreased starch accumulation, with unfilled spaces existing among starch granules during the grain-filling stage. These result in the irregular reflection of light, which gives the appearance of opaque milky spots (Tashiro & Ebata, 1975). Based on the position of the milky portion, chalky kernels are divided into milky-white (MW), white-belly, white-back, white-based and white-core phenotypes. The occurrence of chalky kernels is generally caused by high-temperature (Takata, Sakata, Kameshima, Yamamoto, & Miyazaki, 2010; Yoshida & Hara, 1977) and low solar radiation (Kobata, Uemuki, Inamura, & Kagata, 2004; Takata et al., 2010; Tsukaguchi et al., 2012) conditions during the grain-filling stage, and it significantly differs among cultivars (Wakamatsu, Sasaki, Uezono, & Tanaka, 2007). In addition, its occurrence rate differs among spikelet positions within a panicle; the percentage of the chalky kernels is higher on lower secondary branches than on upper primary branches (Kido & Yanatori, 1968; Nagato & Chaudhry, 1969; Oya & Yoshida, 2008).

The occurrence of chalky kernels may be related to kernel size, because kernel lengths, widths and thicknesses were smaller in MW kernels than those in perfect (PF), white-based and white-back kernels (Wakamatsu et al., 2007). Additionally, the spikelets on the lower secondary branches are shorter than those on the upper primary branches (Ishimaru, Matsuda, Ohsugi, & Yamagishi, 2003; Shimotsubo & Nakayama, 1974). The percentage of chalky kernels was also higher in cultivars with thicker kernel widths or thicknesses (Miyazaki et al., 2014; Nagaoka, Sasahara, Shigemune, Goto, & Miura, 2012).

Kernel size (length, width and thickness) is determined by the endosperm cell number and area of each endosperm cell aligned in each direction, which are influenced by spikelet positions within a panicle and temperature conditions (Ishimaru et al., 2003; Morita, Yonemaru, & Takanashi, 2005). For example, spikelets on lower secondary branches have shorter kernel lengths, widths and thicknesses, with fewer endosperm cell layers, than those on upper primary branches (Ishimaru et al., 2003).

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Kernel width decreases under high temperatures as each cell area decreases, although the number of cells increases (Morita et al., 2005). An endosperm cell has a large number of amyloplasts, as subcellular organelles, that contain a few or a few dozen densely filled starch granules (Tashiro & Ebata, 1975). However, chalky kernels contain small loosely filled starch granules with interspaces between amyloplasts (Ishimaru et al., 2009; Zakaria, Matsuda, Tajima, & Nitta, 2002).

In this study, we analyzed the numbers and areas of endosperm cells, kernel sizes and the occurrence of the chalky kernels at different spikelet positions within a panicle and between different grain appearance quality traits to clarify their relationships.

Materials and methods

Cultivation

The rice cultivar Koshihikari was grown in 2015 in a paddy field of the Field Science Center, Faculty of Agriculture and Marine Science, Kochi University located at 33°33′ N latitude, 133°40′ E longitude and 6 m above sea level. Rice seeds were disinfected and dried, and then soaked in running water for germination. Germinated seeds were sown on 13 May, and seedlings were grown by local conventional methods. The field was tilled, and slow release fertilizer containing N, P_2O_5, and K_2O at a rate of each 100 kg ha^{-1} was applied on 29 May before it puddled. Seedlings with 3.5–4.0 leaves were transplanted on 1 and 2 June. There were two plants per hill and 22.2 hills m^{-2}. The full-heading stage occurred on 5 August. The paddy field was continuously flooded, and agricultural chemicals were appropriately applied. No field treatment was used in this study.

Measurements

Meteorological observations

The meteorological data for August were collected from AMeDAS at the Japan Meteorological Agency. The amount of global solar radiation was collected at the Kochi observation site located 13 km away from the experimental field, and the average of 1981–2010 was used as a normal year value. Air temperatures were collected at the Nankoku observation site located 1 km away from the experimental field, and the average of 2003–2010 was used as a normal year value.

Sampling and measurements of kernel size and grain appearance quality traits

At maturity on 7 September, 20 hills were randomly sampled from each of three different locations, and the samples were dried in a glasshouse. From three hills having average panicle weights and numbers for each location, the longest three panicles per hill were selected. Each panicle was separated equally into upper, middle and lower primary branches, and each 60–90 spikelets were sampled from the tip on the upper primary branch, third to fifth from the tip on the middle primary branch, and second or third from the tip on lower secondary branch. Spikelets were hulled, and the kernel size (length, width and thickness) and grain appearance quality traits of each spikelet were determined by a kernel inspector (RGQI10A, Satake Co., Hiroshima, Japan). Kernels larger than 1.8 mm in thickness were used for all analyses. They were stored in formalin-acetic acid-alcohol (FAA) solution for more than 2 weeks, and then used again for the measurement of kernel size. As kernels swelled in FAA, kernels with an average swelling rate (within ±3.5%) in width and thickness were selected for each panicle position. From each of four categories, PF kernels in the upper, middle and lower branches and MW kernels in the lower branches, six kernels were collected for morphological observation.

Histological analysis of endosperm cells

All measurements and analyses of endosperm cells followed the methods of Morita et al. (2005). Initially, the kernels were stabilized in elder pith (5–8 mm in diameter, Kenis Co., Osaka, Japan). A 15–20-μm transverse section of each kernel was sliced at the center lengthwise using a plant microtome (MTH-1, NK System Co., Osaka, Japan), stained with toluidine blue, and then observed with a biological microscope (4 × 10 times). A photographic image was taken using a USB digital camera system (MacromaX, GOKO Camera Co., Kawasaki, Japan) and was printed. From the printed image, the contour of each endosperm cell was traced on a transparent film with an ultrafine marker (0.3 mm), except for the aleurone layer cells because the difference between cell wall and membrane was unobservable under this magnification. This image was scanned into a computer and analyzed using the program of Morita et al. (2005) after the thinning process. The cell number and each cell's area were analyzed using the distance and angle from the center point of the endosperm.

Statistical analyses

Data collected were analyzed statistically with an analysis of variance (ANOVA) using JMP 7 (SAS institute Inc., Cray, NC, USA.). Statistically significant differences between means were identified by Tukey's honestly significant difference (HSD) test at $p < 0.05$.

Results

The amount of global solar radiation and the air temperature in August during the grain-filling period were compared between 2015 and determined normal year values (Figure 1). During the 7 days after full heading at the beginning of August, they were higher than those in a normal year, while in middle and late August, the values in 2015 were lower than those in a normal year. The average temperature and its difference from that of a normal year were 28.1°C and +0.9°C, respectively, in the former period, and 26.1°C and −0.9°C, respectively, in the latter period.

The percentage of white immature kernel is shown as the total percentage of MW, white-based, white-belly and white-back kernels (Table 1). The percentage of white immature kernels in the lower spikelets (50.0%) was higher than that in the upper (15.8%) and middle (13.2%) spikelets. In the lower spikelets, MW kernels occupied 23.9%, constituting a major proportion. Kernel size (length, width and thickness) in the lower spikelets was significantly smaller than in the upper and middle spikelets, and MW kernels were significantly smaller than PF kernels, even in the same lower spikelets (Table 2). The coefficient of variance for the widths of MW kernels in the lower spikelets was larger than that of PF kernels in the upper, middle and lower spikelets.

After immersion in FAA, kernel size was determined once again, and the swelling rate was calculated. The swelling rate was 7–13% on average for kernel length, width and thickness, and there was no significant difference among panicle positions (data not shown). However, the swelling rate had a significant negative correlation with kernel length, width and thickness before immersion at each panicle position, suggesting that the smaller kernels had a higher swelling rate. Therefore, kernels within an average ±3.5% swelling rate in width and thickness were selected from each panicle position for morphological observation.

From the cross-section analysis of morphological observations, the total area, number and the calculated cell area of the endosperm cells are shown in Table 3. The total area was significantly smaller in the lower

Table 1. Percentage (%) of grain appearance quality traits.

Spikelet position	*n*	Perfect	White-immature[a]	Milky-white	White-based	White-belly and white-back
Upper	57	77.2	15.8	1.8	12.3	1.8
Middle	68	82.4	13.2	0.0	13.2	0.0
Lower	88	21.6	50.0	23.9	21.6	4.5

[a]Total percentage of milky-white, white-based, white-belly and white-back kernels.

Table 2. Rice kernel size.

Spikelet position	Grain appearance	*n*	Length (mm)	Width (mm)	Thickness (mm)
Upper	PF	44	5.27 (2.1)a	2.94 (4.5)a	2.04 (3.5)a
Middle	PF	56	5.27 (2.5)a	2.91 (3.9)ab	2.05 (4.8)a
Lower	PF	19	5.08 (2.8)b	2.83 (4.2)b	1.97 (3.6)b
Lower	MW	21	4.77 (3.6)c	2.62 (7.3)c	1.89 (2.7)c
			***	***	***

Kernels: PF: perfect; MW: milky-white. Values in parentheses indicate coefficients of variance. The same alphabetical letters indicate no significance at the 5% level between different spikelet positions and grain appearance quality traits. *** indicates a 0.1% level of significance.

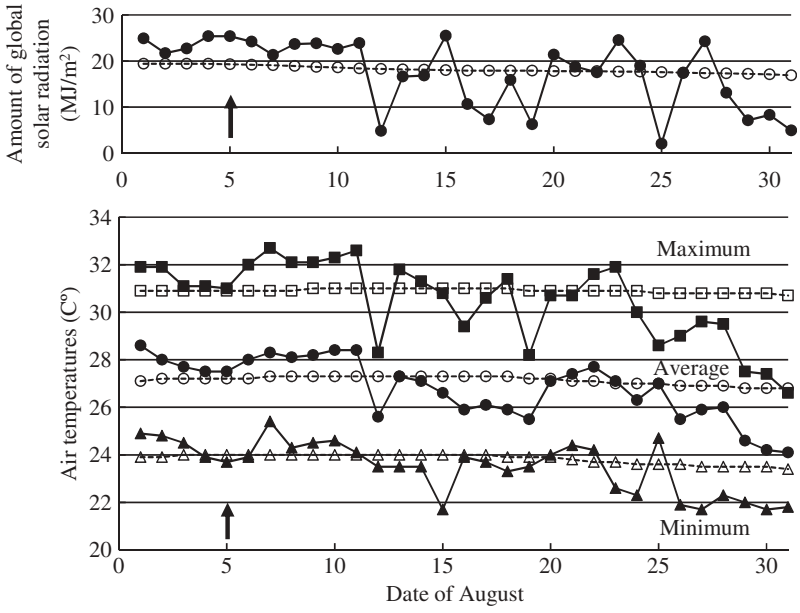

Figure 1. The amounts of global solar radiation and the air temperatures in August 2015 (closed symbols) and a normal year (open symbols) in Kochi, Japan. Arrows indicate the full-heading stage.

Table 3. Total area of endosperm, number of cells and average cell area in cross sections of rice kernels.

Spikelet position	Grain appearance	Total area of endosperm cross sections (mm^2)	Number of cells in endosperm	Average cell area (μm^2/cell)
Upper	PF	5.49a	2502a	2216a
Middle	PF	5.28ab	2888a	1846b
Lower	PF	4.93b	2818a	1762b
Lower	MW	4.53c	2501a	1818b
		***	*	**

Kernels: PF: perfect; MW: milky-white. The total area of endosperm was calculated as the sum of each cell area. The same alphabetical letters indicate no significance at the 5% level among different spikelet positions and grain appearance quality traits. *, ** and *** indicate 5%, 1% and 0.1% levels of significance, respectively.

spikelets than in the upper spikelets, and it was also significantly smaller in MW kernels than in PF kernels, even in the same lower spikelets. The average cell area was significantly smaller in the lower and middle spikelets than in the upper spikelets. However, there was no significant difference in the number of cells among panicle positions and between grain appearance qualities. These relationships are shown in Figure 2 using all kernels analyzed. The total area did not correlate with the number of cells or the cell area. However, there was a significant negative correlation between the number and the area of PF kernels in the upper, middle and lower spikelets, indicating that kernels having a larger number of cells had smaller cells. The cell area in the lower spikelets tended to be smaller than those in the upper and middle spikelets having similar cell numbers.

The cell number and area were analyzed at 10% distance intervals from the center point to the contour in all directions in the endosperm (Figure 3). For example, the total cell number and the average cell area analyzed within 0–10% area are shown as the midpoint, 5% values. PF kernels in the lower and middle spikelets had a significantly larger number of cells than those in the upper spikelets at the distance of 35–55% from the center point, while they had significantly smaller cell areas at all distances. The cell area in the MW kernels was not significantly different from that in the PF kernels, while the number of cells in the MW kernels tended to be less than that in the PF kernels at any distance. A significant decrease in the number of cells in the MW kernels was indicated by a two-way ANOVA of spikelet position and distance.

The cell number and area were also analyzed at angular 30° intervals, with the line connecting the center point and dorsal vascular bundle set as 0° (Figure 4). For example, the average values of the total cell number and the average cell area analyzed from 75° to 105° and from the opposing −75° to −105° are shown as the midpoint, 90° values, although those of each 30° analyzed from −15° to 15° (dorsal side) and from 165° to −165° (ventral side) are shown without averaging because there are no opposing spaces. A two-way ANOVA indicated results similar to those for distance. PF kernels in the lower and middle spikelets had larger numbers of cells and smaller cell areas than those in the upper spikelets, although the difference in the number of cells was not significant at each angle. There was no significant difference in cell area between MW and PF kernels; however, the number of cells in MW kernels was significantly less than in PF kernels at 120–180° around the ventral side.

These results were observed from a comparison of the images in Figure 5; dorsal and ventral radii from the center point of endosperm differed between PF and MW kernels, and the ventral radius in MW kernels was shorter than that in PF kernels in the lower spikelets.

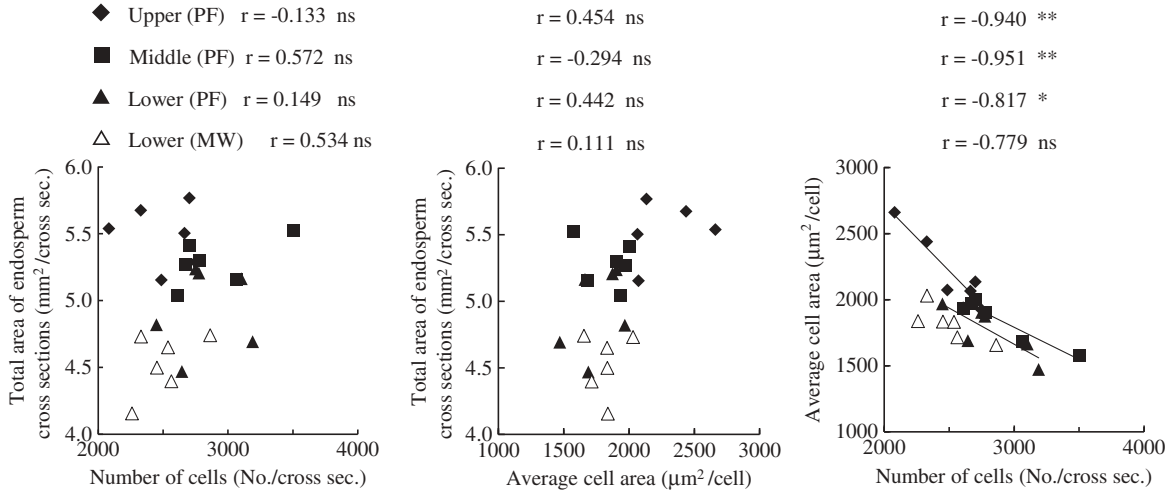

Figure 2. Relationships among the total area of cross sections, number of cells and average cell areas in rice endosperms. * and ** indicate the 5% and 1% levels of significance, respectively. ns indicates no significance at the 5% level. PF: perfect; MW: milky-white.

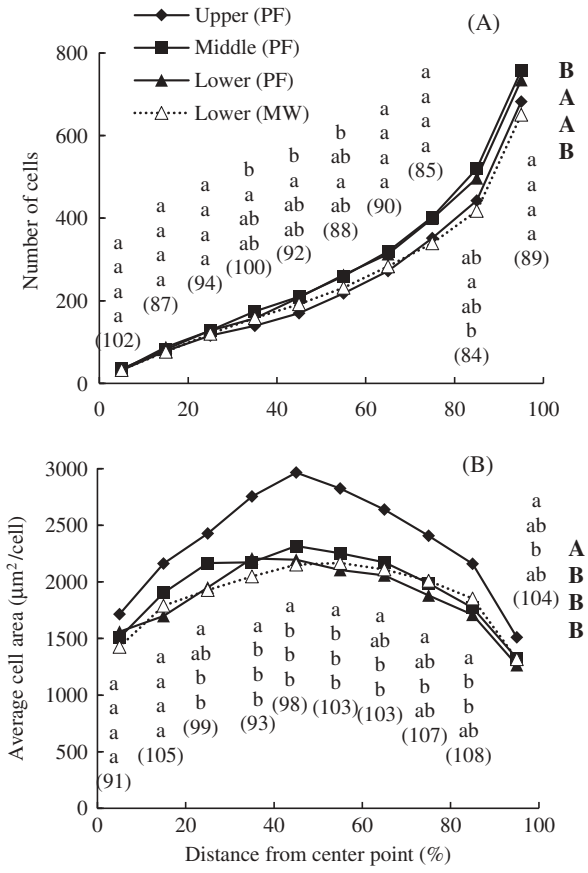

Figure 3. Number of cells (A) and average cell area (B) of endosperm cross sections analyzed in 10% distance intervals from center point to the contour in the endosperm. Values are the averages of six kernels from each spikelet position. The same lowercase and capital letters indicate no significance at the 5% level in each distance and in a two-way ANOVA of spikelet position and distance, respectively. The letters are shown from the top in the order: upper, middle and lower PF kernels and lower MW kernels. Values in parentheses indicate the ratio of MW kernels to PF kernels in the lower spikelets. PF: perfect; MW: milky-white.

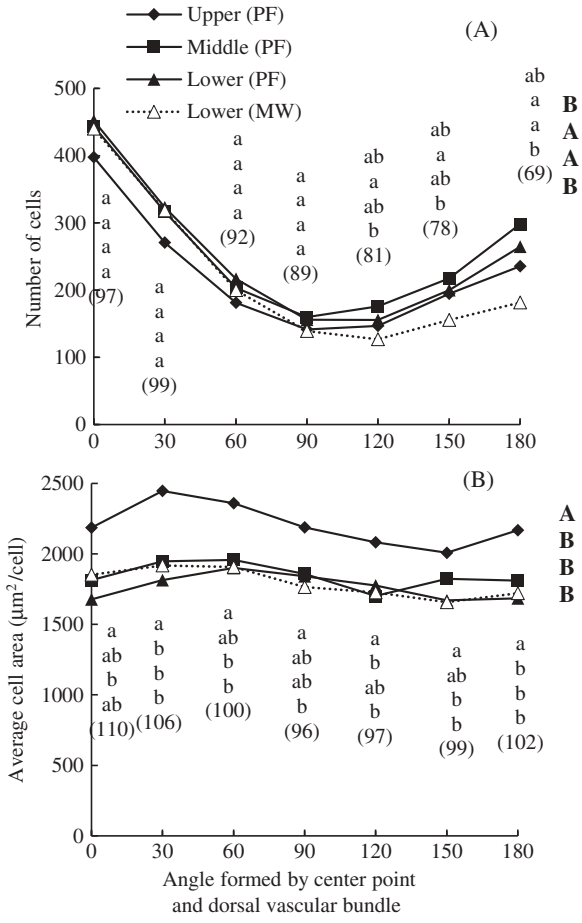

Figure 4. Number of cells (A) and average cell area (B) of endosperm cross sections analyzed for every angular 30° interval formed by the center point in the endosperm and the dorsal vascular bundle. Values are the averages of six kernels from each spikelet position. The same lowercase and capital letters indicate no significance at the 5% level in each angle and in a two-way ANOVA of spikelet position and angle, respectively. The letters are shown from the top in the order: upper, middle and lower PF kernels and lower MW kernels. Values in parentheses indicate the ratio of MW kernels to PF kernels in the lower spikelets. PF: perfect; MW: milky-white.

Discussion

Characteristics of endosperm cell morphology were compared and discussed here among PF kernels at different spikelet positions and between MW and PF kernels at the same spikelet positions to elucidate relationships among endosperm cell morphology, kernel size and the occurrence of chalky kernels.

Endosperm cell morphology among PF kernels at different spikelet positions

PF kernels in the lower spikelets had smaller cell areas with an increased number of cells compared with in the upper spikelets (Figures 2, 3 and 4). This suggests that the decrease in cell area resulted in the decrease in kernel size (width and thickness) in PF kernels. Similar decreases in cell area were reported by Morita et al. (2005) based on analyses of endosperm cells in rice grown under high-temperature conditions.

Endosperm cell morphology between MW and PF kernels of lower spikelets

Cell morphology was compared between MW and PF kernels in the same lower spikelets. MW kernels had significantly fewer cells in the ventral side of the endosperm cross section than PF kernels, although the cell area in MW kernels was not significantly different from that in PF kernels (Figure 4). Ishimaru et al. (2003) reported that kernels on the lower secondary branches

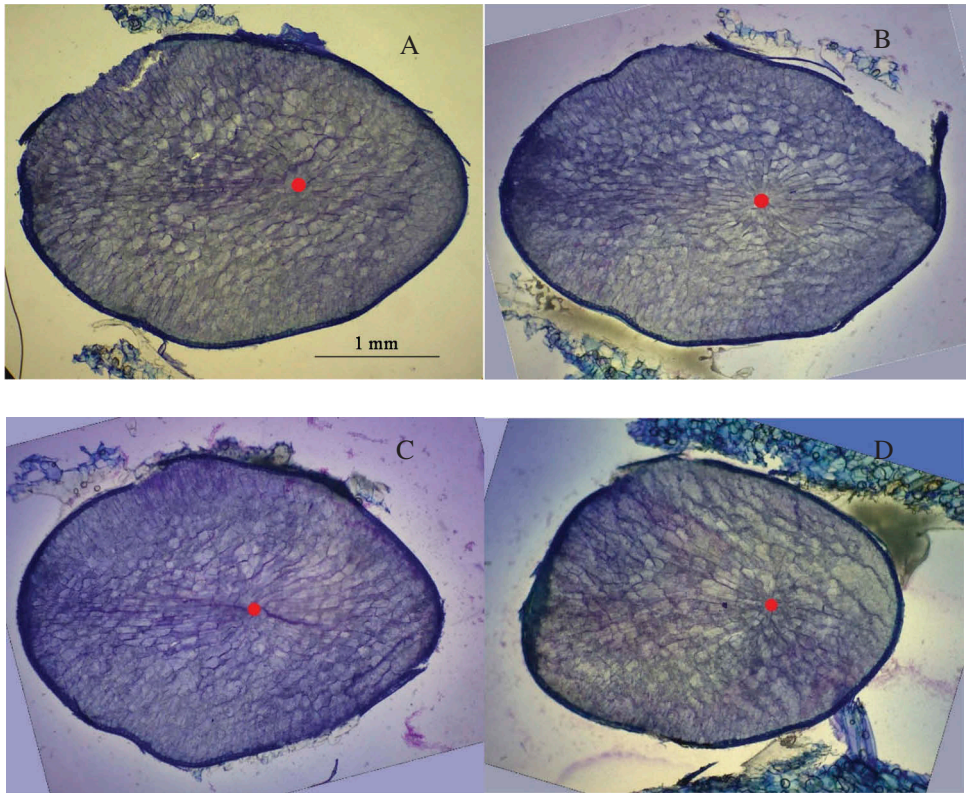

Figure 5. Cross section of upper PF (A), middle PF (B), lower PF (C) and lower MW (D) kernels. A red point indicates the center point of the endosperm. PF: perfect; MW: milky-white.

had fewer endosperm cell layers, with shorter kernel lengths, widths and thicknesses, than those on the upper primary branches. These results suggest that MW kernels have an inferior cell division at the ventral side of the endosperm compared with PF kernels. The decrease in the number of cells in MW kernels is considered to result in the decrease in kernel size (especially in width).

The decrease in the number of ventral cells was observed as the decrease in the ventral radius (Figure 5). Hoshikawa (1967b) observed similar results, in which the radius and the number of cell layers at the ventral side decreased during early cultivation at high temperatures. Conversely, Nagato and Ebata (1965) observed an increase in the ventral radius and a decrease in the dorsal radius under high-temperature conditions during the grain-filling period. High temperatures accelerate an increase in kernel weight at the early grain-filling period, but reduce the length of the period (Nagato & Ebata, 1965). Early grain filling is caused by the development of the ventral and dorsal radii, while late grain filling is mainly caused by the development of the dorsal radius (Hoshikawa, 1967a). These reports suggested that the development of the ventral radius was promoted simultaneously with the increase in kernel weight, which was accelerated by high temperatures at the early grain-filling period, and that the development of the dorsal radius was inhibited by a later shortened grain-filling period (Ebata, 1961; Nagato & Ebata, 1965).

Hoshikawa (1967b) suggested that the difference observed in these studies might result from the different environmental conditions (early cultivation and high-temperature conditions, respectively). In our study, the MW kernels were exclusively observed in the lower position of a panicle (Table 1). Generally, these result from a reduced carbohydrate supply during early grain filling owing to competition from the upper spikelets (Kido & Yanatori, 1968; Oya & Yoshida, 2008). Therefore, it was hypothesized that cell division in the ventral radius was inhibited by a reduced supply of carbohydrates. According to Tsukaguchi et al. (2012), MW kernels are classified into two types: central and ring-shape chalk. The former is induced by high-temperature stress, while the latter is induced by an insufficient carbohydrate supply (i.e. low source/sink ratio and low solar radiation). In our study, imaging indicated that some MW kernels were central chalk type, while others could not be identified well. The amount of global solar radiation and the air temperatures at the beginning of grain filling were higher than those in a normal year (Figure 1). These suggested that the MW kernels at lower spikelets in our study were

induced under simultaneous stresses of low carbohydrate supply and high temperature. Endosperm cell characteristics of central or ring-shape type MW kernels and those of white-belly, white-back and white-based kernels should be clarified in future research, and the relationships between environmental conditions and endosperm cell characteristics should be examined in detail.

The number of cells decreased in the ventral region. The division in endosperm cells begins from the outermost layer centripetally in the embryo sac and is completed within 10 days after fertilization (Hoshikawa, 1967a; Ishimaru et al., 2003). During the early grain-filling period, the carbohydrates required for endosperm development are carried by the nucellus epidermis surrounding the exterior edge of the embryo sac (Hoshikawa, 1968). An electron microscopic study reported that the nucellus epidermis degenerated under high-temperature conditions earlier than under natural conditions (Zakaria et al., 2002). Thus, the delay in carbohydrate translocation through the nucellus epidermis may increase the carbohydrate translocation from the dorsal vascular bundle and result in the decrease in the number of cells in the ventral region located on the opposite side of the dorsal vascular bundle. The relationship between the translocation pathway and the development of the ventral–dorsal radii should be examined in further studies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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