Variations of endoreduplication and its potential contribution to endosperm development in rice (Oryza sativa L.)

Hidekazu Kobayashi

Western Region Agricultural Research Center, National Agriculture and Food Research Organization, Fukuyama, Japan

ABSTRACT

Endoreduplication is the phenomenon by which cells increase their ploidy. Endoreduplication is initiated by the transition from the mitotic cell cycle to the endocycle, in which DNA replication occurs without a subsequent chromosome separation and cytokinesis, and is enhanced by endocycle reiteration. This process appears to play an important role in endosperm development, but the characteristics of endoreduplication in the endosperm of rice (Oryza sativa) remain unclear. To elucidate the features and variations of endoreduplication in rice endosperm, endoreduplication progression in the developing endosperm was compared among 10 cultivars based on flow cytometry and fluorescence microscopy. The flow cytometric analysis detected significant differences among 10 cultivars in the following three parameters: mean ploidy of all nuclei, the proportion of nuclei ≥6C (%E, an estimate of the initiation of the endocycle), and the mean ploidy of nuclei ≥6C (E6P, an estimate of the reiteration of the endocycle). However, no significant correlation between %E and E6P was observed, suggesting that the initiation and reiteration of the endocycle are independently regulated. Fluorescence microscopy revealed that the ploidy of the nuclei was higher in the intermediate region than in the central and peripheral regions of the endosperm. Cells with a higher ploidy were larger in the developing endosperm. Furthermore, the mean ploidy in the developing endosperm was significantly correlated with the mean cell size in the mature endosperm. These results indicate that the cell cycle progression in the endosperm differed significantly among the 10 rice cultivars and such differences may influence endosperm cell size.

Abbreviations: Ak: Akitakomachi; DAP: days after pollination; DAPI: 4′,6-diamidino-2-phenylindole; E6P: mean ploidy of nuclei ≥ 6C; Ha: Habataki; Ho: Hokuriku193; IR: IR64; Ka: Kasalath; Ki: Kinuhikari; Ko: Koshihikari; Ni: Nipponbare; PEG: polyethylene glycol; Sa: Sasanishiki; Ta: Takanari; %E: proportion of nuclei ≥ 6C.

Introduction

Endoreduplication, which is also called endoreplication, is the phenomenon by which cells increase their ploidy (Breuer et al., 2010; Dante et al., 2014; De Veylder et al., 2011; Sugimoto-Shirasu & Roberts, 2003). During endoreduplication, the chromatids are duplicated exponentially, while the number of chromosomes remains unchanged. Endoreduplication is initiated by the transition from the mitotic cell cycle to a modified cell cycle called the endocycle, in which DNA replication occurs without a subsequent chromosome separation and cytokinesis. Ploidy increases are due to the reiteration of the endocycle.

Although the exact role of endoreduplication remains unknown, it is believed to contribute to cell enlargement, increase gene expression levels, accelerate growth, and increase DNA storage (Barow, 2006; Chevalier et al., 2011; Kowles, 2009; Nguyen et al., 2007; Sabelli & Larkins, 2009; Sugimoto-Shirasu & Roberts, 2003). The prevailing hypothesis concerning the role of endoreduplication is that it influences cell enlargement (Chevalier et al., 2014; Orr-Weaver, 2015; Sugimoto-Shirasu & Roberts, 2003). The transition from cell proliferation to cell expansion is often accompanied by the transition from the mitotic cycle to the endocycle. Furthermore, many studies have observed a strong correlation between ploidy and cell size. However, a causal relationship between endoreduplication and cell size has been disputed because some mutants do not exhibit a concurrent decrease in endoreduplication and cell size (Leiva-Neto et al., 2004; Vilhar et al., 2002). Considered together, these findings indicate that endoreduplication may determine the maximum capacity for cell growth instead of strictly regulating cell growth (Breuer et al., 2010).

Endoreduplication occurs in many cell types, especially large, metabolically active, or highly specialized cells (De Veylder et al., 2011). Examples include endosperm cells in...
Poaceae species (Sabelli, 2012; Sabelli & Larkins, 2009). A primary endosperm cell is formed with a 3C nucleus (1C represents the ploidy of a nonreplicated haploid genome) because of the fusion of two polar nuclei in the embryo sac and one sperm nucleus of the pollen grain, but the ploidy of endosperm cells gradually increases during endosperm development via endoreduplication (Sabelli & Larkins, 2009). In Poaceae species, endoreduplication follows several endosperm development phases, such as syncytium formation, cellularization, and mitosis (Sabelli, 2012; Sabelli & Larkins, 2009). Inhibited endoreduplication reportedly impairs endosperm development (Barrôco et al., 2006). Additionally, endoreduplication may influence the yield and/or quality of cereal grains. For example, polyploidy frequency as well as the number of cells per endosperm are correlated with seed weight in wheat (Brunori et al., 1993). The mean ploidy of maize endosperm cells tends to be higher in popcorn than in dent corn (Dilkes et al., 2002). *Brachypodium distachyon* endosperm, in which endoreduplication is less extensive than that in barley, is composed of small cells and contains abundant (1,3;1,4)-β-glucan (Trafford et al., 2013). Therefore, clarifying the features and contribution of endoreduplication in the endosperm of Poaceae species may lead to improvements in cereal grain yield and/or quality.

The intensive investigation of endoreduplication progression in maize endosperm cells has clarified the associated spatiotemporal patterns (Nguyen et al., 2007; Sabelli, 2012; Sabelli & Larkins, 2009). At approximately 8 days after pollination (DAP), maize endosperm cells asynchronously switch from the mitotic cell cycle to the endocycle. The endoreduplication of cells in the central region precedes that of cells in the peripheral region. Consequently, there is a ploidy gradient, with a higher ploidy in the inner region of the maize endosperm. A similar gradient is observed for maize endosperm cell size, and there is a strong correlation between the ploidy and size of endosperm cells (Kowles, 2009). However, endoreduplication progression differs among maize cultivars, and this difference is derived from both the initiation and reiteration of the endocycle (Dilkes et al., 2002).

In contrast to maize, studies of endoreduplication in the developing endosperm of rice are limited. Feulgen microspectrophotometry has been used to reveal the temporal progression of endoreduplication in the rice endosperm (Kono et al., 1979; Ramachandran & Raghavan, 1989). The mean DNA content per nucleus remains low until 4 days after anthesis, and rapidly increases from 4 to 8 days after anthesis (Ramachandran & Raghavan, 1989). Endoreduplication progression in the endosperm varies among the spikelets developing at different panicle positions. The endosperm cells from apical spikelets have more endoreduplicating nuclei than those from basal spikelets (Panda et al., 2015, 2018). The cell cycle regulatory genes, *KRRL* and *CCS2A*, are important for the transition from the mitotic cell cycle to the endocycle, and changes to the expression of these genes hinder endosperm development (Barrôco et al., 2006; Su’udi et al., 2012). Endoreduplication in rice endosperm cells is regulated by phytohormones such as ethylene and cytokinin. The application of 1-methylcyclopropene (an ethylene action inhibitor) and 6-benzylaminopurine (a cytokinin) increases the ploidy of endosperm cells in the dense-panicle rice cultivar ‘OR-1918’ (Panda et al., 2016, 2018). However, to the best of my knowledge, endoreduplication progression has not been compared among rice cultivars. Information regarding the diversity of endoreduplication in rice endosperm among cultivars may be useful for developing methods that apply endoreduplication in breeding programs to improve crops. Additionally, there is a lack of ‘ploidy maps’ revealing the distribution of rice endosperm cells in different ploidy classes. Ploidy maps have been generated for maize, sorghum, and teosinte (Dermastia et al., 2009; Kladnik et al., 2006; Vilhar et al., 2002), and may provide meaningful insights into how the endocycle mechanisms are coordinated with the growth of individual cells in a specific tissue (De Veylder et al., 2011).

The objective of this study was to clarify the features and variations of endoreduplication in rice endosperm. To examine endoreduplication in detail, the three parameters mean ploidy, proportion of nuclei ≥6C (%E), and mean ploidy of nuclei ≥6C (E6P) were evaluated as described by Dilkes et al. (2002) on the basis of flow cytometric data. The mean ploidy of all nuclei is frequently used as an index of endoreduplication, while the parameter %E is used to estimate initiation of the endocycle. For example, a high %E, which indicates a low proportion of 3C nuclei, suggests that mitotic cell division has decreased and that the recruitment of nuclei to the endocycle has increased. The parameter E6P is used to estimate reiteration of the endocycle. For example, a high E6P implies that reiteration of the endocycle has been promoted. The relationship among these three parameters is as follows: mean ploidy = \(3 \times (1 - \%E/100) + E6P \times \%E/100\). To clarify the spatial progression of endoreduplication in rice endosperm, ploidy maps were generated by fluorescence microscopy. In the present study, five *japonica* and five *indica* cultivars were used to evaluate the variation in endoreduplication progression among rice cultivars. The cultivars used are popular in Japan, and chromosome segment substitution lines with some pairs of these cultivars have recently been developed (Ando et al., 2008; Takai et al., 2014). Furthermore, the relationship between endoreduplication and cell size was investigated to clarify whether endoreduplication contributes to the control of cell size in rice endosperm.
Materials and methods

Plant materials for examining the spatiotemporal progression of endoreduplication

In 2014, Oryza sativa cv. ‘Kinuhikari’ (a japonica cultivar) plants were grown under natural conditions in a paddy field at the Western Region Agricultural Research Center, National Agriculture and Food Research Organization (Fukuyama city, Hiroshima, Japan, 34° 29’ N, 133° 23’ E). Caryopses were germinated in a nursery bed on 9 May, and seedlings were transplanted to the paddy field at a hill spacing of 15 cm × 30 cm, with two seedlings per hill, on 29 May. Chemical fertilizer (N:P₂O₅:K₂O = 14%:14%:14%) was applied as a basal dressing at a rate of 28.5 g m⁻², and ammonium sulfate (N = 21%) was applied at a rate of 9.5 g m⁻² at 20 days before heading. Subsequent experiments involved the superior caryopses located at the fourth and fifth nodes from the apex of the upper two primary branches on the panicle. The pollination dates of these caryopses were recorded for the superior caryopses located at the fourth, fifth, and sixth nodes from the apex of the upper two primary branches on the panicle. The caryopses were periodically harvested from several plants in the central row and were preserved as described above. To examine endoreduplication of the endosperm in each cultivar at a similar developmental stage, the caryopses harvested when the dry weight was about 25% of that at maturity were used in subsequent experiments (Supplement 1).

Flow cytometry

Samples were prepared for flow cytometric analysis based on a modified version of the procedures described by Galbraith et al. (1983). Using a disposable scalpel, samples were chopped for 2.5 min in 1.2 mL chopping buffer. The homogenate was filtered through a 70-µm nylon mesh filter, and nuclei were stained with 30 µM propidium iodide. The stained nuclei were analyzed by flow cytometry with a Guava® easyCyte 6–2 L benchtop flow cytometer (Merck Millipore, Billerica, MA, USA). Ten thousand events were counted for the caryopses, and 5,000 events were counted for flag leaves.

Fluorescence microscopy

Samples were prepared for fluorescence microscope analysis using a modified version of the procedures described by Kobayashi et al. (2013). Fixed caryopses were embedded with polyethylene glycol (PEG), and median transverse sections (12 µm thick) were sliced with a microtome. After removing PEG from the sections with phosphate buffer, the sections were mounted in VECTASHIELD® Mounting Medium with DAPI (4’,6-diamidino-2-phenylindole) (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were observed with the IX71 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a WU filter set (excitation wavelength 330–385 nm, emission wavelength 420 nm). Images were captured with a VB-7000 CCD camera (Keyence, Osaka, Japan). To obtain high-resolution images, several endosperm portions were photographed under high magnification (20×) and combined.

To analyze the ploidy in endosperm sections, images were examined based on the modified procedures for trichome nuclei (Kobayashi et al., 2012; Maes et al., 2008). Images captured with a CCD camera were converted to grayscale using Photoshop Elements 12 (Adobe Systems, San Jose, CA, USA), and analyzed using ImageJ 1.44p (National Institutes of Health, USA; http://imagej.nih.gov/ij/). The area of nuclei that had distinct contours was
manually circumscribed, and their integrated density (the product of area and mean gray value) was measured as an index of fluorescence intensity. The integrated density value was log transformed, and histograms of log-integrated density were drawn to evaluate the ploidy of each nucleus. Transverse sectional areas of 30 cells in the respective ploidy classes were then measured using ImageJ 1.44p. In some cultivars, the section contained fewer than 30 cells with 24C nuclei. In such instances, the data for all cells with 24C nuclei were included.

**Measurement of cell size in mature caryopses**

In 2015, mature caryopses were fixed and embedded with PEG as described above. Median and longitudinal transverse sections were sliced with a microtome. After removing PEG from the sections with water, sections were stained with 0.05% toluidine blue solution (Wako Pure Chemical Industries, Osaka, Japan) for 5 min. After rinsing with water for 30 s, images of the sections were captured with a YDU-2 digital stereomicroscope (Yashima Optical Co., Tokyo, Japan). To obtain high-resolution images, several endosperm portions were photographed under high magnification (250x) and combined.

To analyze the cell size in median transverse sections, images were examined using modified procedures described by Morita et al. (2005). Images of the endosperm with an aleurone layer were prepared by tracing cell contours on a Cintiq 13HD graphic tablet (Wacom Co., Saitama, Japan). The area of each cell was analyzed using ImageJ 1.44p, and the mean cell area was determined. To calculate mean cell height, the length of 50–70 cell layers in longitudinal sections was measured. Mean cell volume was calculated by multiplying the mean transverse sectional area of cells by the mean cell height.

**Statistical analysis**

Statistical analysis was carried out using JMP 11 software (SAS Institute Japan Inc., Tokyo, Japan). Data were analyzed by one- or two-way analysis of variance (ANOVA), and then means were compared using a Tukey–Kramer multiple comparison test ($P < 0.05$).

**Results**

**Spatiotemporal endoreduplication progression in rice endosperm**

The temporal progress of endoreduplication in the endosperm of rice ‘Kinuhikari’, a japonica cultivar, was examined using flow cytometry. Flow cytometric analysis detected four or five peaks in fluorescence intensity of the nuclei in developing caryopses (Figure 1(a–c)), whereas a single peak was detected in flag leaves (Figure 1(d)). The peak with the lowest fluorescence intensity in the caryopses coincided with the peak in the flag leaves. Rice leaves reportedly contain only 2C nuclei (Barow & Meister, 2003; Martínez et al., 1994); therefore, the peak with the lowest fluorescence intensity in the caryopses was judged to be 2C, which is derived from the embryo and the pericarp. Using this 2C peak as a reference, the other peaks in the caryopses were judged to be 3C, 6C, 12C, and 24C, which were derived from the endosperm (Figure 1(a–c)).

An analysis of the flow cytometry data enabled the determination of the composition of the nuclei in different ploidy classes in rice endosperm (Figure 2). In this study, the dry weight of a developing caryopsis relative to that of the mature caryopsis was used as a reference for caryopsis development (Figure 2(a)). The 12C nuclei, which were exclusively formed via endoreduplication, were detected as early as 5 DAP (Figure 2(b)), when the relative weight of the caryopsis was about 5% (Figure 2(a)). The proportion of nuclei with an elevated ploidy increased until 8 DAP (Figure 2(b)), when the relative weight of the caryopsis was about 20% (Figure 2(a)). Thereafter, the proportions of nuclei with an increased ploidy was relatively unchanged until 10 DAP (Figure 2(b)), when the relative weight of the caryopsis was about 30% (Figure 2(a)). On the basis of the composition of nuclei in different ploidy classes, the mean ploidy of all nuclei in the endosperm, which is frequently used as an index of endoreduplication, was calculated. The mean ploidy was 4.8C at 5 DAP, and increased significantly to 6.9C at 8 DAP (Figure 2(c)). From 8 to 10 DAP, the mean ploidy did not change significantly. In the present study, endoreduplication in rice endosperm older than 10 DAP could not be measured because the flow cytometer was frequently clogged with developed starch granules.

To clarify the spatial progression of endoreduplication in rice endosperm, median transverse sections of the caryopses stained with DAPI were examined by fluorescence microscopy (Figure 3). Only small nuclei were observed in the endosperm at 5 DAP (Figure 3(a)), whereas many large nuclei were observed at 9 DAP (Figure 3(c)). Image analysis of DAPI fluorescence from endosperm nuclei revealed several peaks in the histograms of integrated density (Figure 3(e,f)). Flow cytometric analysis clarified that the lowest ploidy of rice endosperm nuclei at these stages was 3C (Figure 1). Therefore, the peak with the lowest integrated density was judged to be 3C, and the other peaks were determined as 6C, 12C, and 24C (Figure 3(e,f)). Based on the histograms, the ploidy of each nucleus in the section was determined and superimposed on the images.
to construct ploidy maps (Figure 3(b,d)). At 5 DAP, most of the nuclei in rice endosperm cells were 3C and 6C. However, some nuclei in the intermediate region between the central and peripheral regions were 12C (Figure 3(b)). At 9 DAP, the ploidy varied greatly among the nuclei in the different endosperm regions. The ploidy was higher in the intermediate region than in the peripheral and central regions (Figure 3(d)).

Comparison of endoreduplication among 10 rice cultivars

The temporal progression of endoreduplication based on the relative dry weight of developing caryopses in the indica cultivar ‘Kasalath’ (Supplement 2) exhibited a similar pattern to that in the japonica cultivar ‘Kinuhikari’ (Figure 2). Therefore, endoreduplication progression in the endosperm of 10 rice cultivars was compared at the

Figure 1. Histograms from the flow cytometric analysis of nuclei from developing caryopses and flag leaves of rice. (a) Caryopses at 5 days after pollination (DAP). (b) Caryopses at 7 DAP. (c) Caryopses at 9 DAP. (d) Flag leaves at heading. The ploidy is indicated for each nuclear peak. Cultivar: ‘Kinuhikari’ (japonica).
developmental stage when the dry weight of the developing caryopsis was about 25% of that at maturity (Supplement 1). Significant differences in the mean ploidy, %E, and E6P were observed among the 10 cultivars in 2015 (Figure 4). Similar results were obtained in 2014 (Supplement 3), and highly significant correlations between years were observed (Figure 5). The effects of years on the mean ploidy and E6P were significant, while the interaction of cultivars and years was not significant for each parameter (Supplement 4). The mean ploidy differed among cultivars, with the lowest and highest mean ploidies observed for Ak and Ta, respectively (Figures 4(a) and 5(a)). The following two groups of cultivars were detected based on %E: Ak, Sa, Ko, Ki, and Ka formed a low %E group, whereas Ho, Ha, Ni, IR, and Ta belonged to a high %E group (Figures 4(b) and 5(b)). Differences in E6P were observed among cultivars; E6P was lowest in Ak and highest in Ka and Ta (Figures 4(c) and 5(c)). No significant correlation between %E and E6P was observed in either year (Figure 6).

To compare the spatial progression of endoreduplication in the developing endosperm among cultivars, ploidy maps were constructed at the stage when the dry weight of the developing caryopsis was about 25% of that at maturity (Supplement 1). Significant differences in the mean ploidy, %E, and E6P were observed among the 10 cultivars in 2015 (Figure 4). Similar results were obtained in 2014 (Supplement 3), and highly significant correlations between years were observed (Figure 5). The effects of years on the mean ploidy and E6P were significant, while the interaction of cultivars and years was not significant for each parameter (Supplement 4). The mean ploidy differed among cultivars, with the lowest and highest mean ploidies observed for Ak and Ta, respectively (Figures 4(a) and 5(a)). The following two groups of cultivars were detected based on %E: Ak, Sa, Ko, Ki, and Ka formed a low %E group, whereas Ho, Ha, Ni, IR, and Ta belonged to a high %E group (Figures 4(b) and 5(b)). Differences in E6P were observed among cultivars; E6P was lowest in Ak and highest in Ka and Ta (Figures 4(c) and 5(c)). No significant correlation between %E and E6P was observed in either year (Figure 6).

To compare the spatial progression of endoreduplication in the developing endosperm among cultivars, ploidy maps were constructed at the stage when the dry weight of the developing caryopsis was about 25% of that at maturity (Supplement 1). Significant differences in the mean ploidy, %E, and E6P were observed among the 10 cultivars in 2015 (Figure 4). Similar results were obtained in 2014 (Supplement 3), and highly significant correlations between years were observed (Figure 5). The effects of years on the mean ploidy and E6P were significant, while the interaction of cultivars and years was not significant for each parameter (Supplement 4). The mean ploidy differed among cultivars, with the lowest and highest mean ploidies observed for Ak and Ta, respectively (Figures 4(a) and 5(a)). The following two groups of cultivars were detected based on %E: Ak, Sa, Ko, Ki, and Ka formed a low %E group, whereas Ho, Ha, Ni, IR, and Ta belonged to a high %E group (Figures 4(b) and 5(b)). Differences in E6P were observed among cultivars; E6P was lowest in Ak and highest in Ka and Ta (Figures 4(c) and 5(c)). No significant correlation between %E and E6P was observed in either year (Figure 6).
Figure 3. Arrangement of cells in different ploidy classes in the developing endosperm of rice. (a) and (c) Fluorescence microscopic images of transverse sections of rice caryopses at 5 DAP and 9 DAP, respectively. The section was stained with DAPI, and nuclei are indicated by light blue fluorescence within cells. (b) and (d) Arrangement of nuclei in different ploidy classes in the endosperm at 5 DAP and 9 DAP, respectively. The images of (a) and (c) were converted to grayscale (b and d), and the ploidy of each nucleus was superimposed. Light blue, 3C; green, 6C; yellow, 12C; red, 24C. In each image, the scale bar represents 100 µm. (e) and (f) Histograms of the integrated density of nuclei in transverse sections of rice endosperm at 5 DAP and 9 DAP, respectively. Histogram in (e) corresponds to (a) and is composed of 329 nuclei. Histogram in (f) corresponds to (c) and is composed of 377 nuclei. The ploidy is indicated for each nuclear peak. Cultivar: ‘Kinuhikari’ (japonica).
To evaluate the effects of endoreduplication in the developing endosperm on the mature caryopsis, the dry weight and cell size of the mature caryopsis in 2015 were examined (Table 2, Supplements 7 and 8). One-way ANOVA detected significant differences in caryopsis weight, the mean transverse sectional area, and the mean volume of endosperm cells among the 10 rice cultivars (Table 2). Although no significant correlation between the mean

Figure 4. Comparison of endoreduplication in the developing endosperm among 10 rice cultivars in 2015. (a) Mean ploidies of all nuclei in the developing endosperm. (b) Proportion of nuclei ≥6C (%E). (c) Mean ploidies of nuclei ≥6C (E6P). The E6P was calculated as described for the mean ploidy, except the 3C class was excluded. Data represent the mean ± SE of four replicates. Bars with the same letter are not significantly different according to the Tukey–Kramer multiple comparison test (P < 0.05). Cultivars are arranged in the order of increasing mean ploidy. (I) indicates indica cultivars, while (J) indicates japonica cultivars.

Figure 5. Correlation of endoreduplication parameters between 2014 and 2015. (a) Mean ploidy. (b) %E. (c) E6P. Open and closed symbols represent data from indica and japonica cultivars, respectively. ***Significant at 0.001 probability level.

To evaluate the effects of endoreduplication in the developing endosperm on the mature caryopsis, the dry weight and cell size of the mature caryopsis in 2015 were examined (Table 2, Supplements 7 and 8). One-way ANOVA detected significant differences in caryopsis weight, the mean transverse sectional area, and the mean volume of endosperm cells among the 10 rice cultivars (Table 2). Although no significant correlation between the mean
Figure 6. Correlation between %E and E6P. (a) 2014. (b) 2015. Open and closed symbols represent data from indica and japonica cultivars, respectively. ns not significant at 0.05 probability level.

Figure 7. Arrangement of cells in different ploidy classes in the developing endosperm of japonica rice cultivars. (a–e) Fluorescence microscopic images of transverse sections of rice caryopses. The section was stained with DAPI, and nuclei are indicated by the light blue fluorescence within cells. (f–j) Arrangement of nuclei in different ploidy classes in the endosperm. Light blue, 3C; green, 6C; yellow, 12C; red, 24C. (a) and (f) Ak. (b) and (g) Ki. (c) and (h) Ko. (d) and (i) Ni. (e) and (j) Sa. The scale bar represents 100 µm. Figures correspond to the results from 2015.
ploidy in the developing endosperm and the weight of the mature caryopsis was observed (Figure 11(a)), a significant correlation was detected between the mean ploidy in the developing endosperm and the mean transverse sectional area or volume of endosperm cells in the mature caryopsis (Figure 11(b,c)).

Discussion

**Spatiotemporal progression of endoreduplication in rice endosperm**

Endoreduplication was detected as early as 5 DAP in rice endosperm (Figures 1(a), 2(b) and 3(b)), although the mean ploidy of all nuclei was low at 5 DAP (Figure 2(c)). The mean ploidy of endosperm cells increased until 8 DAP, and thereafter remained relatively constant (Figure 2(c)). This temporal progression is consistent with the results for rice endosperm examined using Feulgen microspectrophotometry (Ramachandran & Raghavan, 1989). In rice endosperm, mitotic cell division

Table 1. Two-way ANOVA of the transverse sectional area of endosperm cells in 10 rice cultivars.

| Year | Ploidy (P) | Cultivar (C) | P*C |
|------|------------|--------------|-----|
| 2014 | ***        | ns           | *   |
| 2015 | ***        | ns           | *** |

*Significant at 0.05 probability level; **significant at 0.001 probability level; ***not significant at 0.05 probability level.
Figure 9. Comparison of the transverse sectional area of cells in the same ploidy class among rice cultivars. (a) 3C. (b) 6C. (c) 12C. (d) 24C. Data represent the mean ± SE of four replicates. Bars with the same letter are not significantly different according to the Tukey–Kramer multiple comparison test ($P < 0.05$). Figures correspond to the results from 2015. Cultivars are arranged in the order of increasing mean ploidy. (I) indicates indica cultivars, while (J) indicates japonica cultivars.

Figure 10. Correlation between endoreduplication and transverse sectional area of cells within the 3C and 6C ploidy classes in 2015. (a) %E and area of 3C cells. (b) E6P and area of 3C cells. (c) %E and area of 6C cells. (d) E6P and area of 6C cells. Open and closed symbols represent data from indica and japonica cultivars, respectively. *Significant at 0.05 probability level; **significant at 0.01 probability level; ***not significant at 0.05 probability level.
begins at 4 DAP and ceases at 8 or 9 DAP (Hoshikawa, 1967). These observations indicate that endoreduplication overlaps temporally with mitosis in rice endosperm. A similar overlap of mitosis and endoreduplication occurs in maize, in which endosperm cells gradually and asynchronously switch from the mitotic cycle to the endocycle (Sabelli & Larkins, 2009). Thus, cells in the mitotic cycle and in the endocycle coexist in the developing endosperm of Poaceae species, and the degree of endoreduplication differs among nuclei.

Ploidy maps clearly revealed the distribution of nuclei with varying degrees of endoreduplication in the rice endosperm (Figure 3). The ploidy was higher in the intermediate region than in the central and peripheral regions of the rice endosperm (Figure 3(d)), with similar spatial patterns observed in all 10 analyzed rice cultivars (Figures 7 and 8). Interestingly, this spatial progression of endoreduplication differed from that seen in previous reports of maize, sorghum, and teosinte; the ploidy is reportedly higher in the inner region of the endosperm (Dermastia et al., 2009; Kladnik et al., 2006; Vilhar et al., 2002). In maize endosperm, the ploidy gradient is due to the spatiotemporal pattern of the mitosis/endoreduplication switch. This switch from the mitotic cycle to the endocyte starts with cells in the central region and spreads outward (Nguyen et al., 2007; Sabelli & Larkins, 2009). Cells in the central region of rice endosperm are also formed earlier than those in the outer region (Hoshikawa, 1967). Therefore, cells in the central region of the rice endosperm should cease mitosis earlier than those in the outer region. However, 12C nuclei were not detected in cells in the central region of the rice endosperm at 9 DAP, when the nuclei in the intermediate region were ≥12C (Figure 3(d)). Furthermore, the cells in the central region of rice endosperm reportedly commence the process of programmed cell death around 10 DAP (Kobayashi et al., 2013). These results suggest that nuclei in the central region retain a low ploidy throughout rice endosperm development.

Table 2. Dry weight and cell size of the mature caryopsis in 10 rice cultivars in 2015.

| Cultivars | Caryopsis weight (mgDW) | Mean transverse sectional area of endosperm cells (×10^2 μm^2) | Mean volume of endosperm cells (×10^3 μm^3) |
|-----------|-------------------------|---------------------------------------------------------------|------------------------------------------|
| Ak (J)    | 21.1                    | 19.6                                                          | 71.2                                     |
| Ki (J)    | 21.6                    | 22.0                                                          | 80.7                                     |
| Ko (J)    | 20.7                    | 21.1                                                          | 75.9                                     |
| Ni (J)    | 22.8                    | 23.4                                                          | 85.2                                     |
| Sa (J)    | 22.7                    | 21.2                                                          | 74.8                                     |
| Ha (I)    | 19.1                    | 23.1                                                          | 94.2                                     |
| Ho (I)    | 22.9                    | 24.2                                                          | 96.9                                     |
| IR (I)    | 22.2                    | 20.6                                                          | 85.9                                     |
| Ka (I)    | 15.8                    | 23.9                                                          | 99.5                                     |
| Ta (I)    | 19.8                    | 25.8                                                          | 103.6                                    |

***Significant at 0.001 probability level.

Data represent the mean of four replicates. The mean volume of endosperm cells was calculated by multiplying the mean transverse sectional area of cells by the mean cell height.

Figure 11. Correlation between the mean ploidy in the developing endosperm and parameters of the mature caryopsis. (a) Caryopsis weight. (b) Mean transverse sectional area of endosperm cells. (c) Mean volume of endosperm cells. Open and closed symbols represent data from indica and japonica cultivars, respectively. **Significant at 0.01 probability level; ns : not significant at 0.05 probability level.
**Comparison of endoreduplication among 10 rice cultivars**

Endoreduplication progression in endosperm cells varied among the 10 examined rice cultivars (Figure 4, Supplement 3). For example, the mean ploidy, which is a typical parameter representing endoreduplication progression in whole endosperm cells, differed significantly among the cultivars, with the highest and lowest mean ploidies observed in Ta and Ak, respectively. The rank order of mean ploidy among the cultivars was similar in 2014 and 2015 (Figure 4, Supplement 3), and highly significant correlations between years were observed (Figure 5). These results imply that endoreduplication progression is genetically regulated in rice endosperm.

To examine endoreduplication progression in rice endosperm in detail, %E and E6P were calculated. The parameter %E is applied to estimate the initiation of the endocycle, while E6P is used to estimate the reiteration of the endocycle. As reported for maize (Dilkes et al., 2002), no significant correlation between %E and E6P was observed in rice endosperm (Figure 6). For example, on the basis of %E, Ka belonged to the lowest group, whereas on the basis of E6P, it belonged to the highest group (Figure 4(b,c)). Additionally, ploidy maps revealed that Ka had more 24C nuclei than Ni, Ha, and IR, which were similar to Ka regarding mean ploidy (Figures 7 and 8). These results suggest that the initiation and reiteration of the endocycle are independently regulated in rice endosperm cells. This independence probably enables cells to cease mitosis early and to remain within a low ploidy class (≤6C), as observed in the cells located in the central region of rice endosperm (Figures 3, 7 and 8). Previous studies characterized some regulatory mechanisms underlying the initiation of the endocycle in rice endosperm. For example, the CDK inhibitor OsRya;KRP1 is important for the exit from the mitotic cell cycle (Barróco et al., 2006). Meanwhile, OsCCS52A, which is an activator of the anaphase-promoting complex that initiates cyclin degradation, also mediates the exit from the mitotic cell cycle (Su’udi et al., 2012). The application of 1-methylcyclopropene increased the frequency of nuclei ≥6C by promoting the production of CDKAs and CYCD2:2, while inhibiting the production of CYCB2:2 (Panda et al., 2016). In contrast to the emerging understanding of endocycle initiation, reiteration of the endocycle is less well understood in rice endosperm. In *Arabidopsis thaliana*, some mechanisms for terminating the reiteration of the endocycle have been identified (Breuer et al., 2010). For example, a transcriptional regulator, GT-2-LIKE1 (GTL1), is produced only during the post-branching stages of leaf trichomes, and GTL1 loss-of-function mutations lead to an additional round of the endocycle. Future rice endosperm studies should focus on the reiteration of the endocycle.

In the developing rice endosperm, proportional relationships between ploidy and cell size were observed. Cells with a higher ploidy had a larger transverse sectional area (Table 1, Figure 9 see the vertical axis). Similar relationships have been reported in the developing endosperm of maize and sorghum (Kladnik et al., 2006; Vilhar et al., 2002). Furthermore, Panda et al. (2018) reported that a 6-benzylaminopurine treatment simultaneously increases the ploidy and size of rice endosperm cells. Accordingly, endoreduplication appears to affect rice endosperm cell size. In addition to the variation in cell size reflective of ploidy variation, significant differences in cell size were observed among cultivars within the same ploidy classes, such as 3C and 6C (Figure 9(a,b)). The transverse sectional area of endosperm cells with 3C nuclei was negatively correlated with %E and E6P, and that of cells with 6C nuclei was negatively correlated with E6P (Figure 10). Endosperm cells in the cultivars with low %E or E6P probably remained within the same ploidy class for a relatively long period. Therefore, a prolonged duration within the same ploidy class might promote cell expansion. Variations in cell size associated with ploidy were greater than those associated with cultivars (Figure 9(a,b)). For example, the Ak cells with 3C nuclei, which were the largest cells with 3C nuclei among the 10 rice cultivars studied (648 µm²), were smaller in area than the Ka cells with 6C nuclei, which were the smallest cells with 6C nuclei (1017 µm²). These results suggest that endoreduplication considerably influences rice endosperm cell size. Furthermore, the effects of endoreduplication on cell size in the developing endosperm appeared to continue in the mature endosperm, because the mean ploidy in the developing endosperm was significantly correlated with the mean mature endosperm cell size (Figure 11(b,c)).

Although endoreduplication appeared to play an important role in determining rice endosperm cell size, there was no significant correlation between the mean ploidy in the developing endosperm and the weight of the mature caryopsis (Figure 11(a)). Because organ size is determined not only by cell size, but also by cell number (Orr-Weaver, 2015), the variation in cell size associated with endoreduplication is probably cancelled by the diversity in cell number in the examined rice cultivars. However, the genetic variation related to the endoreduplication of endosperm cells might be useful for improving caryopsis weight. For example, new rice genotypes with heavy caryopses may be developed by crossing genotypes with high ploidies and cell numbers per endosperm, as proposed for wheat (Brunori et al., 1993).
The enlargement of endosperm cells associated with endoreduplication may affect the quality of rice as food. The surface area of a sphere \((4\pi r^2)\) generally does not keep pace with increases to the volume \((4/3\pi r^3)\). Therefore, for endosperms with an identical volume, the endosperm composed of large cells will contain less cell wall material than the endosperm containing small cells. Endosperm cell walls are considered to affect the texture of cooked rice (Shibuya & Iwasaki, 1984). These results imply that endoreduplication in endosperm cells affects the texture of cooked rice via changes in the amount of cell wall material. Thus, rice quality may be improved by modifying the endoreduplication in endosperm cells.

In conclusion, this study clarified the characteristics of endoreduplication in the developing rice endosperm and their variations among rice cultivars. Rice endosperm exhibited a typical spatial progression of endoreduplication, with the ploidy of nuclei higher in the intermediate region than in the central and peripheral regions of the endosperm. Endoreduplication progression differed significantly among 10 rice cultivars. This diversity reflects the variation in the initiation and reiteration of the endocycle, which are two processes that are independently regulated in rice endosperm. There were proportional relationships between ploidy and cell size in the developing rice endosperm. Furthermore, the mean ploidy in the developing endosperm was significantly correlated with mature endosperm cell size. These results suggest that endoreduplication influences rice endosperm cell size. Therefore, the genetic variation mediating the endoreduplication of endosperm cells may be applicable for improving rice yield and/or quality via changes in cell size.

Acknowledgments

The author thanks Kenji Nagata, Takeshi Saito, and Tatsuya M. Ikeda [Western Region Agricultural Research Center, National Agriculture and Food Research Organization (NARO)] for their advice regarding rice cultivation, flow cytometry, and fluorescence microscopy. The author is also grateful to Osamu Ideta (NARO) for providing rice seeds. I thank Robert McKenzie, PhD, from Edanz Group (www.edanzediting.com/ac), for editing a draft of this manuscript.

Disclosure statement

No potential conflict of interest was reported by the author.

Funding

This work was supported by the Japan Society for the Promotion of Science KAKENHI [Grant Number 26850010].

References

Ando, T., Yamamoto, T., Shimizu, T., Ma, X. F., Shomura, A., Takeuchi, Y., … Yano, M. (2008). Genetic dissection and pyramiding of quantitative traits for panicle architecture by using chromosomal segment substitution lines in rice. *Theoretical and Applied Genetics*, 116, 881–890.

Barow, M. (2006). Endopolyploidy in seed plants. *BioEssays*, 28, 271–281.

Barow, M., & Meister, A. (2003). Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. *Plant Cell & Environment*, 26, 571–584.

Barróco, R. M., Peres, A., Droual, A. M., De Veylder, L., Nguyen, L. S. L., De Wolf, J., … Frankard, V. (2006). The cyclin-dependent kinase inhibitor OrysaKRP1 plays an important role in seed development of rice. *Plant Physiology*, 142, 1053–1064.

Breuer, C., Ishida, T., & Sugimoto, K. (2010). Developmental control of endocycles and cell growth in plants. *Current Opinions in Plant Biology*, 13, 654–660.

Brunori, A., Forino, L. M. C., Frediani, M., & Ruberti, F. (1993). Cell number and polyplody in the starchy endosperm of *Triticum aestivum* in relation to seed weight. *Journal of Genetics & Breeding*, 47, 217–220.

Chevalier, C., Bourdon, M., Pirrello, J., Cheniclet, C., Gévaudant, F., & Frangne, N. (2014). Endoreduplication and fruit growth in tomato: Evidence in favour of the karyoplastic ratio theory. *Journal of Experimental Botany*, 65, 2731–2746.

Chevalier, C., Nafati, M., Mathieu-Rivet, E., Bourdon, M., Frangne, N., Cheniclet, C., … Hernould, M. (2011). Elucidating the functional role of endoreduplication in tomato fruit development. *Annals of Botany*, 107, 1159–1169.

Dante, R. A., Larkins, B. A., & Sabelli, P. A. (2014). Cell cycle control and seed development. *Frontiers in Plant Science*, 5, 493.

De Veylder, L., Larkin, J. C., & Schnittger, A. (2011). Molecular control and function of endoreplication in development and physiology. *Trends in Plant Science*, 16, 624–634.

Dermastia, M., Kladnik, A., Dolenc Koce, J., & Chourey, P. S. (2009). A cellular study of teosinte *Zea mays* subsp. *parviglumis* (Poaceae) carvopsis development showing several processes conserved in maize. *American Journal of Botany*, 96, 1798–1807.

Dilkes, B. P., Dante, R. A., Coelho, C., & Larkins, B. A. (2002). Genetic analyses of endoreduplication in *Zea mays* endosperm: Evidence of sporophytic and zygotic maternal control. *Genetics*, 160, 1163–1177.

Galbraith, D. W., Harkins, K. R., Maddox, J. M., Ayres, N. M., Sharma, D. P., & Firoozabady, E. (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science*, 220, 1049–1051.

Hoshikawa, K. (1967). Studies on the development of endosperm in rice. 1. Process of endosperm tissue formation. *Japanese Journal of Crop Science*, 36, 151–161. (In Japanese with English summary).

Kladnik, A., Chourey, P. S., Pring, D. R., & Dermastia, M. (2006). Development of the endosperm of *Sorghum bicolor* during the endoreduplication-associated growth phase. *Journal of Cereal Science*, 43, 209–215.

Kobayashi, H., Ikeda, T. M., & Nagata, K. (2013). Spatial and temporal progress of programmed cell death in the developing starchy endosperm of rice. *Planta*, 237, 1393–1400.
Kobayashi, H., Yanaka, M., & Ikeda, T. M. (2012). 6-Benzylaminopurine treatment induces increased pubescence on wheat leaves. Plant Growth Regulation, 67, 19–25.

Kono, Y., Tashiro, T., & Inagaki, N. (1979). Studies on the developmental physiology of the endosperm tissue in rice plants. II. Nuclear DNA content and endosperm structure in early developmental stage of caryopsis. Japanese Journal of Crop Science, 48, 163–171. (In Japanese with English summary).

Kowles, R. V. (2009). The importance of DNA endoreduplication in the developing endosperm of maize. Maydica, 54, 387–399.

Leiva-Neto, J. T., Grafi, G., Sabelli, P. A., Dante, R. A., Woo, Y., Maddock, S., … Larkins, B. A. (2004). A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm. Plant Cell, 16, 1854–1869.

Maes, L., Inzé, D., & Goossens, A. (2008). Functional specialization of the transparent testa glabra1 network allows differential hormonal control of laminal and marginal trichome initiation in Arabidopsis rosette leaves. Plant Physiology, 148, 1453–1464.

Martínez, C. P., Arumuganathan, K., Kikuchi, H., & Earle, E. D. (1994). Nuclear DNA content of ten rice species as determined by flow cytometry. The Japanese Journal of Genetics, 69, 513–523.

Morita, S., Yonemaru, J., & Takanashi, J. (2005). Grain growth and endosperm cell size under high night temperatures in rice (Oryza sativa L.). Annals of Botany, 95, 695–701.

Nguyen, H. N., Sabelli, P. A., & Larkins, B. A. (2007). Endoreduplication and programmed cell death in the cereal endosperm. In O. A. Olsen (Ed.), Endosperm – plant cell monographs (Vol. 8, pp. 21–43). Berlin, Heidelberg: Springer-Verlag.

Orr-Weaver, T. L. (2015). When bigger is better: The role of polyploidy in organogenesis. Trends in Genetics, 31, 307–315.

Panda, B. B., Badoghar, A. K., Sekhar, S., Kariai, E., Mohapatra, P. K., & Shaw, B. P. (2015). Biochemical and molecular characterisation of salt-induced poor grain filling in a rice cultivar. Functional Plant Biology, 43, 266–277.

Panda, B. B., Badoghar, A. K., Sekhar, S., Shaw, B. P., & Mohapatra, P. K. (2016). 1-MCP treatment enhanced expression of genes controlling endosperm cell division and starch biosynthesis for improvement of grain filling in a dense-panicle rice cultivar. Plant Science, 246, 11–25.

Panda, B. B., Sekhar, S., Dash, S. K., Behera, L., & Shaw, B. P. (2018). Biochemical and molecular characterisation of exogenous cytokinin application on grain filling in rice. BMC Plant Biology, 18, 89.

Ramachandran, C., & Raghavan, V. (1989). Changes in nuclear DNA content of endosperm cells during grain development in rice (Oryza sativa). Annals of Botany, 64, 459–468.

Sabelli, P. A. (2012). Replicate and die for your own good: Endoreduplication and cell death in the cereal endosperm. Journal of Cereal Science, 56, 9–20.

Sabelli, P. A., & Larkins, B. A. (2009). The development of endosperm in grasses. Plant Physiology, 149, 14–26.

Shibuya, N., & Iwasaki, T. (1984). Effect of cell wall degrading enzymes on the cooking properties of milled rice and the texture of cooked rice. Nippon Shokuhin Kogyo Gakkaishi, 31, 656–660.

Su’udi, M., Cha, J. Y., Jung, M. H., Ermawati, N., Han, C., Kim, M. G., … Son, D. (2012). Potential role of the rice OsCCS2A gene in endoreduplication. Planta, 235, 387–397.

Sugimoto-Shirasu, K., & Roberts, K. (2003). “Big it up”: Endoreduplication and cell-size control in plants. Current Opinion in Plant Biology, 6, 544–553.

Takai, T., Ikka, T., Kondo, K., Nonoue, Y., Ono, N., Arai-Sanoh, Y., … Kondo, M. (2014). Genetic mechanisms underlying yield potential in the rice high-yielding cultivar Takanari, based on reciprocal chromosome segment substitution lines. BMC Plant Biology, 14, 295.

Trafford, K., Haleux, P., Henderson, M., Parker, M., Shirley, N. J., Tucker, M. R., … Burton, R. A. (2013). Grain development in Brachypodium and other grasses: Possible interactions between cell expansion, starch deposition, and cell-wall synthesis. Journal of Experimental Botany, 64, 5033–5047.

Vilhar, B., Kladnik, A., Blejec, A., Chourely, P. S., & Dermastia, M. (2002). Cytometrical evidence that the loss of seed weight in the miniature! seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. Plant Physiology, 129, 23–30.