Development and Application of a Plate Method for Visualizing Aleurone Layers in Mature Rice Grains

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**ABSTRACT**

Rice bran oil, a valuable ingredient of rice bran (composed mainly of embryo and aleurone), is gaining increasing attention for its abundance and benefits for human health. To increase production, breeders have selected for enlarged embryos and thickened aleurone layers. However, breeding for the latter is impeded by the time-consuming and labor-intensive process of observation of aleurone traits. Here, we established a new method for visualizing aleurone layers comprising embedding of mature grains in a plate, dichromatic staining of half grains, and computer-assisted image analysis. With this ‘plate method’, a batch of up to 100 grains fixed on the plate can be handled and examined more efficiently than the standard cryomicrotome method, which only processes the grains individually. In addition, the results obtained from the plate method were highly correlated with that of the cryomicrotome method in terms of aleurone area \((r = 0.92)\) and mean aleurone thickness \((r = 0.93)\). This new method allowed us to rapidly assess the aleurone phenotypes of more than 22,000 mutagenized grains of ‘Mizuhochikara’, with 700–1000 grains per day. As a result, one mutant line with thickened aleurone layer was successfully isolated.

**Introduction**

Rice (\textit{Oryza sativa} L.) is the staple cereal of more than half of the world’s population. Usually, harvested rice grains are milled, and the resulting white rice (isolated endosperm) is cooked and eaten. During milling, the embryo and outer layers, which account for about 10\% of the weight, are removed as rice bran (Champagne \textit{et al.}, \textit{2004}). Although most of the bran produced is used in animal feed or becomes crop waste, it has considerable potential value. It is rich in vitamins, proteins, minerals, antioxidants, and lipids (Gul \textit{et al.}, \textit{2015}). Extracted rice bran oil (RBO) has the highest value owing to its abundance and favorable properties, including a desirable fatty acid balance, the high smoking temperature in cooking, and oxidative stability (Gul \textit{et al.}, \textit{2015}; Manickavasagan \textit{et al.}, \textit{2017}).
Moreover, RBO seems to have moderate but significant effects in reducing blood low-density lipoprotein cholesterol levels (Pal & Pratap, 2017). These qualities are driving demand for RBO.

To meet market and industry needs, breeders have worked to increase RBO production. Increasing the size of the embryo by using ‘giant embryo’ mutation is one of the most successful examples (Sakata et al., 2016). Aleurone is also a promising target. Aleurone predominates among rice bran components and holds the most RBO (Khin et al., 2013). The aleurone layers lie between the starch endosperm and the seed coat, which are composed of quadrangular parenchyma cells. Their thickness is positively correlated with RBO content (Khin et al., 2013). Although rice aleurone is generally monolayered, some cultivars have up to 7 layers (Juliano & Tuanho, 2004), indicating the presence of genetic factors controlling aleurone thickness. Nevertheless, there is a shortage of studies and breeding programs targeting rice aleurone, most likely owing to the difficulty in observing the tissue (Khin et al., 2013).

In general, morphological and histochemical characterization of cereal grains requires sectioning, methods of which depend on the study purposes. Examination of rice aleurone needs an intact cross-section because the thickness of aleurone layers varies across the grain, being thicker on the dorsal side than on the ventral and lateral sides. Although thin sections can be obtained from mature rice grains, differences in moisture content and hard tissues of the endosperm create difficulties (Saito et al., 2008), and complicated steps are needed to prepare thin sections on a cryomicrotome (Chiou et al., 2018; Xu & Wei, 2020). On the other hand, a thick-sectioning method is a more simple and practical approach in which aleurone is distinguished from other tissues by using staining techniques on the grain cross-section. In the preceding studies, examination of aleurone layer using half-cut grain under a microscope (Khin et al., 2013) or half-seed assay under a stereomicroscope (Liu et al., 2018) was performed on a single grain basis. However, they are not efficient enough for large-scale analyses since sample preparation and microscopic examination of each single rice grain are inherently painstaking.

Here, we optimized the thick-sectioning method for a much greater number of samples. The accuracy of the new method was validated by comparison with the cryomicrotome (thin sectioning) method. Subsequently, the new method was employed to screen mutagenized populations of ‘Mizuhochikara’ for thickened-aleurone grains.

**Materials and methods**

**Plant materials**

Several cultivars of rice (Oryza sativa L. ssp. japonica) and N-methyl-N-nitrosourea (MNU) mutant populations of ‘Mizuhochikara’ were used in this study. Mizuhochikara is a widely adaptable, high-yielding cultivar used widely as animal feed, in rice bread making, as forage grain, and in shochu (Japanese distilled liquor) brewing (Sato et al., 2017; Tamura et al., 2012). Also, giant embryo mutants of Mizuhochikara, which have increased lipid content, have already been obtained (Sakata et al., 2016).

**Experiment 1. Plate method (modified half-grain method) and validation against the cryomicrotome method**

**Plate method preparation and staining**

We used mature japonica rice grains harvested on Kyushu University Farm, Japan. An acrylic 100-well-template plate (Figure 1(a)) designed by Kett Electric Laboratory, Tokyo, Japan), which can hold 100 grains, was used for this study. Each well with a long oval shape opening is 4.0 mm in length, 3.0 mm in width, and 3.0 mm in depth. Using clear nail polish, intact mature brown rice grains were half-embedded (embryo-end up) in the wells so that their longitudinal centers were at the same level as the surface of the plate. The plate was soaked in distilled water (DW) at room temperature overnight (Figure 1(b)). The softened grains were cut transversely at the plate surface with a sharp single-edge razor blade (Olfa, Osaka, Japan) into equal halves. The upper halves would be kept for germination. The lower halves were retained in the plate (Figure 1(b)). The cut surfaces were carefully rinsed with DW, which was gently blotted with Kimwipers S-200 mini tissue (Nippon Paper Crecia Co., Ltd., Tokyo, Japan).

The cut surfaces were then stained. First, the whole plate was immersed in ‘New MG solution, methanolic’, a readymade (May-Grünwald) solution (Kanto Chemical Co., Inc., Tokyo, Japan), which was diluted 1:3 (v/v) with methanol just before use. After 4 to 5 min of incubation at room temperature, the surfaces were rinsed with DW 4 or 5 times. Second, the plate was washed gently with an iodine solution (0.05 mol L⁻¹, Wako, Tokyo, Japan) for 30 to 60 s to stain the endosperm, followed by washing with DW. Finally, the stained surfaces were wetted with drops of DW, covered with a coverslip, and
photographed under a digital microscope at 85X magnification (MSX-500Di, Moritex Schott, Saitama, Japan). In each image, the total cut surface area, endosperm area, and circumference of the cut surface were determined by image-analysis software (WinROOF 2018, Mitani Corporation, Tokyo, Japan). Aleurone traits were then calculated as below, assuming that the outer and inner aleurone layers have the same circumference (Khin et al., 2013). The total cut surface area did not include seed coat or pericarp.

\[
\text{Aleurone area (mm}^2\text{)} = \left[\text{total cut surface area (mm}^2\text{)} - \text{endosperm area (mm}^2\text{)}\right]
\]

\[
\text{Mean aleurone thickness (\text{\textmu}m)} = \frac{\text{aleurone area (mm}^2\text{)}}{\text{aleurone outer circumference (mm)}} \times 1000
\]

**Validation of new method against cryomicrotome method**

To verify the accuracy of the new method, we compared the results with those of the cryomicrotome (thin sectioning) method, which is often used for detailed and accurate measurement of aleurone. Eleven *japonica* cultivars were used for this comparison, namely ‘Mizuhochikara’, ‘Hinohikari’, ‘Hitomebore’, ‘Koshihikari’, ‘Reihou’, ‘LO1050’, ‘Nikomaru’, ‘Tsukushihomare’, ‘Tsukushimochi’, ‘Tsukushiroman’, and ‘Yumeikkon’. The cultivars varied in aleurone layer thickness, from ‘Tsukushihomare’, ‘Mizuhochikara’, and ‘Nikomaru’ in the thinnest group to ‘LO1050’ in the thickest. Six grains of each cultivar were examined by both methods. The grains were embedded in the wells on the plate using nail polish as above and soaked in DW before being sliced in half. The lower (non-embryo) halves retained in the plate were stained as above. The upper (embryo) halves were prepared for tomography by Kawamoto’s film method with some modifications (Khin et al., 2013). A halved grain was placed in a single embedding well with its cut surface facing the well’s floor, and the well was filled with Super Cryo Embedding Medium (Section-Lab Co., Ltd., Hiroshima, Japan). All embedded grains were frozen inside the cryomicrotome chamber (Leica CM1100, Wetzlar, Germany) at −20°C and then fixed on the sample holder for sectioning. Sections 10 μm thick were made and collected on adhesive film (10 mm × 20 mm: Section-Lab Co. Ltd., Hiroshima, Japan). The sections on the films were dried at room temperature for 20s and then stained as in the modified half-grain method, but for half the time owing to their thinness. Stained sections were mounted between the film and a glass slide, wetted with drops of DW, covered with a coverslip, and microphotographed as in the plate method. Aleurone traits were calculated as above.

**Experiment 2. Use of plate method in isolating mutant lines for thickened aleurone layer**

To demonstrate the method’s effectiveness, we screened a mutagenized population of ‘Mizuhochikra’ for thicker aleurone. The mutant population was induced by treating fertilized egg cells with N-methyl-N-nitrosourea (Sakata et al., 2016). We harvested ~2,200 M1 plants that were self-pollinated to produce M2 grains. Using the plate method described above, at least 10 M2 grains from each M1 plant (~22,000 grains in total) were assessed in terms of aleurone thickness. The grains were dried-cut before soaking to preserve the embryo halves for the next-
generation advancement. Then, the whole plate was subjected to soak in DW, later, the grains on the plate were cut again to make finer surfaces for staining. M2 candidate lines with thickened aleurone were raised from saved embryos to obtain progenies.

**Statistical tests**

Analysis of variance (ANOVA) was performed in Excel to assess the difference between the two methods and among cultivars. The relationship between aleurone traits measured by plate method and by cryomicrotome method was examined through correlation analysis.

**Results**

**Experiment 1. Development of new plate method and validation against standard cryomicrotome method**

**Screening aleurone layers by the plate method (modified half-grain method)**

We established a modified, straightforward half-grain method to observe the aleurone layer. It incorporates the preparation of half-grain samples in a plate (Figure 1 (a,b)), staining (Figure 1(c)), and observation under a digital microscope (Figure 1(d)). As shown in Figure 2(b), an intact transverse section of grain was obtained. The aleurone layer was dyed pale blue, which was distinguished from the seat coat that was dyed in the dark blue and from the starchy endosperm that was dyed pink (Figure 2(b,c)). The aleurone layer was thicker on the dorsal side (2 layers) and thinner on the ventral and lateral side (single layer). The structure of the aleurone layer and starchy endosperm were well-maintained throughout the process.

**Examination of aleurone layer by plate method and cryomicrotome-sectioning method**

We compared the effectiveness and reliability of the plate method against the cryomicrotome method using the matching halves of each grain (Figure 2).

ANOVA found no significant interaction between cultivars and methods in the measured values, which comprised the total cut surface area, starchy endosperm area, and outer circumference (Table 1). The methods were the main factor causing the differences in all these measured values (P < 0.001, Table 1). The total cut surface area, starchy endosperm area, and circumference were larger by the plate method than by the cryomicrotome method. On the other hand, the calculated values, comprising the aleurone area and mean aleurone thickness, were smaller in the plate method. In addition, there was a significant interaction between cultivars and methods in aleurone area and mean aleurone thickness.

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**Figure 2.** Grains processed by two methods. (a–c) by the plate method. (d–f) by the cryomicrotome method. (b, e) Stained section of halved grain obtained by plate and cryomicrotome, respectively. Bars = 200 µm. (c, f) Enlargements of rectangles in (b and e), respectively. Bars = 100 µm. White arrowheads indicate aleurone layers. SE, starchy endosperm.
Table 1. Aleurone parameters of 11 cultivars determined by the plate method and the cryomicrotome method.

|                          | Total cut surface area (mm²) | Starchy endosperm area (mm²) | Outer circumference (mm) | Aleurone area (mm²) | Mean aleurone thickness (µm) |
|--------------------------|------------------------------|------------------------------|--------------------------|---------------------|------------------------------|
|                          | Plate method | Cryo-method | Plate method | Cryo-method | Plate method | Cryo-method | Plate method | Cryo-method | Plate method | Cryo-method |
| Mean (11 cultivars)      |              |              |              |              |              |              |              |              |              |              |              |
| Range                    |              |              |              |              |              |              |              |              |              |              |              |
| Cultivars (F-value)      | 1.70 ns      | 1.62 ns      | 2.81**       | 37.42***     | 42.55***      | 79.89***     |
| Methods (F-value)        | 96.49***     | 116.10***    | 103.15***    | 33.76***     | 1.99*         | 2.10*        |
| Cultivar × method (F-value) | 0.67 ns    | 0.77 ns      | 0.94 ns      | 1.99*        | 2.10*         |              |

Values indicate mean ± standard deviation (n = 6). Significance: *P < 0.05, **P < 0.01, ***P < 0.001; ns, non-significant.

(P < 0.05, Table 1). However, overall, high correlations were detected between the methods in estimated aleurone area (r = 0.92***)) and mean aleurone thickness (r = 0.93***; Figure 3).

**Experiment 2. Screening and isolating thickened-aleurone phenotypes from a mutant population**

In Experiment 1, the accuracy of the plate method has been validated. It was then used for large-scale screening of rice mutants. The plate method is tremendously efficient in isolating the grains with thicker aleurone, making the mutant screening much easier. In practice, the cryomicrotome method takes 5–10 minutes to prepare a grain sample. In contrast, the plate method can process 100-grain samples (one plate) in 40–45 minutes, except soaking time. Accordingly, it takes only about 25 seconds for one grain. By using the plate method, image capturing of 700–1,000 grains could be carried out within a day.

Out of 2,200 lines, one promising mutant line with a significantly thickened aleurone layer compared with wild type (WT) was successfully isolated. The distribution of mean aleurone thickness in a population of M₃ grains (n = 153) from the promising M₂ line showed a wide range of mean aleurone thickness from approximately 19 µm to 63 µm (Figure 4). Overall, the mean aleurone thickness of M₃ grains increased by almost 50% relative to that of WT: 28.71 versus 19.36 µm. Remarkably, some grains had nearly triple the aleurone thickness compared to that of WT grains. Regarding the number of aleurone layers, WT mainly had one single layer (Figure 5(a,b)) while M₃ mutant comprised several layers that varied across the grain, increased either in the dorsal side and until half of the lateral side (Figure 5(c,d)), or uniformly across the grain (data not shown).

**Discussion**

In a rice grain, aleurone traits are quick and reliable indicators of the amount of lipids, which are mainly stored in the form of triacylglycerol (TAG; Khin et al., 2013). TAG quantification is feasible for both single grain and a large number of grains that are bulked (Nagamoto et al., 2018; Sakata et al., 2016). However, large-scale analyses of aleurone traits for thousands of grains have been difficult because it requires observation of every single grain.

In this study, we developed a plate method that enables the efficient handling of many sample grains during sectioning and staining. Immobilized grains are

![Figure 3](image-url)  **Figure 3.** Relationships between the plate and cryomicrotome method in aleurone area and mean aleurone thickness of 11 cultivars. ***Significant at P < 0.001.
easily cut on the plate without the need for a microtome. The resulting cut grains can be stained altogether by immersing the plate in the dye solution. Then, the stained grain dissections on the plate can be clearly observed under a digital microscope. Furthermore, the corresponding embryo halves can be stored and used to obtain subsequent generations. Improvements were also made in the staining process by using two coloring reagents, ‘New MG solution’ and iodine solution. New MG solution is typically used to test the degree of polishing of cereal grains, in which the pericarp is dyed green or blue, and endosperm is dyed pink (Serna-Saldivar, 2012). The iodine solution was used to stain starch, which is absent in the aleurone, to

Figure 4. Distribution of mean aleurone thickness of M3 grains and ‘Mizuhochikara’ (wild-type) determined by the plate method.

Figure 5. Cross-sections of WT ‘Mizuhochikara’ and an M3 grain of mutant. (a, c) Whole cross-section of WT ‘Mizuhochikara’ and an M3 mutant grain and. Bars = 200 µm. (b, d) Enlargements of rectangles in (a) in (c), respectively. Bars = 100 µm. The white arrows indicate aleurone layers (AL). SE: Starchy endosperm.
distinguish the tissues better. As a result, the aleurone (pale blue) is clearly distinguished from the pericarp and seed coat (dark blue), as well as from the endosperm (purple; Figure 2). Accordingly, the new method allows for more precise measurement of aleurone layers, compared with those that use Oil Red O staining (Khin et al., 2013).

We compared the results between the new plate method and the cryomicrotome (thin sectioning) method, as in the preceding study (Khin et al., 2013). Although the measured values (total cut surface area, starchy endosperm area, and outer circumference) were significantly larger in the plate method than in the cryomicrotome method, these values were not significant among the cultivars in each method. Presumably, these higher values in the plate method were caused by the swelling of the dissections due to the higher water content. When samples were cut, the surfaces of half-grains embedded in the plate retained large amounts of water. On the other hand, in the cryomicrotome method, sample swelling would be minimal because they were frozen-fixed 10 mm-thick dissections, which contained much lesser amounts of water.

However, in terms of the calculated values (aleurone area and mean aleurone thickness), the ANOVA test detected interactions between the methods and the cultivars. We believe these interactions were caused by the differences in degrees of water-swelling of grains among cultivars. These differences could be derived from the variation in components (amylose-amylopectin ratio) and the molecular structures of endosperm starch, the predominant constituent (70 ~ 80%) of a rice grain. It is widely known that the water absorption of a rice grain is strongly influenced by the characteristics of its endosperms starch. In any case, further improvements of the method are needed to detect subtle differences in quantitative traits.

However, despite the systematic errors pertaining to the plate method, the method is of good use for mutant screenings and segregation analyses. The errors in the plate method could be negligible considering the increased thickness of the aleurone layers in the isolated mutant as described in the Results section. In this study, the plate method mainly focuses on reducing time for sample preparation and observation for large-scale analyses rather than an accurate measurement. Moreover, there were strong correlations between the two methods in aleurone area \( r = 0.92^{**} \) and average thickness \( r = 0.93^{***} \) (Figure 3). Compared with the result of Khin et al. (2013), in the present study, the differences between the plate method and thin-sectioning method were reduced from 30% to 12% in aleurone area and from 45% to 16% in average thickness. From these results, we can safely conclude that our new plate method is of great practical use to characterize rice aleurone.

The approach of whole-grain cross-section analysis allows for quick examination of aleurone layer morphology throughout the cross-section of a grain. More importantly, this method enables simultaneous examination of many grains in a wide range of aleurone thickness (Figure 4). The method is recommended for mutant screenings and segregation analyses on aleurone traits in rice.

Using this new plate method, we identified one mutant with thicker aleurone from 2,200 M_1 populations of MNU-induced mutant lines. Subsequently, the M_2 grains of the isolated mutant were grown to produce M_3 grains that had thick aleurones with an increased number of aleurone layers (Figure 5). Notably, they exhibited a wide range of variations in aleurone; the grains with thickest aleurones surpass the previously reported thick-aleurone rice cultivar, ‘LO1050’ (Khin et al., 2013). Using the method developed in the present study, genetic mapping of the causal gene(s) is underway, which would lead to the development of new rice cultivars with higher lipid content.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Data availability statement**

The authors confirm that the supporting data included in this finding are available within the article.

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