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A method for obtaining high integrity RNA from developing aleurone cells and starchy endosperm in rice (*Oryza sativa* L.) by laser microdissection

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Abstract

Laser microdissection (LMD) is now becoming a powerful tool to elucidate the spatial profiling of gene expression. However, a remaining difficulty is obtaining high integrity RNA from fixed and embedded tissues in order to obtain accurate and reliable expression analyses. This study aimed to develop methods for the preparation of high integrity RNA from aleurone cells and starchy endosperm of developing rice endosperm by LMD. Acetone or 3:1 ethanol:acetic acid (AA), was used for the fixative and 2% carboxymethyl cellulose (CMC) or paraffin, was used for the embedding reagent. AA and CMC were better for identification of cell types and for recovery of intact RNA, although CMC-embedding did not preserve morphology as well as paraffin-embedding. Quantitative RT-PCR revealed that *OsSUT1* mRNA encoding a sucrose transporter was localized in aleurone cells and that *SDBE* mRNA encoding a starch debranching enzyme (pullulanase) was localized in starchy endosperm. Taken together, these results show that our LMD method is suitable for preparing high integrity RNA from aleurone cells and starchy endosperm in rice. mRNA obtained with this method should help to understand the molecular basis for endosperm development.

Keywords: Aleurone cells; High integrity RNA; Laser microdissection; *Oryza sativa* L.; Starchy endosperm

1. Introduction

Laser microdissection (LMD) is a powerful tool for isolating individual cell types from sections of heterogeneous tissue viewed under a microscope with the assistance of an intense laser beam [1]. LMD was initially developed for the isolation of animal cells, but it has recently been adapted to plant cells. The dissected cells can be used to extract RNA, and the extracted RNA has recently been employed for global gene expression profiling in plant LMD research [2–8]. Thus, LMD is a method for combing conventional histology with the latest methods of molecular biology.

For LMD, a suitable experimental procedure must be developed for each tissue of interest, taking into consideration the preservation of morphological structure, the precise cutting of tissues by laser, the recovery of intact RNA from fixed and embedded tissues. Preparing high integrity (intact) RNA from fixed and embedded tissues is difficult with LMD. Because low integrity (partially degraded) RNA provides variable results in gene expression analyses such as quantitative RT-PCR analyses, RNA quality control prior to downstream expression analyses is essential [9–11]. In this regard, the novel methods for fixation and embedding of tissues have been newly developing in plant LMD research to obtain high integrity of RNA [7,12].

Cereal endosperm is the tissue that is the most important food for humans and live stocks because it accumulates large

Abbreviations: AA, 3:1 ethanol:acetic acid; CMC, 2% carboxymethyl cellulose; LMD, laser microdissection; RIN, RNA integrity number

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amounts of storage compounds. Inside the integuments, the cereal grain consists of aleurone cells enclosing starchy endosperm. The aleurone cells are symplastically isolated from the adjacent maternal tissues, and thus the aleurone layer has an essential role in the uptake of assimilates from maternal tissues [13–14]. Aleurone cells contain large amounts of lipids, storage proteins (albumin and globulin) and phytin but little starch, whereas starchy endosperm accumulates large amounts of starch and storage proteins (glutelin, globulin and prolamin) during maturation [15]. Therefore, understanding the molecular basis for endosperm development into aleurone cells and starchy endosperm helps to improve the yields and grain quality of cereal crops. Kurita et al. [16] demonstrated that LMD could be used to isolate aleurone cells and starchy endosperm of developing rice endosperm. However, it was not clear which combination of fixative and embedding reagent yielded the highest integrity of RNA.

This paper aimed to establish a suitable method to prepare high integrity RNA from aleurone cells and starchy endosperm of developing rice endosperm by LMD. Because cereal endosperm accumulates large amounts of storage compounds, we focused on developing a method for precise cutting that does not scatter storage compounds. To demonstrate that the RNAs obtained by our method could be used for the global profiling of genes of aleurone cells and starchy endosperm, we compared the abundances of mRNAs of some genes involved in the conversion of sucrose to starch and showed that they were different between the two cell types.

2. Materials and methods

2.1. Plant material

Oryza sativa cv. Koshihikari (Japonica rice variety) was grown in 0.02 m^2 pots in a greenhouse until the booting stages. At the booting stage, plants were transferred into a naturally illuminated temperature-controlled chamber. Day (13 h) and night (11 h) air temperatures were maintained at 26 °C and 20 °C, respectively. Developing rice caryopses at 6–8 days after flowering (c.a. 6.0 mm in length, 2.5 mm in width, 1.8 mm in thickness) were used for the following experiments. Developing rice caryopses were immediately immersed in ice-cold fixative, and then kept at −20 °C for 1–2 days to minimize the change in gene expression during penetration of the fixative. Infiltration was not done at any step for the better morphological preservation.

2.2. Fixation and embedding for cryo-sectioning

Two combinations of fixative and embedding reagents were tried for cryo-sectioning (Table 1). In plant LMD, acetone [16–17] or 3:1 ethanol:acetic acid (AA) [2,3,4,12,18] is used as a fixative, thus these two fixatives were compared in this study. Because the use of an aldehyde in the fixative is known to severely decrease the extraction efficiency of RNA [3], FAA (formalin:acetic acid:ethanol = 1:1:18), which is frequently used as a fixative in plant histological research, was not used in this study. As an embedding reagent, 2% carboxymethyl cellulose (CMC) was used. The embedding reagent, CMC, is slightly sticky. This study is the first trial in plant LMD research to use CMC as an embedding reagent. We modified the method of Kurita et al. [16] by replacing Optimal Cutting Temperature compound (O.C.T. compound; Sakura Finetechnical Co., Ltd., Japan) with CMC as the embedding reagent. The reason why we used CMC as the embedding reagent is described in Section 2.4. When we used a combination of acetone + CMC, specimens were washed twice with CMC for 1 h, and then embedded in CMC. When we used a combination of AA + CMC, specimens were washed with CMC, once for 3 h and overnight. Washing time was longer when AA was used as a fixative, because penetration of washing reagent into specimen needed longer time. The washing was conducted with swirling rotator at 4 °C. Specimens were embedded in CMC into 2 cm × 3 cm frozen block on dry ice-cooled hexane. Specimen blocks were stored at −80 °C until sectioning.

Table 1
Combinations of fixative and embedding reagent attempted in this study

	Fixative	Embedding reagent	Reference
1	Acetone	2% carboxymethyl cellulose (CMC[a])	Modification of Kurita et al. [16][b]
2	3:1 Ethanol:acetic acid (AA)	CMC	This study
3	AA	Paraffin	Kerk et al. [3]

[a] The use of CMC as a cryo-embedding reagent is referred to Kawamoto [19].
[b] O.C.T compound was used as embedding reagent in the method of Kurita et al. [16].

2.3. Fixation and embedding for paraffin-sectioning

When we used a combination of AA + paraffin, the experimental procedure was based on that of Kerk et al. [3]. After fixation, the specimens were dehydrated in a graded ethanol series (1 h each; 80%, 90% 95%, 100%, 100%, and 100% v/v) and ethanol:xylem series (3 h each; 75%:25%, 50%:50%, 25%:75%, 100% xylen, 100% xylen, and 100% xylen v/v) at room temperature. Dehydrated samples were gradually replaced with paraffin (Paraplast plus, Oxford Labware) through xylen:paraffin series (over 6 h each; 75%:25%, 50%:50%, 25%:75%, 100% paraffin, 100% paraffin and 100% paraffin) at 58 °C, and finally embedded in 100% paraffin. Specimen blocks were stored at 4 °C until sectioning.

2.4. Sectioning

Cryo-sectioning was conducted following the method of Kawamoto [19]. A synthetic adhesive (Cryoglue Type I:hexane = 4:6 v/v, FINETEC Co. Ltd, Japan) was brushed on thin film (Leica). This adhesive film (c.a. 5.4 cm × 2.0 cm in a piece) was placed on a cutting surface of frozen specimen block in a cryo-microtome (Leica, CM1850) at −20 °C. This step avoids thin section curling and helps to make a section without artifacts. Kurita et al. [16] used O.C.T. compound as an embedding reagent, but synthetic adhesive, Cryoglue Type I,

did not stick to the O.C.T compound blocks and the sectioning could not be conducted. The section on adhesive film was attached to pre-cooled glass slide with double-sided adhesive tape. Transversal sections were made at the median part of developing caryopsis. The cryo-sections were air-dried for 5 min, and used for LMD within 5 h after sectioning. The paraffin section was cut with a rotary microtome. The paraffin section was de-paraffinized by immersing in a xylene and ethanol series and air-dried before use in LMD. The thickness of both the cryo- and paraffin sections were 8 μm. The specimen blocks were sectioned within 10 days after embedding for the first replication, and again at 30 days after embedding for the remaining two replications with acetone + CMC and AA + CMC.

2.5. LMD and the extraction of RNA

Aleurone cells at the dorsal side and starchy endosperm at the central position were microdissected with an AS LMD system (Leica Microsystems, Germany). Laser conditions for microdissection were: aperture set at 14, intensity at 46 (highest value) and speed at 7. Care was taken to minimize shattering of the storage compounds, and we stopped to dissect the cells if storage compounds shattered. Fifty to hundred pieces of dissected aleurone cells and central endosperm were collected into 500 μl PCR tube cap filled with 20 μl mineral oil. Note that 20 μl mineral oil without containing dissected cells was also prepared to make a blank solution for RNA quantification. Total RNA was extracted with a Picopure RNA isolation kit (Molecular Devices) according to the manufacturer's instructions using DNase I.

2.6. Assessment of RNA integrity

Integrity of RNA was assessed using a 2100 Bioanalyzer (Agilent technologies). RNA integrity was expressed as RNA Integrity Number (RIN) with a capillary electropherogram and virtual gel image. RIN was calculated with 2100 expert software (version B.02.02, Eukaryote total RNA Pico mode). The software algorithm allows the categorization of total RNA quality on a scale from 1 to 10, in which 10 corresponds to the most intact RNA and 1 corresponds to the most degraded RNA [11]. Fleige and Pfaffl [20] analyzed the effect of RNA integrity on quantitative RT-PCR performance using various kinds of mammalian total RNA with different RINs, and they regarded a RIN higher than five as good total RNA quality and higher than eight as perfect total RNA for downstream expression analyses.

2.7. RNA quantification and T7 RNA polymerase-based RNA amplification

Quantification of total RNA was determined by the fluorescence based method, RiboGreen RNA Quantification kit (Molecular Probes, Eugene, OR). Because a solution of PicoPure RNA isolation kit had fluorescence resulting in the overestimation of RNA quantity, blank solution prepared at the step of extraction of RNA was used to determine the net RNA quantity. 10 ng of total RNA was used for T7 RNA polymerase-based RNA amplification based on the method of Nakazono et al. [2] except that second-round RNA amplification was not conducted in this study.

2.8. Quantitative RT-PCR

35 ng of amplified mRNA was used for the synthesis of cDNA. The analyzed genes were *Actin*, *RINO1* encoding a *Myo* inositol-1-phophate synthase [21], *OsSUT1* encoding a sucrose transporter [22] and *SDBE* encoding a starch debranching enzyme [23]. For the quantification of gene expression, QuantiTect SYBER green PCR kit (Qiagen) was used with a real time RT-PCR system (7500 Real Time PCR System, Applied Biosystems). The designed PCR primers

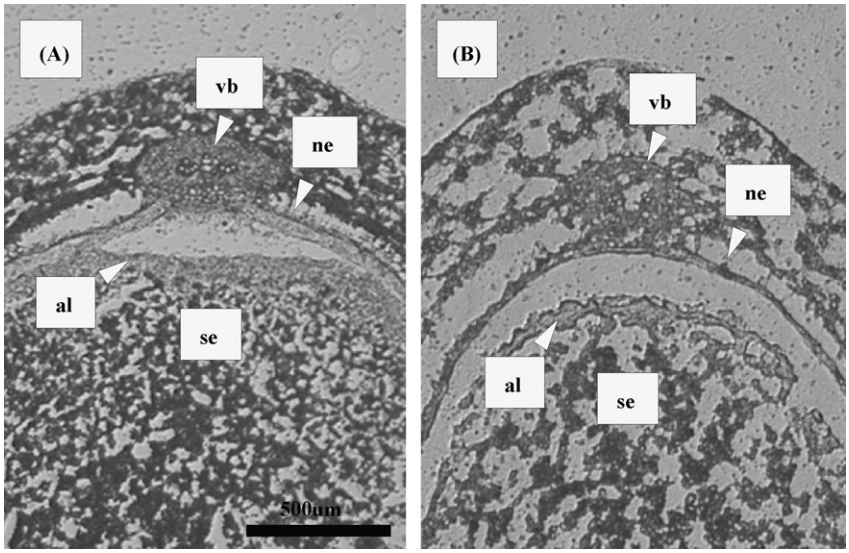

Fig. 1. Median transversal section of developing rice caryopsis embedded in paraffin (A) and CMC (B). Fixative was AA in both sections. Magnification of panel A and B is the same. al, Aleurone cells; ne, nucellar epidermis; se, starchy endosperm; vb, vascular bundle.

for expression analysis of *Actin*, *RINO1*, *OsSUT1* and *SDBE* were as follows: *Actin*: TCTTGGCATCTCTCAGCACA and CTTAGCATTCTTGGGTCCGA, *RINO1*: AGTACAAGTGAGGGAGGCTG and TACCATAGTAAGGCTGCTGC, *OsSUT1*: TTGTGTTGTGTGTGTGTGCAG and TGATGATGATGGAAGCTCACTT, *SDBE*: CCCAAGTTTCGACATGCTTT and AGCATGCCATATTTCCATGT.

3. Results and discussion

3.1. Morphological preservation, laser cutting of developing endosperm and integrity of extracted RNA

In both the paraffin sections and cryo-section, aleurone cells and starchy endosperm could be easily identified due to the difference in cell structure (Fig. 1A and B). Morphological preservation of maternal tissues was better in the paraffin section; the form of vascular tissues and thin single-cell layer of nucellar epidermis were well preserved in the paraffin sections (Fig. 1A), while these structures were slightly collapsed in the cryo-sections (Fig. 1B).

Aleurone cells could be micro-dissected easily in both the cryo-sections (Fig. 2A and A') and paraffin sections (data not shown). The central endosperm could be easily dissected in the cryo-sections (Fig. 2B and B'), but storage compounds were sometimes scattered during the laser cutting in the paraffin sections (Fig. 2C). The slight stickiness of CMC and/or the use of synthetic adhesive, Cryoglue Type I, may have prevented the storage compounds from scattering during the laser cutting in the cryo-sections.

In terms of the integrity of extracted RNA indicated by capillary electropherogram, the baseline was at a low level in

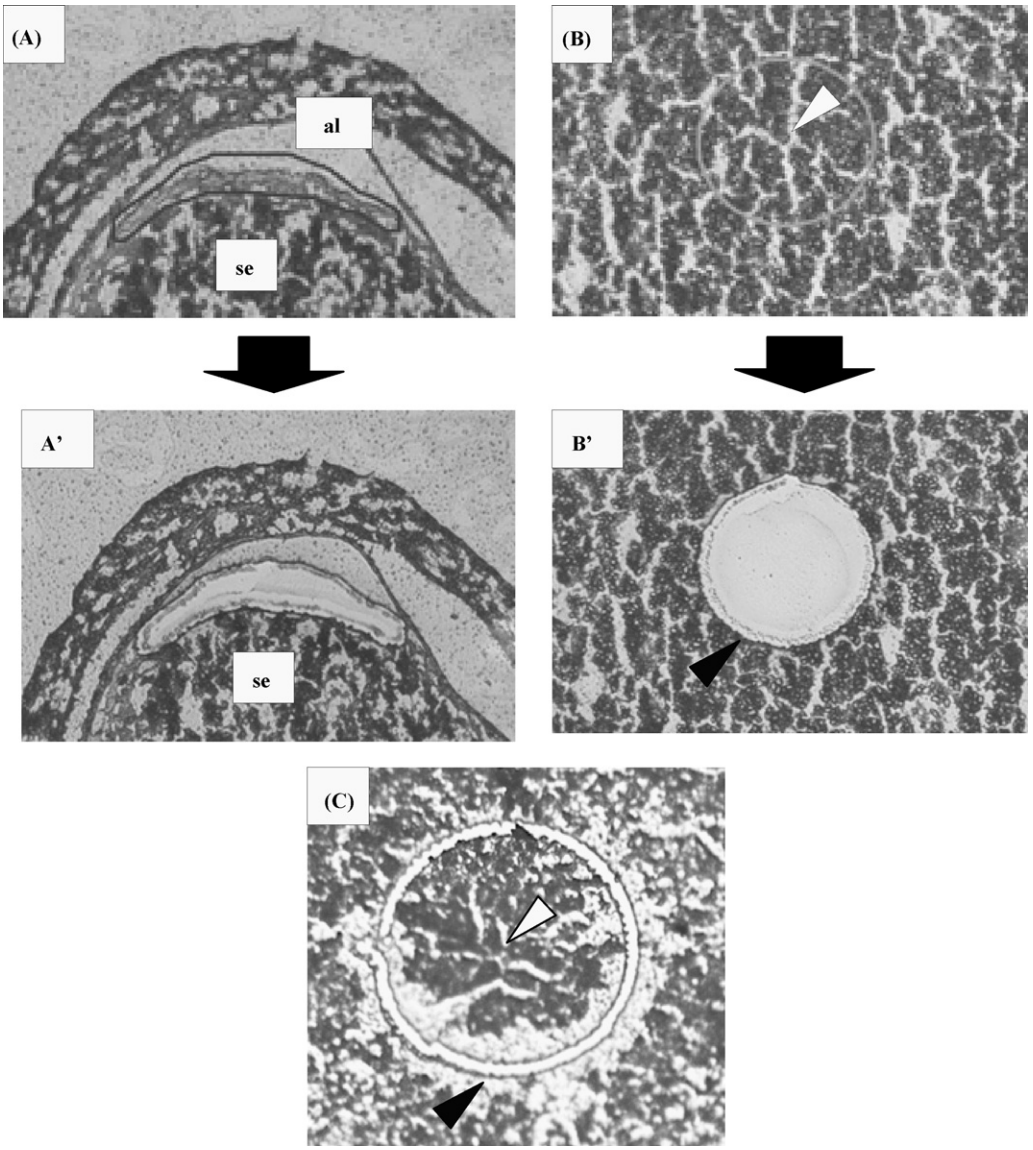

Fig. 2. Laser microdissection of aleurone cells (A and A') and central endosperm (B, B' and C). Areas marked by lines in A and B were cut by the laser and are shown missing in A' and B'. The sections in A and B were prepared with the AA + CMC method and the section in C was prepared with the AA + paraffin method. Laser cutting was precisely performed in panel B', whereas storage compounds are irregularly scattered along the laser cutting in panel C (black arrowheads in panel B' and C). White arrowheads indicate the center of starchy endosperm. Abbreviations are the same as in Fig. 1.

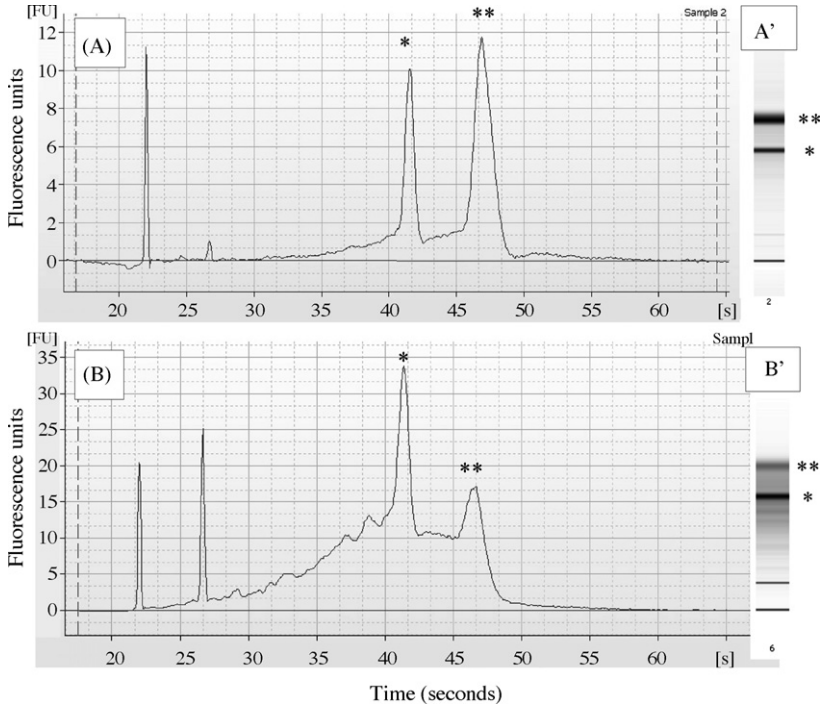

Fig. 3. (A and B), Electropherogram of total RNA extracted from the central endosperm of sections prepared with the AA + CMC method (A) and AA + paraffin method (B). (A' and B'), virtual gel images of A and B. These figures were obtained with Agilent 2100 Bioanalyzer. Single and double asterisk(s) correspond to 18S rRNA and 28S rRNA, respectively. RNA in B (RIN = 6.1) is more degraded than that in A (RIN = 8.6).

RNA from the cryo-sections (Fig. 3A). The virtual gel image had two clear bands corresponding to 28S rRNA and 18S rRNA (Fig. 3A'). In contrast, the baseline of the electropherogram in the paraffin sections was high (Fig. 3B) with a diffuse band corresponding to 28S rRNA (Fig. 3B'). The differences in the baseline of electropherogram indicated that there was little degradation of RNA in the cryo-sections, whereas there was degradation of RNA in the paraffin sections compared to cryo-section. Thus, in the cryo-embedding method, the RNA was well-preserved during all the steps from fixation to RNA extraction.

In comparison of the RIN in different combinations of fixative and embedding reagents, the maximum RINs obtained with the acetone + CMC method and the AA + CMC method were almost the same, while the minimum RIN was much higher in AA + CMC method. The ability to reliably obtain RINs around 8.0 with AA + CMC method (Table 2) is important because in mammalian studies, such values are regarded as excellent for downstream expression analyses [20]. These results suggested that AA is superior to acetone with respect to preserving RNA in developing rice endosperm. Thus, the integrity of RNA obtained by the AA + CMC method appears to be sufficient for transcription analyses in developing rice endosperm.

3.2. Spatial expression of *RINO1* and genes related to carbohydrate metabolism between aleurone cells and starchy endosperm

The RNA extracted from specimens prepared with the AA + CMC methods was used for the expression analysis after the synthesis of cDNA. The expression of *RINO1* was five fold higher in the aleurone cells than in the central endosperm (Fig. 4). Because *RINO1* is known to be localized in aleurone cells by *in situ* hybridization [21], this result verified that the aleurone cells were microdissected without significant contamination of the neighboring starchy endosperm.

Furthermore, the mRNA localizations of *OsSUT1*, which encodes a sucrose transporter [22], *SDBE*, which encodes a starch debranching enzyme [23], were compared between aleurone cells and central endosperm (Fig. 4). *OsSUT1* was preferentially expressed in aleurone cells. By i*n situ* hybridization, Furbank et al. [24] reported the *OsSUT1* mRNA localized in aleurone cells, whereas Hirose et al. [25] reported it

Table 2
Comparison of RNA integrity number (RIN) extracted from the sections of cryo- and paraffin embedding

Combination	Cell type	Average (RIN)	Replication(s)	Maximum (RIN)	Minimum (RIN)
Acetone + CMC	Aleurone cells	7.1	3	9.0	5.1
	Central endosperm	7.8	3	8.6	7.1
AA + CMC	Aleurone cells	8.2	3	8.6	7.8
	Central endosperm	8.5	3	8.6	8.3
AA + paraffin	Aleurone cells	5.0	1	–	–
	Central endosperm	6.1	1	–	–

For details on algorithm and calculation for RIN, see Schroeder et al. [11].

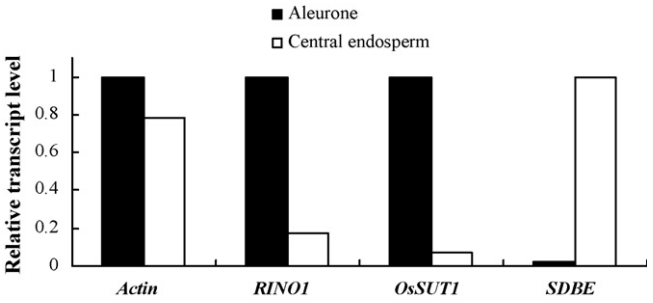

Fig. 4. Spatial expression of *Actin*, *RINO1*, *OsSUT1* and *SDBE* between aleurone cells and starchy endosperm. *Actin* mRNA was equally expressed between aleurone cells and starchy endosperm.

both in aleurone cells and starchy endosperm. Our expression analysis with LMD technique clearly supported the result of Furbank et al. [24]. In contrast to *OsSUT1*, we found *SDBE* was specific to central endosperm, but hardly expressed in aleurone cells. Because the sucrose transporter is involved in the uptake of sucrose from maternal tissues into endosperm *via* apoplast, whereas starch debranching enzyme is involved in forming the amylopectin fine structure in developing endosperm, these results suggested that each mRNA would be distributed depending on its function in aleurone cells and starchy endosperm.

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