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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,