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Separation of Specific Cells Using a Laser Micro-dissection System and Detection of mRNA in Developing Rice Endosperm Tissue

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Abstract

Cloning of the expressing gene in the target cell is important for understanding the molecular mechanism of specific gene expression. Laser micro-dissection techniques allow the precise separation of a single target cell from morphologically distinct cells. We describe here an excellent method for separating a single cell or a cell cluster consisting of a few cells from a small target area of plant tissues. In rice endosperm tissues, the aleurone layer and starchy endosperm tissue are differentiated from a single endosperm mother cell. We succeeded in dissecting a specific cell area from endosperm and aleurone tissue (7 days after flowering). This information will be useful for studying cell differentiation and developing mechanisms of endosperm tissue. RT-PCR analysis for mRNAs for actin and glutelin confirmed the purity of the dissected cells.

Keywords: aleurone layer, laser micro-dissection, rice seed development, starchy endosperm tissue

Abbreviations

cDNA, complementary DNA; DAF, days after flowering; RT-PCR, reverse transcribed-polymerase chain reaction.

Introduction

Rice is one of the most important crops in the world and it therefore, rice has been selected as a model plant for study in the field of genome science (Sasaki, 1998). An understanding of the formation mechanisms of endosperm tissue is extremely important in stabilizing world crop production, and rice endosperm is an ideal tissue for basic studies of plant seed endosperm development.

The starchy endosperm tissue and the aleurone layer, which have quite different functions in seeds, are differentiated from a single endosperm mother cell in rice during its development. There have been reported extensive studies on the morphology of the developing process (Hoshikawa, 1973; Brown *et al.*, 1997), the accumulation mechanisms of storage substances (Tanaka *et al.*, 1980; Yamagata *et al.*, 1986), and the physiological role of these cells after germination (Okamoto *et al.*, 1982). Molecular biological knowledge about development of the endosperm cell, however, is still very limited (Kranz *et al.*, 1998; Olsen, 1998).

In order to clarify the mechanisms of the seed formation process at the molecular level, we have been studying a laser micro-dissection system that allows the isolation of specific cells from endosperm tissues. This technique will be very useful to constructing a cDNA library from only a few endosperm cells. The isolation of target cells without contamination from other tissues is very important for the analysis of cell-specific gene expression (Dresselhaus *et al.*, 1994). Recently, an effective laser micro-dissection system has been developed in mammalian cells (Emmert-Buck *et al.*, 1996); however, this system has not been applied to plant cells because plant cells have rigid cell wall structures, which are absent in mammalian cells.

In this study, we modified the laser micro-dissection process to allows easy dissection of plant cells and succeeded in separating a few starchy endosperm cells from endosperm tissue from specific cell regions. The purity of isolated cells was confirmed by RT-PCR using cell-specific primers.

Materials and Methods

Plant materials

Rice (*Oryza sativa* L. cv. Nipponbare) was cultivated in the experimental greenhouse of the Kyoto Prefectural Institute of Agricultural Biotechnology.

Fixation of rice tissue and laser micro-dissection

Rice roots or seeds were excised into thin sections (2- to 3-mm long) so that fixing solution and embedding agent could penetrate them quickly. The excised tissue was fixed in cold acetone overnight at 4 ℃, and then the acetone was removed by air drying the samples for 10 min. The tissue was soaked in Optical Cutting Temperature compound (O.C.T compound; Sakura Finetechnical Co., Ltd., Japan) for 10 min and then frozen at −80 ℃ until the O.C.T. compound hardened. Using a cryostat (HM500M; Microm, Germany), we cut the frozen root into sections 10 to 40 μm thick and the seed tissue into sections 20 μmthick. Tissue sections were mounted on non-adhesive glass slides and stored at −80 ℃ until use.

The glass slides were placed on the stage of a Laser Micro Cutter (LSC-0355UV, Sigma Koki Co., Ltd., Japan). The tissue section of the target cell area was dissected with irradiating laser light (1.5-5.0 mJ, wavelength 355 nm), and then the dissected tissue was launched with the irradiating laser light (4 mJ). A launched tissue section was captured directly in a micro-tube cap containing lysis buffer.

RNA extraction from laser micro-dissected tissues

The captured endosperm cells were suspended in 20 μl of denaturing solution [4 M guanidium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 6.25 mM PEX (potassium ethylxanthogenate, Fluka), 1%(v/v) β-mercaptoethanol, and 0.5%(v/v) N-lauroylsarcosine]. The cell lysate was spun down at 4 ℃ and then incubated at 65 ℃ for 10 min. The cell extract was infiltrated with denaturing solution in a vacuum chamber for 10 min, and then the infiltrated cell extract was incubated again at 65 ℃ for 10 min. Total RNA was purified from the cell extract using Pinpoint Slide RNA Isolation System I (Zymo Research, USA). Total RNA was precipitated with cold ethanol, washed with RNA washing buffer two times, and then resuspended in 10 μl of RNase-free buffer. DNase treatment was performed for 30 min at 37 ℃ using 0.1 U of DNase (Nippongene, Japan) and 10 U of RNase inhibitor (Invitrogen, USA). After DNase treatment, total RNA was heated at 90 ℃ for 10 min to inactivate the DNase. Total RNA was precipitated with cold ethanol, and then the pellet was resuspended in 4 μl of RNase-free H_2O. RT-PCR was performed with 1 μl of the total RNA, and cDNA synthesis was performed with 3 μl of the total RNA using SMART cDNA synthesis kit (Clontech Laboratories, USA).

RT-PCR from laser micro-dissected tissue

RT-PCR for rice glutelin mRNA was performed using the Titan One Tube RT-PCR system (Roche, Germany). Partial coding sequences of glutelin were used as PCR primers; the forward primer (G491F) was 5'-AGGAGATATCGTTGCACTGC-3', and the reverse primer (G1526R) was 5'-CTCTAA-GGCCTCGTTCTCCGACTC-3'. One micro-liter of total RNA from micro-dissected tissue was used as a template, and 40 pmol of each primer was used. The first RT reaction was performed for 30 min at 42 ℃ . Amplification reaction of cDNA was performed as follows: preheat at 94 ℃ for 2 min; 30 cycles at 94 ℃ for 1 min, 55 ℃ for 1 min, and 68 ℃ for 2 min; and a final extension at 68 ℃ for 7 min. A control reaction was also performed with the same template and primers but without reverse transcriptase. Products of RT-PCR were electrophoresed using 1.2%(w/v) agarose gel.

Amplification of the cDNA pool from laser micro-dissected endosperm tissue

We performed cDNA amplification using SMART PCR cDNA Synthesis Kit with minor modifications. For cDNA synthesis, 3 μl of total RNA from laser micro-dissected tissue was incubated for 60 min at 42 ℃, and then the reaction mixture was placed on ice to terminate first-strand synthesis. Subsequently, second-stranded cDNA was amplified using the Long Distance-PCR (LD-PCR) method. The LD-PCR reaction mixture included 10 μl of synthesized first-strand cDNA mixture, 74 μl of RNase-DNase-free H_2O, 10 μl of 10 x Klen Taq buffer, 2 μl of dNTP mix (10 mM), 2 μl of PCR primer (20 μM), and 2 μl of 50 x Advantage Klen Taq polymerase mix. The LD-PCR was performed under the following conditions: preheat at 95 ℃ for 1 min; 35 cycles at 95 ℃ for 15 s, 65 ℃ for 30 s, and 68 ℃ for 6 min. Amplified cDNA was precipitated with the addition of 2.5 volume of ethanol, cooled at −80 ℃ for overnight, and then centrifuged at 20,000 *g* for 30 min at 4 ℃. The pellet was washed with cold 70%(v/v) ethanol and then dried by air. The pellet was resuspended in 10 μl of RNase-DNase-free H_2O and electrophoresed using a 1.0%(w/v) agarose gel.

Results

Laser micro-dissection of rice tissue

We tested the laser micro-dissection system using root tissue from germinating seed (3 days after germinatinon) of rice. The root tissue was embedded using O.C.T. compound after fixation with acetone. The embedded root tissues were cut off at 10, 20, 30, and 40 μm in thickness. The sections were micro-dissected by laser beam at 1.5, 1.75, 2.5, and 5.0 mJ, respectively. To check the most effective laser power for the suitability of tissue thickness, we examined the morphology of dissected roots.

Fig. 1 shows that a thickness of 10 or 20 μm is best for single cell dissection, because an endosperm cell is 10 to 20 μm in diameter. **Fig. 1C** and **D** show that our laser micro-dissection system can be applied to thicker sections, but a thickness of 30 or 40 μm was not suited to cut off the target cell area clearly. Based on our observations, we chose to use laser power at 1.5 to 1.75 mJ and sections 20 μm thick to dissect a target cell area.

We tried to cut off a rice egg cell, because the egg cell is one of the most important cells in plant biology. **Fig. 2A** shows the shape of an ovule from a developing rice seed (1 DAF). The ovule was fixed with acetone, and a tissue section was prepared using the same method described above. We successfully cut off a rice egg cell (**Fig. 2B** and **C**) using our laser micro-dissection system.

RNA extraction from laser micro-dissected tissues

We sought to determine which RNA fixating agent would sloe or stop the degradation of active mRNA molecules. To that end, rice seed (7 DAF) was fixed at 4℃ overnight with three kinds of fixing agent (acetone, ethanol, or 4% paraformaldehyde-phosphate buffered saline). Target cell areas from endosperm tissue (**Fig. 3**) were collected by laser micro-dissection, and total RNA was extracted. A partial fragment of actin 1 cDNA was amplified by RT-PCR, and the amplified cDNA fragment was detected only from the laser micro-dissected sample fixed with acetone (data not shown). Therefore, acetone is suitable as an RNA fixing agent for tissues dissected by the laser micro-dissection technique.

RT-PCR analysis of rice glutelin expression

We used RT-PCR to identify rice glutelin mRNA from laser micro-dissected starchy endosperm tissue (**Fig. 3B**) and aleurone layer (**Fig. 3C**). Fifty to one hundred target cells were found to be needed to isolate enough total RNA for RT-PCR analysis.

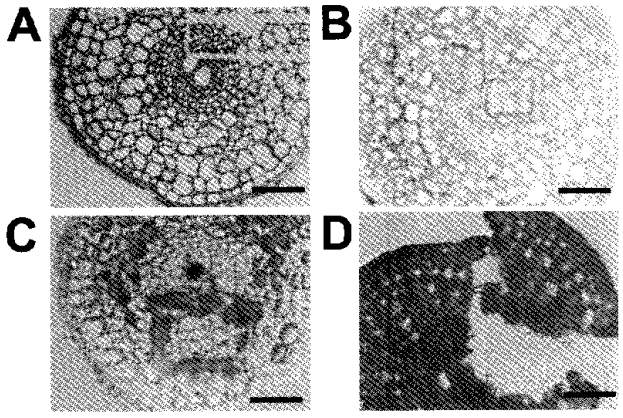

Fig. 1 Laser micro-dissection of rice root tissues. The thickness of rice root tissue is 10 (A), 20 (B), 30 (C), and 40 (D) μm, and the laser power of the Laser Micro Cutter is 1.5 (A), 1.75 (B), 2.5 (C), and 5 (D) mJ, respectively. Scale bar indicates 50 μm.

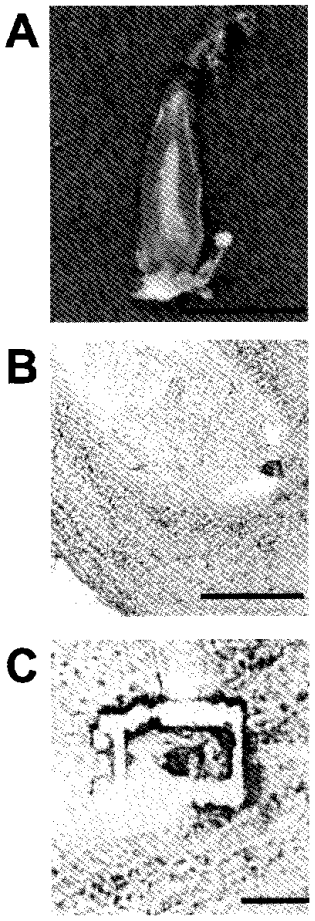

Fig. 2 Laser micro-dissection of rice egg cell. (A) The ovule and pericarp from rice seed (1 day after pollination). Scale bar indicates 500 μm. (B) The microscopic cross section of a rice ovule egg cell. Scale bar indicates 100 μm. (C) A laser micro-dissected egg cell. Scale bar indicates 50 μm.

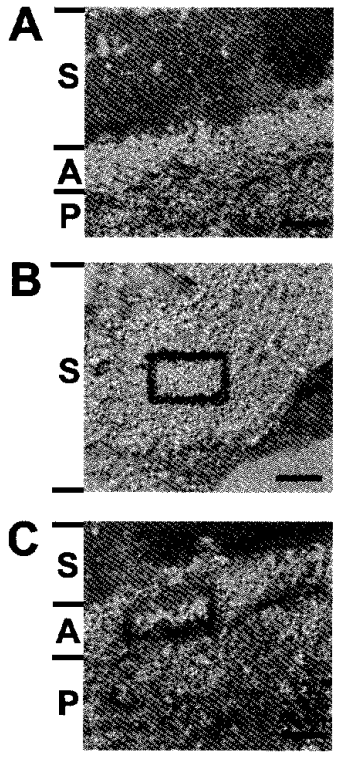

Fig. 3 Laser micro-dissection of rice starchy endosperm cells and aleurone layer cells.

(A) Cross section of freshly frozen rice seed (7 DAF). (B) Laser micro-dissection of endosperm cells (7 DAF). (C) Laser micro-dissection of aleurone cells (7 DAF). S; starchy endosperm tissue. A; aleurone layer. P; pericarp. Scale bar indicates 20 μm on each panel.

Fig. 4 (lane 1) shows that a partial glutelin cDNA sequence (1 kbp) was amplified using total RNA from starchy endosperm cells as a template. No amplified glutelin cDNA fragment was detected when total RNA from aleurone cells was used as a template (lane 3). On the other hand, a partial glutelin genomic DNA sequence (1.3 kbp) was amplified when we used rice genomic DNA of an embryo as a template (lane 2). Note that no bands was a negative control of RT-PCR product without reverse transcriptase using total RNA from starchy endosperm cells as a template (lane 4). These results make it clear that glutelin mRNA is expressed specifically in starchy endosperm cells, not in aleurone layer cells. Glutelin cDNA was specifically amplified from total RNA for starchy endosperm cells. This means that complete separation was performed using our laser micro dissection system.

Amplification of a cDNA pool from laser micro-dissected endosperm tissue

One of our primary objectives was to synthesize a specific cDNA fragment from a small amount of dissected cells. Another objective was to synthesize

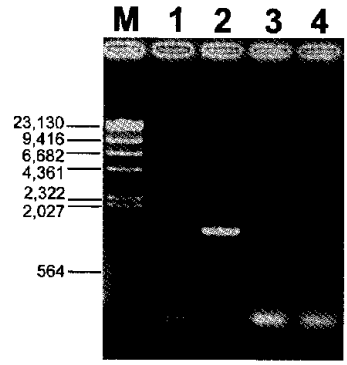

Fig. 4 RT-PCR analysis of glutelin cDNA fragment from laser micro-dissected tissue.

All reaction mixtures contain reaction buffer, deoxy nucleotide mixture, glutelin primers, Taq DNA polymerase, and reverse transcriptase (except for lane 4). Lane 1 contains mRNA from starchy endosperm cells. Lane 2 contains genomic DNA from rice embryo. Lane 3 contains mRNA from aleurone cells. Lane 4 contains mRNA from starchy endosperm cells with no reverse transcriptase. Agarose gel electrophoresis was performed by 1% (w/v) agarose.

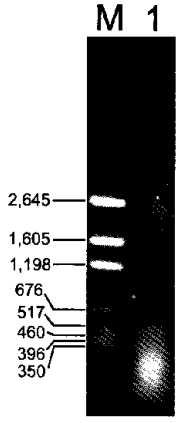

Fig. 5 RT-PCR analysis of synthetic cDNA molecules from laser micro-dissected endosperm cells.

Lane M is the DNA size marker; Lane 1 shows synthetic cDNA from micro-dissected starchy endosperm cells.

a long length of cDNA from a small amount of laser micro-dissected cells. An amplification of a cDNA pool was performed with PCR after we synthesized cDNA using the SMART system. We found that the synthesized cDNA molecules from the target cells, which we collected using the laser micro-dissection technique, were spread over approximately 2 kbp (**Fig. 5**). This result suggested that the synthesized cDNA molecules were of sufficient length to enable us to construct a specific cDNA library from small amounts of target cells dissected by the laser micro-dissection system.

Discussion

We showed that the laser micro-dissection system could be adopted as a useful apparatus for plant cell separation. Unlike animal cells, plant cells have a cell wall, which has led researchers to believe that strong laser power was necessary to cut the cell wall. The maximum output of our Laser Micro Cutter is 5 mJ, which is 10 times as strong as the currently used laser micro-dissection system. Using our laser micro-dissection system on plant cells, we were able to cut a target cell area from root tissue and collect the dissected part.

There are two types of laser micro-dissection apparatus, one of which is the indirect dissection system using the laser micro-dissection apparatus combined with special film. Because the output energy of the laser rays is weak, it is difficult to dissect directly most plant tissues containing moisture. If the plant tissue sample has a lot of moisture, the moist tissue is dried up on the special stick-on film (for example, EVA film). Then the outskirts (the special stick-on film) of the target tissue are melted by the laser ray, so the sample on the film is dissected (Bohm *et al.*, 1997; Matsunaga *et al.*, 1999). A second type of apparatus, our Laser Micro Cutter, can cut a frozen sample containing moisture directly on a slide glass, because the output of the laser is strong. Micro-dissection can be accomplished easily with the device, because it can be used without thinking about congeniality between the sample and the film. We successfully amplified glutelin cDNA by RT-PCR using mRNA that was extracted from the micro-dissected starchy endosperm cells at the seed developing stage. But, glutelin cDNA was not amplified using mRNA from micro-dissected aleurone cells, which were in contact with the starchy endosperm cells at the same stage. This result clearly shows that the genetic expression in the target cells was reflected precisely by the cells collected with our laser micro-dissection system. It is possible to analyze the gene expression mechanisms of endosperm cell differentiation using this laser micro-dissection system.

Recently, there was a report of an in vitro mRNA amplification technique using T7 RNA polymerase (Luo *et al.*, 1999). Our laser micro-dissection methods combined with in vitro mRNA amplification will be expanded to form an improved cDNA synthesis method, in which the uniform quality of the cDNA group is superior. Other current developments include gene expression profiling methods in which specific cellular mRNAs are collected using laser micro-dissection following immunohistochemical analysis (Fend *et al.*, 1999). We hope that our research will contribute to the variety of methods available for successfully analyzing plant genetic expression.

Acknowledgment

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