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REGULAR ARTICLE

Cryosection preparation for histological study, gene expression analysis and imaging mass spectrometry

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ABSTRACT

In recent years, genetic expression analysis and molecular weight measurements in the microregions using cross-sections has become possible as the performance of research equipment has improved. Good-quality sections that retain analytical information are required for accurate analyses, and cryosections that do not require chemical fixation or dehydration embedding processing are ideal. However, hard lignified regions and soft cell regions are mixed in plant samples, and thus, preparing cryosections of plant samples is much more challenging than it is with animal tissues. In this study, we implemented the cryosection preparation method used in animal samples to cryosection preparation of plant samples and established a slicing preparation method suited to plant studies that preserves tissue morphology and can be used for gene expression analysis and specific molecular weight measurements at specific microregions.This method for preparation of sections is faster than the conventional paraffin-embedding method or the resin embedding method. The results show that thus prepared sections have a superior performance in gene expression and imaging mass spectrometry analyses compared with those prepared by the conventional methods.In this report we provide the reader with a method to markedly improve plant research through morphology that can be applied to biochemical studies, such as gene analysis and molecular weight measurements.

Keywords: *ctyosection, woody plant preparation, Kawamoto method.*

INTRODUCTION

In recent years, gene expression analysis and molecular weight measurements in microregions using tissue sections has become possible with the improvement of research equipment and its performance. Methods for section preparation that preserve morphology while preventing nucleic acid and protein degeneration are required for conducting accurate analyses. Cryosections that do not require chemical fixation or dehydration and embedding processing are ideal, and they have been used for sample collection for analysis of gene expression and for mapping specific are composed of mixed lignified hard regions and soft cell regions, rendering the preparation of cryosections from plant samples much more challenging compared with animal tissues. Until present, no method for preparing cryosections suited for studies using microsampling by laser microdissection (LMD) and imaging mass spectrometry has been reported (retrieved in Web of Science, June 2018).

A cryosection preparation method developed by Kawamoto (the Kawamoto method), which uses adhesive film to support cryosections, has been used successfully in laboratory animals [1, 2]. Introducing this method for cryosection preparation of plant samples that will retain

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morphological properties will lead to marked developments in plant research. Therefore, we aimed to establish a cryosection preparation method suited to plant studies that improves upon the Kawamoto method, preserves tissue morphology, and can be used for gene expression analysis and specific molecular weight measurements at specific microregions.

In this study, we have improved the method described by Kawamoto in order to make cryosections with well-preserved tissue structures from a woody plant sample and successfully prepared 3 μm thick sections composed of a mixture of soft and hard tissue. Gene expression and imaging mass spectrometry analyses using the sections showed superior to those of sections prepared by conventional methods.

This method should greatly advance plant research, because it can produce sections more rapidly than the conventional paraffin or resin embedding methods, and, owing to preserved sample morphology, it can be applied in biochemical research such as gene expression analysis and molecular weight measurements.

Plant sections are typically prepared after embedding in resin or paraffin to prevent damages to the tissues during cutting. This method is problematic because it requires several steps such as fixation, dehydration, embedding, or other processing operations[3], during the course of which information related to gene expression, cells, and tissues of the target sample is lost. Also, sample processing is time-consuming as it takes more than one week, in some cases it takes 10 days. However, these problems can be completely resolved using a cryosectioning preparation method in which a fresh sample is cryoembedded and then immediately cut into sections.

Cryosectioning preparation method has produced many favorable results with soft tissues, owing to developments in medical and animal research. However, it is extremely difficult to prepare a frozen sample from the lungs, lymph nodes, and a whole body of laboratory animals in general, etc., while preserving morphology. Also, cryosections from hard tissues such as bone and teeth were previously impossible to prepare. To prepare good cryosections from these samples, Kawamoto developed a method for preparing cryosections that preserve morphology through the application of an adhesive film on the cutting surface of the sample. That method enabled the acquisition of frozen samples that retained tissue morphology 10 min after sample collection, even from a bone sample, from which cryosection preparation had previously been impossible.

MATERIALS & METHODS

Preparation of cryosection

Cryosections were prepared according to the Kawamoto method [2]. The freezing method was modified because the morphology of soft cell regions during freezing of the entire sample in cryogen is hard to retain. A cryosection preparation kit (SECTION-LAB Co. Ltd., Japan) and a very sharp disposable tungsten carbide blade (SL-T30UF; SECTION-LAB Co. Ltd.) were used to prepare cryosections with unaltered morphology of the soft and hard cell regions of woody samples. Japanese cedar (*Cryptomeria japonica*) and Japanese zelkova (*Zelkova serrata*) were used as hard woody samples, liriodendron (*Liriodendron tulipifera*) was used as a general woody substance, and Japanese camellia leaves and uncooked rice were used as soft plant samples. Shoots of the three woody species were cut to a length of 1 cm and camellia leaves were cut into 5 mm wide sections in the direction of leaf veins; the cut samples were placed in 20% sucrose solutions. Then, to remove air bubbles from the samples, they were placed at low pressure until bubbles were removed. Then the samples were put into the proper amount of embedding medium (SCEM, SECTION-LAB) in the embedding container, and only the base of the containers was placed in hexane/dry ice, which is a cryogen, to freeze the samples and embedding medium from the base. After freezing, the frozen samples were taken out of the containers and installed in a cryomicrotome (CM3050S; Leica Microsystems, Germany) kept at -20ºC (a chamber temperature) and -25ºC (a sample holder temperature). After being left approximately 20 min, the sample was trimmed with the tungsten carbide blade until the target sample surface appeared.

To prepare sections for histological observation, an adhesive film was applied to the cutting surface (Cryofilm type 3C [16UF], SECTION-LAB), and

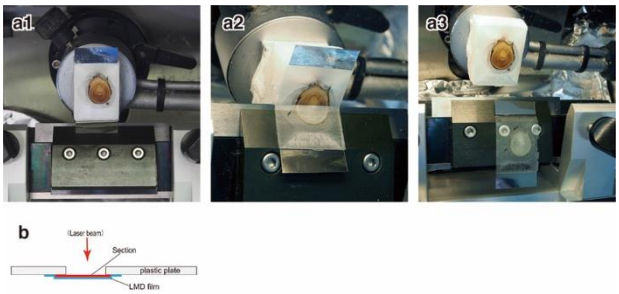

Figure 1. Sample cryoembedding and cryosection preparation using film. **a1.**Samples were cryoembedded in the cryoembedding agent SCEM and installed in a cryomicrotome, trimming was performed until the target sample surface appeared, and then the adhesive film was attached.**a2.**Appearance during cutting.**a3.**The section is recovered while attached to the film.**b.**When using laser microdissection, the laser should hit the sample from the section side.

a 3 μm thick cryosections were cut (Fig. 1-a). The cryosections were recovered attached to the cryofilm. They were moved out of the cryomicrotome chamber, thawed at room temperature for approx. 30 seconds, and placed in 100% ethanol. Approximately 20 s later, they were fixated for 3 min in 4% paraformaldehyde phosphate buffer solution (pH 7.4), and rinsed with water for 5 min. After rinsing, the sections were stained for 30 s in 0.2% toluidine blue phosphate buffer solution (pH 7.4), and subsequently rinsed with water for 4 min. The cryofilm sections were placed on glass slides with the section side facing upward, and a mounting medium (SCMM-R1T) was dropped on the section and left to air for 1-2 minutes. To prepare a slide with the section that is stored between the cryofilm and the slide glass, the surface of the cryofilm section was turned downward and placed on the glass slide dropped with the mounting media. After removing the excess encapsulation media with filter paper, the slides were observed and recorded under an optical microscope.

Utilization of the method in total RNA extraction and quality evaluation for LMD-RTqPCR

To prepare cryosections from the Japanese zelkova samples for LMD, an adhesive film for LMD (LMD film type 2(16)) was affixed to the cut surface of the sample, which was then cut into10 μm thick zelkova cryosections. The cryosections attached to the LMD adhesive film were allowed to stand for 12 h in a cryomicrotome chamber and freeze-dried. After freeze-drying, the sections were placed in air-tight containers, removed from the cryomicrotome, and left to reach room temperature. Later, the adhesive film to which the section was attached was adhered to a plastic plate with a hole in it (Fig. 1-b). This is equivalent to when the section is attached to a foil membrane slide. The entire section was sampled under a Leica LMD7000. For comparison, two types of sections were prepared: frozen sections thawed at room temperature and frozen sections that were chemically fixed after being thawed at room temperature. Furthermore, to study the storage stability of the sections, the scenario where RNA was extracted within 1 week after sample preparation and the scenario where RNA was extracted after storing the samples at room temperature for 2 months were compared. Four sections were analyzed under each condition.

A section sampled with LMD was placed in a 1.5 mL tube, and an RNeasy Micro Kit (QIAGEN, Germany) was used to extract total RNA. The extracted RNA was quantitatively and qualitatively evaluated using an RNA 6000 Pico Kit (Agilent Technologies, USA) and a 2100 Bioanalyzer (Agilent Technologies).

Application of the method in IMS

Cryosections of Japanese zelkova and unpolished rice were prepared for IMS. Electrically conductive adhesive film (Cryofilm type IMS-C1, SECTION-LAB) was affixed to the cut surface of the frozen samples, which was then cut into 10 μm thick cryosections. The cryosections attached to the IMS adhesive film were kept for 12 h in a cryomicrotome chamber and freeze-dried. After freeze-drying, the sections were placed in air-tight containers, removed from the cryomicrotome, and left to reach room temperature. Later, the sections were fixed on glass slides using double-sided adhesive tape, with the surface of the section facing upward. The sample of zelkova or rice section exposed to the laser irradiation was determined by light microscopic observations. Then, 660 mg ofα-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich, Japan) was deposited on the glass slide at 250 °C for 8 min for zelkova or 6.5 min for rice in an iMLayer (Shimadzu, Japan). The sample was immediately analyzed by matrix assisted laser desorption/ionizationï imaging mass spectrometry (MALDI-IMS) using iMScope (Shimadzu). The mass spectra of the designated areas on a specimen

photographed before matrix application were acquired in the positive ion mode.

Zelkova: The scanning mass range on the sample was from *m/z* 100 to 300, *m/z* 300 to 600, or *m/z* 600 to 900. The laser irradiation times, laser power, laser irradiation diameter, laser frequency, sample voltage, and accumulated number of MALDI-IMS were 100 shots, 62.0, 50 ε m, 1000 Hz, and 1/pixel, respectively. The detection voltage of each mass range was 1.80 kV for *m/z* 100 to 300, 1.65 kV for *m/z* 300 to 600, and 2.00 kV for *m/z* 600 to 1000. A laser scan on the root surface was performed automatically (83 × 81 pixels per scan on average). The spatial interval of data points was 50 ε m, giving 6723 data points in the section.

Rice: The scanning mass range on the sample was from *m/z* 100 to 490 or *m/z* 490 to 900. The laser irradiation times, laser power, laser irradiation diameter, laser frequency, sample voltage and accumulated number of MALDI-IMS were 100 shots, 67.0, 40 ε m, 1000 Hz, and 1/pixel, respectively. The detection voltage of each mass range was 1.95 kV for *m/z* 100 to 490 or 2.00 kV for *m/z* 490 to 900. A laser scan on the rice surface was performed automatically (76 × 47 pixels per scan on average). The spatial interval of data points was 80 ε m, giving 3572 data points in the section.The data collected through the microscopic system were digitally processed by Imaging MS solution (Shimadzu) software.

RESULTS & DISCUSSION

We modified the procedure and established a method capable of preparing good cryosections from plant materials. We attempted to prepare cryosections from woody plants that included the hard woody layer and soft cell layer, and we verified the superiority of this method in tissue observations.

First, we improved sample freezing to prevent tissue damages caused by freezing. Next, to prevent damages caused by cutting of frozen samples, we selected a knife that was very sharp and capable of cutting hard material.

By the improvements, we prepared 3 μm thick sections without damages from a frozen sample of the conifer Japanese cedar (a diameter of approximately 1 cm) (Fig. 2-a1). The outermost layer, the outer bark, was retained, and a series of structures toward the center of the sample, such as the soft inner bark, soft cambial zone, and hard xylem, were clearly observed. In the inner bark, cell layers of phloem fibers with hard, thick cell walls, soft phloem parenchyma, and sieve cell formation were systematically observed (Fig. 2-a2). The soft cells surrounding the hard phloem fibers, which cannot be observed in conventional cryosections, were discerned. The assimilation products in the phloem parenchyma could also be clearly viewed, and it was possible to observe tissue structure in the same manner as with sections prepared by conventional means.

Almost complete sections of 3μm thickness was also obtained from a broad-leaved tree Japanese zelkova of approximately 1 cm in diameter (Fig. 2-b1). As with Japanese cedar tree, the outermost layer composed of the outer bark was retained, and a series of structures toward the inside of the cross-section were well-preserved, including the soft inner bark, soft cambial zone, and hard xylem. In the inner bark, the hard phloem fibers and soft phloem parenchyma and sieve tube elements were observed without damage (Fig. 2-b2). The cambial zone and differentiation zone, which regulate cell differentiation and exist in the inside of the inner bark, were retained; in the differentiation zone, it was possible to observe the process of differentiation into xylem cells, i.e., the expansion of the cell diameter and the thickening of the cell wall (Fig. 2-a2, b2). The Japanese zelkova has a G-layer where cellulose accumulates on the inner side of the cell wall. With conventional sample preparation methods, the G-layer contracts during the preparation process, altering the tissue structure (Fig. 2-b3). In contrast, with the present method the structure was observed without contractions (Fig. 2-b2).

A 3 μm thick complete cryosection was also obtained using a sample approximately 1 cm in diameter prepared from liriodendron, a relatively soft broad-leaved tree, in a manner similar to that employed for cedar and zelkova. The tissues in the cryosection retained their morphology, and a series of structures could be observed in detail from the outermost layer, the outer bark, to the inner bark and woody layer (Fig. 2-c1, c2).

Preparation of leaf cross-sections with intact tissues using conventional sectioning methods has been challenging owing to the presence of many intercellular spaces in leaves. However, the present method allows cross-sections of the entire leaf to

be easily prepared with completemorphology (Fig. 2-d1, d2). In the cross-section, all leaf structures were satisfactorily preserved, from the cuticular layer, upper epidermis, and palisade tissue, to intercellular space in the spongy tissue and lower epidermis along with stomata. This confirms that the present method is suitable for samples such as leaves.

To test the applicability of this method in cereal grain, cryosections of rice grain were successfully conducted immediately before germination. The morphology of the germ, endosperm, and starch layers was conservedwell, and the embryo before germination could be observed in the germ. This confirmed the suitability of this method for preparing cryosections of cereals (Fig. 4-b). As shown above, this method can be used to prepare cross-sections with intact tissue structures from various plant samples not only in a short amount of time, but also easily, and therefore, it is better suited for preparing cross-sections from plant samples than the conventional methods.

To use the sections obtained by this method for in-situ analysis, the present method was applied for gene expression analysis by laser microdissection (LMD-quantitative reverse transcription PCR [RTqPCR]) and imaging mass spectrometry. Both results prepared using present sections were

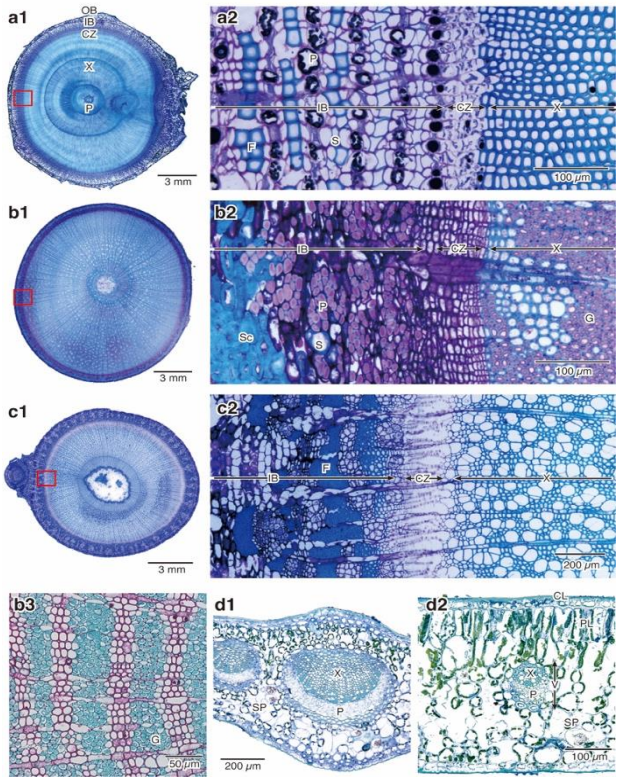

Figure 2. Cryosections of Japanese cedar, Japanese zelkova, liriodendron, and Japanese camellia, stained with toluidine and with thickness of 3 μm.**a1**. Cross section of Japanese cedar trunk. OB: outer bark, IB: inner bark, CZ: cambial zone, X: xylem, P: pith. **a2**. Magnified image of the region framed in red in figure a1. F: phloem fiber, P: phloem parenchyma, S: sieve cell, IB: inner bark, CZ: cambial zone, X: xylem.**b1**. Japanese zelkova trunk cross section.**b2**. Magnified image of the region framed in red in figure b1. Sc:sclereid, G: G-fiber = gelatinous fiber.**b3**. Magnified image of the xylem in a paraffin embedded section. G layer is contracting and peeling.**c1**. Liriodendron trunk cross section.**c2**. Magnified image of the red frame in figure c1.**d1**,**d2**. Cross section of Japanese camellia leaf. X: xylem, P: phloem, V: leaf vein, SP: spongy parenchyma, PL: palisade layer, CL: cuticle.

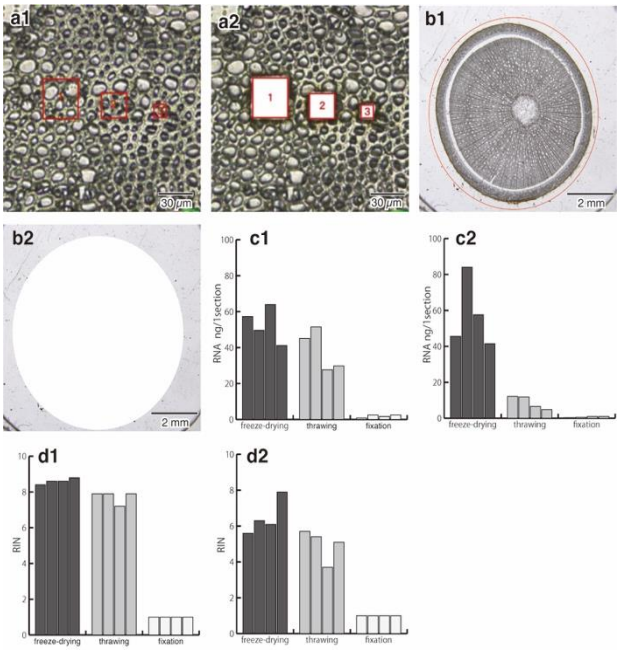

Figure 3. Section cutting test of cross-sections by laser dissection method and yield by reverse transcription DNA amplification quantitation.**a1**. Before cutting the Japanese zelkova freeze-dried section by laser microdissection method. 10 μm thick xylem region. Red circle indicates the cut part.**a2**. After the cutting of the section in figure a1. The width of the squares is 30 μm (square 1), 20 μm (square 2), and 10 μm (square 3).**b1**. Japanese zelkova using the reverse transcription DNA amplification quantitation method. Before cutting. The area inside the red circle is the region that was dissected and recovered.**b2**. After recovering the dissected sample in figure b1.**c1**. Total RNA yield extracted within 1 week after section preparation; four sections were measured, freeze-drying: freeze-dried slice, thawing: thawed slice, fixation: chemically fixed slice.**c2**. Total RNA extracted from sections stored at room temperature for 2 months.**d1**. RNA integrity number (RIN) of total RNA extracted within 1 week after section preparation.**d2**. RIN value of total RNA extracted from sections stored at room temperature for 2 months.

superior to those of the sections prepared conventional methods in regard to information preserved in the samples.

RNA extraction for LMD-RTqPCR was used on a section of a shoot of Japanese zelkova. First, a cutting test using laser microdissection was performed on the shoot section to assess whether it can be applied for sample collection at a cell level by cutting a piece 10× 10 μm in size. The results showed that samples could be accurately collected (Fig. 3-a1, a2).

To assess RNA yield recovery, a single 10 μm thick cross-section of the entire shoot was used to prevent sample error (Fig. 3-b1, b2). RNA was extracted from each of the following four sections: a freeze-dried section, a thawed cryosection, a chemically fixed section, and a long-term-stored section to study and compare the yield and quality of the RNA.

Figure 3-c1 shows the total RNA quantity extracted from a single section when the RNA was extractedwithin the first week of section preparation. The RNA yield obtained from freeze-dried cryosections (Fig. 3-c1 freeze-drying group) and cryosections thawed at room temperature (Fig. 3-c1 thawing group) was higher than that from cryosections that had been chemically fixed after thawing at room temperature (Fig. 3-c1 fixation group). Very small amount of RNA was recovered from chemically fixed sections, and chemical fixation measures aimed at preserving tissue structure were found to hinder in-situ analysis of gene expression. The RNA yield from sections stored at room temperature for 2 months after preparation (Fig. 3-c2) decreased greatly in thawed sections and chemically-fixed sections. However, an adequate RNA yield was obtained from the sections that were freeze-dried, despite having been stored for 2 months at room temperature.

The RNA integrity number (RIN value), which is used to indicate the RNA quality, showed that the quality of the RNA was higher when it was extracted within 1 week since section preparation for both the freeze-dried and thawed sections (Fig. 3-d1, d2). The lowest RIN value of 1 was identified for chemically fixed samples, even when extraction was performed within the first week after section preparation. Approximately 139 ng/mm^3 of RNA was extracted from freeze-dried sections when the extraction was conducted within the first week of sample preparation. This amount was similar to 150 ng/mm^3 of RNA extracted from a non-embedded spruce sample [4].

Generally, a RIN value of 5.0 or greater is required for real-time PCR analysis, and a RIN value of 7.0 or greater is required when a cDNA library is prepared [4,5]. The RIN value of the RNA extracted from the freeze-dried section prepared by the present method was high, at approximately 8.6, if extraction was performed within 1 week of sample preparation, and approximately 6.5 when the RNA was extracted from sections stored for approximately 2 months at room temperature. The RIN value for extractions performed from paraffin-embedded sections is usually 5 or below [6]. Various analyses require RNA with a RIN value of 8 or more [5]. These results show the significant advantage of the present method for research of gene expression in plant samples, because it can be used to efficiently recover RNA for high-quality gene expression analyses from microregions.

For the IMS, samples were prepared from Japanese zelkova shoot and unpolished rice. This sectioning method also did not hinder mass spectrometry, because the section was prepared with an electrically conductive adhesive film (Fig. 4-a2, b2). In the mass/charge ratio (*m/z*) of 100ï 600, signal was detected only in the cambial layer at *m/z* = 205 and 409; no signal was present only in the cambial layer at *m/z* = 228 and 320 (Fig. 4-a2). At *m/z* = 116 and 417, the signal originated in the cells. In the cross-sections of unpolished rice, signal distribution differed between the germ and endosperm (Fig. 4-b2). The obtained images

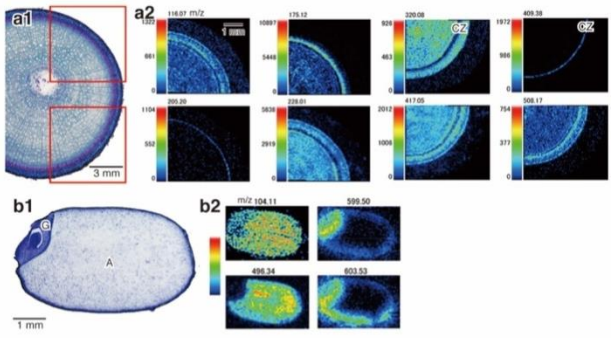

Figure 4. Results of imaging mass spectrometry (IMS).**a1**. Histological picture of a cross-section of a Japanese zelkova shoot. The area in the red frame is the IMS analytical region.**a2**. Japanese zelkova IMS analysis image. The numbers above the images are the mass-to-charge ratios (*m/z*). CZ: cambial zone. **b1**. Histological picture of unpolished rice. G: Germ, A: Albumen.**b2**. IMS analytical image of unpolished rice.

showed signals only in the germ, the endosperm, or in both. Signal distribution was confirmed in the endosperm and continued from the germ to one side of the endosperm, which was estimated to greatly help IMS in plants.

This report describes the method used for section preparation that preserves tissue structure and tissue information. The present method can be expected to significantly contribute to the functional analysis of plants because it enables not only detailed observations of tissue structures, but also biochemical analyses in specific microregions.

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REFERENCES

[1] Kawamoto, T. 2003. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. J. Korean Soc. Archives of Histology and Cytology. 66: 123-143.

[2] Kawamoto, T and Kawamoto, K. 2012.Preparation of thin frozen sections from nonfixed and undecalcified hard tissues using Kawamotôs film method-2012. Methods in Molecular Biology. 1130:149-164.

[3] McDonald, K. 2014.Rapid embedding methods into epoxy and LR White resins for morphological and immunological analysis of cryofixed biological specimens. Microscopy and Microanalysis. 20:152-163.

[4] Abbott, E., Hall, D., Hamberger, B. andBohlmann, J. 2010. Laser microdissection of conifer stem tissues: isolation and analysis of high quality RNA, terpene synthase enzyme activity and terpenoid metabolites from resin ducts and cambial zone tissue of white spruce (*Piceaglauca*). BMC Plant Biology. 10:106.

[5] Fleige, S. andPfaffl, M.W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine. 27: 126-139.

[6] Cañas, R. A., Canales, J., Gómez-Maldonado, J. Ávila, C. andCánovas, F. M.2014. Transcriptome analysis in maritime pine using laser capture microdissection and 454 pyrosequencing. Tree Physiology. 34: 1278-1288.