Video Article

Hybrid-Cut: An Improved Sectioning Method for Recalcitrant Plant Tissue Samples

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Abstract

Maintaining plant section integrity is essential for studying detailed anatomical structures at the cellular, tissue, or even organ level. However, some plant cells have rigid cell walls, tough fibers and crystals (calcium oxalate, silica, *etc.*), and high water content that often disrupt tissue integrity during plant tissue sectioning.

This study establishes a simple Hybrid-Cut tissue sectioning method. This protocol modifies a paraffin-based sectioning technique and improves the integrity of tissue sections from different plants. Plant tissues were embedded in paraffin before sectioning in a cryostat at -16 °C. Sectioning under low temperature hardened the paraffin blocks, reduced tearing and scratching, and improved tissue integrity significantly. This protocol was successfully applied to calcium oxalate-rich *Phalaenopsis* orchid tissues as well as recalcitrant tissues such as reproductive organs and leaves of rice, maize, and wheat. In addition, the high quality of tissue sections from Hybrid-Cut could be used in combination with *in situ* hybridization (ISH) to provide spatial expression patterns of genes of interest. In conclusion, this protocol is particularly useful for recalcitrant plant tissue containing high crystal or silica content. Good quality tissue sections enable morphological and other biological studies.

Video Link

The video component of this article can be found at http://www.jove.com/video/54754/

Introduction

The paraffin-based sectioning method is a widely-used technique for anatomical studies. Preservation of intact tissue anatomy is important for morphological and biological studies. The paraffin-embedded sectioning technique is advantageous because paraffin-embedding retains cell and tissue morphology. In addition, paraffin blocks can be stored conveniently for long periods of time. However, paraffin-embedded sectioning is not suitable for plant tissues containing intracellular crystals. Crystals within the cells often tear paraffin ribbons and damage tissue integrity during sectioning.

Unlike paraffin sectioning, cryosectioning is relatively fast and sections can be obtained without fixation, serial dehydration or embedding medium infiltration [1]. Cryosections are compatible with many applications such as immunohistochemistry, *in-situ* hybridization, and enzyme histochemistry. The other advantage of cryosectioning is that this method does not go through a potential denaturation processes such as high temperature and chemical treatments, so cellular molecules are well preserved within the tissue sections [2]. Cryosectioning is generally preferred over paraffin-based sectioning for studies in animal tissues. However, cryosectioning is not the first choice in plants because freezing temperature sometimes causes formation of ice crystals that affect the quality of section integrity. Although osmoregulation such as sucrose solutions, polyethylene glycol (PEG), or glycerol [3] have been reported to reduce crystal ice formation under freezing conditions, the improvement is less than optimal.

To adapt to different environments, different plants often have distinct tissue texture and plant cells have evolved to form rigid cell walls, tough fibers, and crystals [4,5]. For example, insoluble calcium oxalate crystals and silica bodies are fairly common in plants [6]. Silicate bodies/crystals have been reported to help maintain plant architecture, erectness, and prevent disease or pest in cereal plants [7-9].

The potted orchids and cut-flower orchid market is flourishing and it is a growing industry. *Phalaenopsis aphrodite* (moth orchid) is one of the most important export ornamental plants in Taiwan. Significant efforts have been made to understand the morphological and physiological changes of the flowering processes in *Phalaenopsis* orchids. Floral spikes of *Phalaenopsis* orchids are initiated from axillary buds at the leaf base. After a period of cool ambient temperature (approximately one and half months), axillary buds enlarge, break dormancy, and protrude from the leaf base to develop into young floral spikes. To understand the physiological, cellular, and molecular processes of spike initiation, it

is essential to develop a robust anatomical technique to visualize tissues or markers in a timely manner. However, the presence of ubiquitous crystals in orchid tissues, particularly in axillary buds, makes anatomical work difficult.

Here we sought to improve section integrity of recalcitrant plant tissues that have been hitherto regarded as technically challenging. Here we show an improved protocol named Hybrid-Cut. It is a paraffin-based sectioning method that is performed using a cryostat. Paraffin embedding resolves high water content in plant tissue. Sectioning under low temperature hardens the paraffin block, reduces crystal tearing problem, and improves tissue integrity significantly. This protocol significantly improves tissue integrity for recalcitrant plant samples.

Protocol

1. Fixation and Embedding

1. Reagent preparation
    1. 10x Phosphate-buffered saline (PBS)
        1. Add 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 ml double distilled water (ddH_2O) and adjust the pH to 7.4 with HCl. Add ddH_2O to a total volume of 1 L, then add 1,000 µl diethyl pyrocarbonate (DEPC) and shake vigorously. Store PBS overnight at room temperature and autoclave at 121 °C for 20 min the following day.

    2. 1x PBS
        1. Dilute the stock 10x PBS at 1:10 ratio in ddH_2O to obtain a final concentration of 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.003 M KCl, and 0.13 M NaCl.

    3. Paraformaldehyde (PFA) fixative
    Caution: Paraformaldehyde is toxic. Prepare it under a fume hood. Wear gloves.
        1. To prepare 250 ml PFA fixative, heat 100 ml 1x PBS to 70 - 80 °C and add 1,750 µl of NaOH. Then add 10 g of paraformaldehyde and mix thoroughly until dissolved. Place the solution on ice and then adjust the pH to 7.2 with H_2SO_4 (260 - 270 µl for 100 ml) after cooling.
        2. Adjust the volume to 250 ml with 1x PBS, add 625 µl glutaraldehyde to a final concentration of 0.25%. Add 250 µl Triton X-100 and 250 µl Tween 20 to facilitate the infiltration of the fixative.
        NOTE: The PFA fixative can be stored at 4 °C for one month without losing its fixation ability.

    4. Prepare different concentrations of ethanol (30%, 50%, 70%, 85%, and 95%) with DEPC-treated water.

2. Plant sample collection
    1. Axillary buds
        1. Use mature *Phalaenopsis* orchid plants at the four-leaf stage. Carefully remove leaves by tearing the leaf following the midrib using hands.
        2. Use a sharp scalpel to carefully remove axillary buds from the base of the third or fourth leaf on the monopodial stem.

    2. Seed sample
        1. Harvest mature orchid seed pods at 4 months after pollination. Cut seed pods longitudinally with a scalpel. Shake the opening seed pod gently and release the dry seeds on filter paper.

    3. Protocorm
        1. Sow mature orchid seeds on a 1/2x Murashige and Skoog agar plate, and grow in a tissue culture room in a 12 hr light period and constant temperature of 25 °C. Sample green protocorm at 7 weeks after sowing.

    4. Protocorm-like bodies (PLBs)
        1. Grow orchid PLBs on T2 regenerating agar plates as described previously [10] and place under the same growth conditions as in the step 1.2.3.1. Collect 10 PLBs at a height of 5 - 8 mm.

    5. Leaf sample
        1. Collect leaf tissue from a mature *Phalaenopsis* orchid plant as described in step 1.2.1.1.
        2. Use a sharp scalpel to cut a small piece of leaf tissue (7 mm length x 5 mm width) from the second newly developed leaf.

    6. Root sample
        1. Using the same plant described in the step 1.2.1.1, dissect 1 cm length of root tip tissue using a sharp scalpel.

    7. Young spike
        1. Use a sharp scalpel to cut young spike tissue from the tip portion of the flower stalk 10 cm in length.

    8. Flower bud
        1. Excise a small flower bud of 5 mm in diameter from orchid flower stalk. Cut part of the flower bud tissue longitudinally (3 mm in thickness).

    9. Leaf tissues and spikelet tissues of cereal crops
        1. Cut 1 cm length leaf blade tissue from the first newly developed leaves from rice, wheat and maize plants.
        2. Collect 10 spikelets from plants (rice, wheat and maize) one day before anthesis.

3. Fixation

1. Fix plant samples immediately by transferring them into a glass scintillation vial containing 15 ml ice-cold PFA fixative.
2. Apply a vacuum (~ 720 mm Hg) to plant samples in a 4 °C cool room. Hold the vacuum for 15 - 20 min (small bubbles should be released from the samples). Repeat this step until most of the tissues sink after release of vacuum. Hold the vacuum overnight and release the vacuum slowly the next day.

4. Dehydration
NOTE: Use the same vial from the fixation through the infiltration steps. Pour or use a pipette to drain off the solution in the previous step and replace with 15 ml of new solution into the vial. Depending on the texture of tissues, treat harder tissues such as orchid axillary bud, seed, protocorm, and PLB, and the spikelet of rice, wheat, and maize for a longer time; treat softer tissues such as orchid flower bud, young spike, leaf and root, and the leaf of rice, wheat, and maize for a shorter time.
    1. After fixation, immerse the sample in 15 ml 1x PBS for 10 min on ice.
        1. Dehydrate samples in 15 ml ethanol series at room temperature as follows: 30% ethanol for 30 min, 50% ethanol for 30 min, 70% ethanol for 1 hr, 85% ethanol for 54 min for harder tissues and 30 min for softer tissues, 95% ethanol for 54 min for harder tissues and 30 min for softer tissues, and 100% ethanol twice for 54 min for harder tissues and 30 min for softer tissues.
        NOTE: Plant samples can be stored in 70% ethanol at 4 °C for several months.

5. Paraffin infiltration
    1. Infiltrate samples with 15 ml ethanol and xylene substitute mixture for 54 min each for harder tissues and 30 min for softer tissues, at room temperature as follows: ethanol/xylene substitute (2:1, v/v), ethanol/xylene substitute (1:1, v/v), ethanol/xylene substitute (1:2, v/v), and pure xylene substitute twice. Caution: Xylene is toxic. Do this step in fume hood.
    2. Infiltrate sample in the vial with 15 ml xylene substitute and paraffin mixture in an oven at 60 °C, overnight for harder tissues, and for 60 min for softer tissues as follows: xylene substitute/paraffin (2:1, v/v), xylene substitute/paraffin (1:1, v/v), and xylene substitute/paraffin (1:2, v/v).
    3. Infiltrate sample with 15 ml pure paraffin twice a day and incubate at 60 °C in an oven.
    4. Repeat step 1.5.3 the next day.

6. Tissue embedding
    1. Switch on the power of the tissue embedding center 1 hr in advance to melt the wax in the paraffin reservoir before embedding tissues in a paraffin block.
    2. Warm up the metal molds (size of base 3.3 cm length x 2 cm width) on the warming tray at 62 °C, and pour approximately 13 ml of molten wax into the mold base.
    3. Transfer one tissue sample from section 1.5.4 into the mold with warmed forceps and orient it into the desired position.
    4. Move the mold onto the cool plate carefully, and leave until the wax is solidified.

2. Tissue Sectioning

1. Paraffin sectioning method
    1. Make a wax base by placing an embedding cassette on the top of a mold (same size in the section 1.6.2), fill with molten wax and remove the mold after the wax is solidified.
    NOTE: The embedding cassette will form a wax base to anchor the sample paraffin block.
    2. Trim the paraffin block into an appropriate shape and size, and place some wax pieces on a flat spatula. Heat the wax pieces using an alcohol burner until the wax melts and then place the molten wax on the wax base in section 2.1.1 to adhere the block. Clamp it in the microtome.
    3. Place a new blade onto the microtome, and adjust the angle to 5 degrees to facilitate the sectioning in the microtome.
    4. Cut the sample paraffin block into thin slices (10 µm), as described previously [11].

2. Hybrid-Cut sectioning method
    1. Trim the sample paraffin block from step 1.6.4 to a column with a trapezoid surface at the top to an appropriate size using a razor blade.
    2. Add some Optimal Cutting Temperature compound (OCT) to the center of the cryostat stage. Attach the paraffin block to the cryostat stage and then quickly orient the tissue block into the desired position.
    3. Transfer the paraffin block/stage to a cryostat chamber. Allow OCT to solidify on quick freeze bar at -42 °C for 10 min. Do not move the block during solidification of OCT (**Figure 2C**).
    4. Allow the cryostat adaptor and chamber temperature to cool down to -20 °C and -16 °C respectively before attaching the paraffin block/stage to the cryostat adaptor. Section tissues to 10 µm in thickness.
    5. Pick tissue sections with forceps. Float the sections on 800 µl DEPC-treated water in a Poly-L-lysine coated slide and transfer the slides onto a hot plate at 42 °C.
    6. Allow the sections to flatten on DEPC-treated water at 42 °C. Use filter paper to drain off the water from the edge.
    7. Mount tissue on the slide by placing the slide on a 42 °C hot plate overnight.

3. Tissue Staining

1. Deparaffinization
    1. Collect the slides from the hot plate and place them in a staining rack. Add 150 ml xylene into a staining jar in fume hood and immerse the rack in xylene for 5 min.

2. Rehydration

1. Prepare different concentrations of ethanol solutions (100%, 95%, 70%, 50%, and 30%) in DEPC-treated water and fill 150 ml of solution into distinct staining jars, respectively.
2. Rehydrate the samples through a series of decreasing ethanol concentration, each step for 3 min at room temperature. Transfer the slides in the staining rack one jar to another jar containing different ethanol concentrations: 100% Ethanol, 95% Ethanol, 70% Ethanol, 50% Ethanol, and 30% Ethanol.
3. After rehydration, immerse the specimens in ddH_2O for 3 min.

3. Hematoxylin staining
   1. Stain tissue samples by immersing tissue slides in hematoxylin solution for 1.5 min.
   2. Rinse briefly in ddH_2O containing 1 - 2 drops of 12 N hydrochloric acid (HCl) for a few seconds, and then wash briefly in ddH_2O.

4. Dehydration
   1. Dehydrate the samples through a series of increasing ethanol concentrations for 3 min at room temperature: 30% Ethanol, 50% Ethanol, 70% Ethanol, 95% Ethanol, and 100% Ethanol. Clear the specimens with xylene in the fume hood for 5 min.

5. Mounting
   1. Drop appropriately 600 µl of xylene-based mounting medium onto slide. Carefully place a coverslip over the specimen. Avoid air bubbles forming to get good quality images.
   2. Allow the slides to air dry overnight. Observe the specimen under a microscope the next day.
      Note: Images were captured at 25X to 400X magnification depending on the size of specimens.

4. *In Situ* Hybridization

1. Probe synthesis
   1. Clone *Phalaenopsis aphrodite Actin* gene specific coding sequence using primers 5'-GGCAGAGTATGATGAATCTGGTCC-3' and 5'-AGGACAGAAGTTCGGCTGGC -3' (to get 242 bp PCR amplicon) and *CyclinB1;1* gene specific coding sequence using primers 5'-TCGTAGCAAGGTTGCTTGTG-3' and 5'- ATGAGCATGGCGCTAATACC-3' (to get 327 bp PCR amplicon) as described [12].
   2. Ligate the specific coding sequences into vectors (*e.g.,* pGEMT) according to the manufacturer's protocol.
   3. Generate digoxigenin (DIG) labeled sense and antisense probes using SP6/T7 DIG RNA labeling kit according to manufacturer's instructions.

2. *In situ* hybridization
   1. Cut the 2nd and 3rd axillary bud tissue slices (10 µm thickness) generated using the Hybrid-Cut method, and mount slices onto coated slide.
   2. Deparaffinize tissue sections in xylene (see 3.1.1), rehydrate in decreasing concentrations of ethanol (see 3.2.2), and digest with 2 mg/ml proteinase K at 37 °C for 30 min.
   3. Perform *in situ* hybridization according to the protocol previously described [11,13] with some modifications, *i.e.*, with hybridization temperature for *Actin* as 59 °C and 60 °C for *CyclinB1;1*. Hybridize slide with 40 ng DIG-labeled RNA probe.
   4. Use nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution to detect hybridization signals as described [11].

Representative Results

Hybrid-Cut Improves the Integrity of Tissue Sections

Understanding the anatomy of the reproductive floral structure is important for investigating the underlying mechanism of flower initiation in orchids. However, the accumulation of intracellular calcium oxalate crystals in *Phalaenopsis* orchids makes such studies a challenging task. To circumvent the problems associated with tearing caused by crystals during the sectioning process (**Figure 1**), we developed a system we named Hybrid-Cut. This protocol combines traditional paraffin embedding and cryosection techniques. We first tested Hybrid-Cut in axillary buds of *Phalaenopsis* orchid because they are notorious for tissue rigidity and high crystal content. Axillary bud tissue was fixed in 4% PFA (see Protocol, step 1.3). After serial ethanol dehydration and paraffin wax infiltration, the axillary bud was embedded in a paraffin block. The block was trimmed to a suitable size before sectioning (**Figure 2A**). A small amount of OCT was then applied to the center of the cryostat stage (**Figure 2B**). The paraffin block was then adhered to the stage via OCT. The stage was incubated in a cryostat for 10 min to allow complete solidification of OCT before sectioning (**Figure 2C**) and then cryosection was performed in a -16 °C chamber (**Figure 2D**).

As a comparison, a paraffin block containing an axillary bud of *Phalaenopsis* orchid was subjected to regular microtome sectioning. As shown in **Figure 1A**, severe tearing of the paraffin ribbons was observed after microtome sectioning. The tissue integrity and cellular structure was also compromised (**Figure 1B**). Hybrid-Cut, on the other hand, produced intact tissue sections (**Figure 3A**) with preserved structural integrity (**Figure 3B**).

Application of Hybrid-Cut to Various Tissues of *P. aphrodite*

To further test the amenability of Hybrid-Cut, we tested different tissues of *Phalaenopsis* orchids. It is often difficult to obtain sections of seeds with good tissue integrity because of the hardened seed coats. Using this protocol, detailed structures of seeds were preserved after sectioning (**Figure 4A**). As shown in **Figure 4A**, protein bodies, the common storage products [14], could be clearly identified. Hybrid-Cut also worked successfully and showed the detailed structures of the shoot apical meristem of the one-month old protocorm (the germinated structure from a seed) (**Figure 4B**) and protocorm-like-bodies (PLBs, **Figure 4C**). The intracellular crystals were observed in sections of PLB. *Phalaenopsis* orchids have thick and succulent leaves and they perform Crassulacean acid metabolism (CAM)-type photosynthesis [15]. The transverse section of leaf blade showed large mesophyll cells and vascular bundles containing xylem and phloem (**Figure 4D**). Few stomatal openings were observed on the abaxial leaf surface during the daytime (**Figure 4D**). In fact, CAM plants evolved to maximize carbon gain but simultaneously minimize water loss by opening their stomates in the night under arid conditions [15,16]. The root apical meristem of *P. aphrodite* is shown in **Figure 4E**. The root tip cells appeared to contain a significant number of crystals, which were very well preserved after sectioning (**Figure 4E**). Longitudinal sections of young floral spikes provided information about the architecture of young floral primordials (**Figure 4F**). Moreover, sepals, petals, labellum, and pollinia could be clearly identified from the longitudinal section of young flower buds that have completed differentiation in this 5 mm flower bud (**Figure 4G**). Notice that crystals were accumulated in the sepals of young floral buds. In short, this protocol works consistently to maintain tissue integrity and produces intact morphology enabling anatomical and possible cellular studies.

Hybrid-Cut Preserves Tissue Integrity in Cereal Crops

We also tested Hybrid-Cut on cereal crops such as rice, wheat and maize that contain high silica content [17,18]. As shown in **Figure 5**, the tissue integrity of transverse sections of rice, wheat, and maize leaves were significantly improved by the Hybrid-Cut method. Xylem, phloem, mesophyll cells, stomates, and bulliform cells that control rolling of the leaf blade to avoid water loss were clearly identified from sections of rice leaves. The Kranz anatomy, mesophyll cells, and vascular bundles of the maize leaf [19,20] were clearly identified. It was intriguing to find a high density of stomates on both the adaxial and abaxial sides of maize leaves (**Figure 5**). The ratio of adaxial and abaxial stomata of 0.7 in maize has been reported previously [21]. In addition, this protocol also worked successfully to provide the detailed cell morphology of spikelets from rice, wheat, and maize (**Figure 6**). Spikelets are known to contain abundant silica [9]. Normally, that causes difficulty in conducting tissue sectioning.

***In Situ* Hybridization**

Genomic and transcriptomic approaches are commonly used to annotate the functions of genes. Providing spatial distribution of transcripts of the genes of interest in developmental or environmental contexts is important to add new insight into the possible functions of the genes. *In-situ* hybridization (ISH) has been developed to localize gene expression patterns at the tissue level [11,22-25]. In addition, ISH can provide cellular, and in some cases sub-cellular, resolution of mRNA distribution in multicellular organisms [26]. During ISH, RNA and tissue integrity is essential to obtain reliable spatial information about the selected transcript. We tested ISH using the Hybrid-Cut protocol. *Actin* gene (PATC157348) was cloned using primers 5'-GGCAGAGTATGATGAATCTGGTCC-3' and 5'- AGGACAGAAGTTCGGCTGGC -3' to get 242 bp PCR amplicon. *Cyclin B1:1* (PATC146999) gene specific coding sequence using primers 5'-TCGTAGCAAGGTTGCTTGTG-3' and 5'- ATGAGCATGGCGCTAATACC-3' to get 327 bp PCR amplicon. After ISH, *Actin* and *Cyclin B1:1* gene expression was monitored in young and mature axillary buds (**Figure 7**). Both genes were expressed in meristematic cells *of* 2nd and 3rd axillary buds, with stronger signals detected in 3rd axillary buds. These results demonstrated that Hybrid-Cut retain good anatomy and provide spatial gene expression pattern.

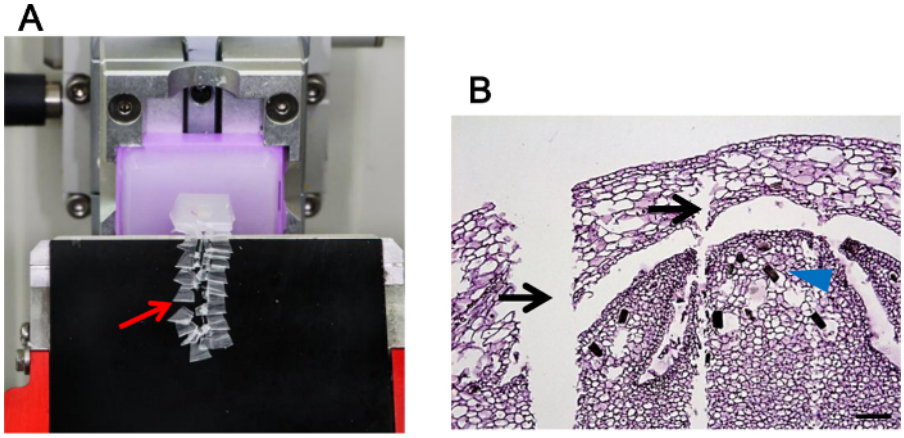

Figure 1: Traditional Paraffin Section Causes Severe Tearing of Tissue. A paraffin block containing an axillary bud of *Phalaenopsis* orchid was subjected to regular microtome sectioning. Severe tearing of the paraffin ribbons was observed after traditional microtome sectioning **(A)**. The tissue integrity and cellular structure was also compromised **(B)**. The arrows show the severe tearing of the tissue slice. Arrowhead shows the crystal bodies. Scale bar 100 µm. Please click here to view a larger version of this figure.

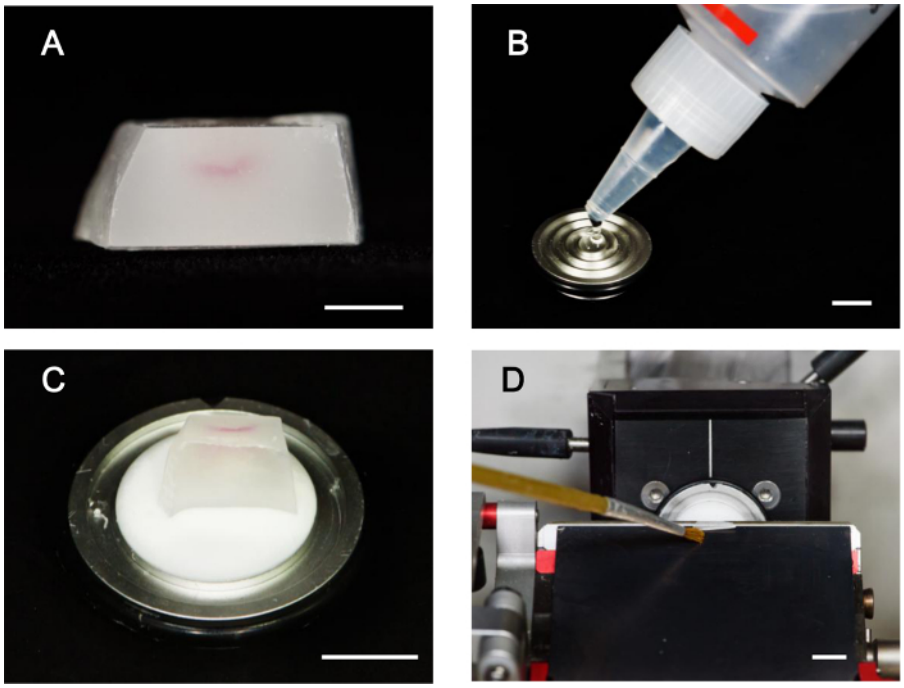

Figure 2: Hybrid-Cut Sectioning Method. An axillary bud tissue was fixed in PFA and tissues were dehydrated, infiltrated with paraffin wax, and embedded in a paraffin block. The paraffin block was trimmed to an appropriate size **(A)**. Optimal Cutting Temperature compound (OCT) was applied to the center of the cryostat stage **(B)**. The paraffin block was attached to the OCT on the cryostat stage. Under low temperature, the paraffin block was adhered to the cryostat stage via OCT **(C)**. Tissue slices were sectioned in the cryostat chamber at -16 °C **(D)**. Scale bars represent 0.5 cm (A), and 1 cm (B-D). Please click here to view a larger version of this figure.

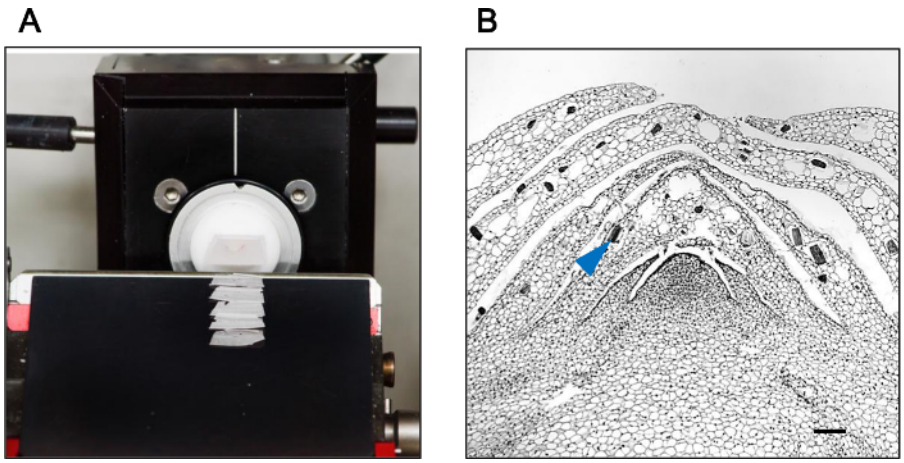

Figure 3: Hybrid-Cut Improves Section Integrity. A paraffin block containing an axillary bud of *Phalaenopsis* orchid was subjected to Hybrid-Cut sectioning **(A)** and the Hybrid-Cut method produced sections with excellent tissue integrity **(B)**. The arrowhead shows the endogenous crystal bodies embedded in the axillary bud tissue. Scale bar 100 µm. Please click here to view a larger version of this figure.

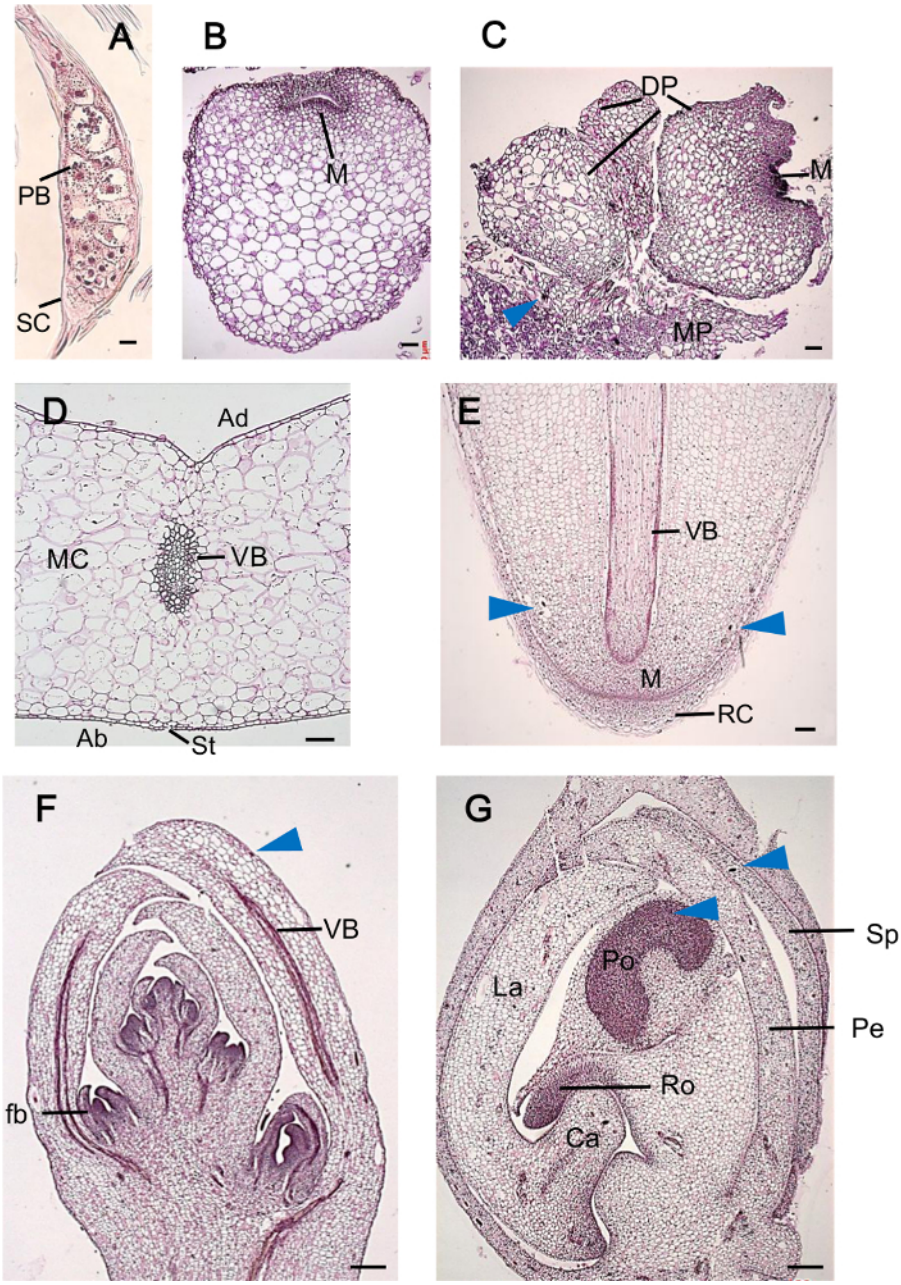

Figure 4: Application of Hybrid-Cut to Various Tissue Sections of *Phalaenopsis* orchid. Different orchid tissues were sectioned using the Hybrid-Cut method, such as longitudinal section of orchid seed during the mature stage **(A)**, longitudinal section of protocorm **(B)**, longitudinal section of protocorm-like bodies (PLB) **(C)**, transverse section of leaf blade **(D)**, longitudinal section of root **(E)**, longitudinal section of young flower spike **(F)**, and longitudinal section of young flower buds **(G)**. Tissue sections were stained by hematoxylin. SC, seed coat; PB, protein body; M, meristem; MP, mother PLB; DP, daughter PLB; Ad, adaxial leaf surface; Ab, abaxial leaf surface; St, stomata; MC, mesophyll cell; VB, vascular bundle; RC, root cap; fb, flower bud; Se, sepal; Pe, petal; La, labellum; Po, pollinia; Ro, rostellum; Ca, callus. Arrowheads show crystals. Scale bars represent 20 μm (A-E) and 200 μm (F-G). Please click here to view a larger version of this figure.

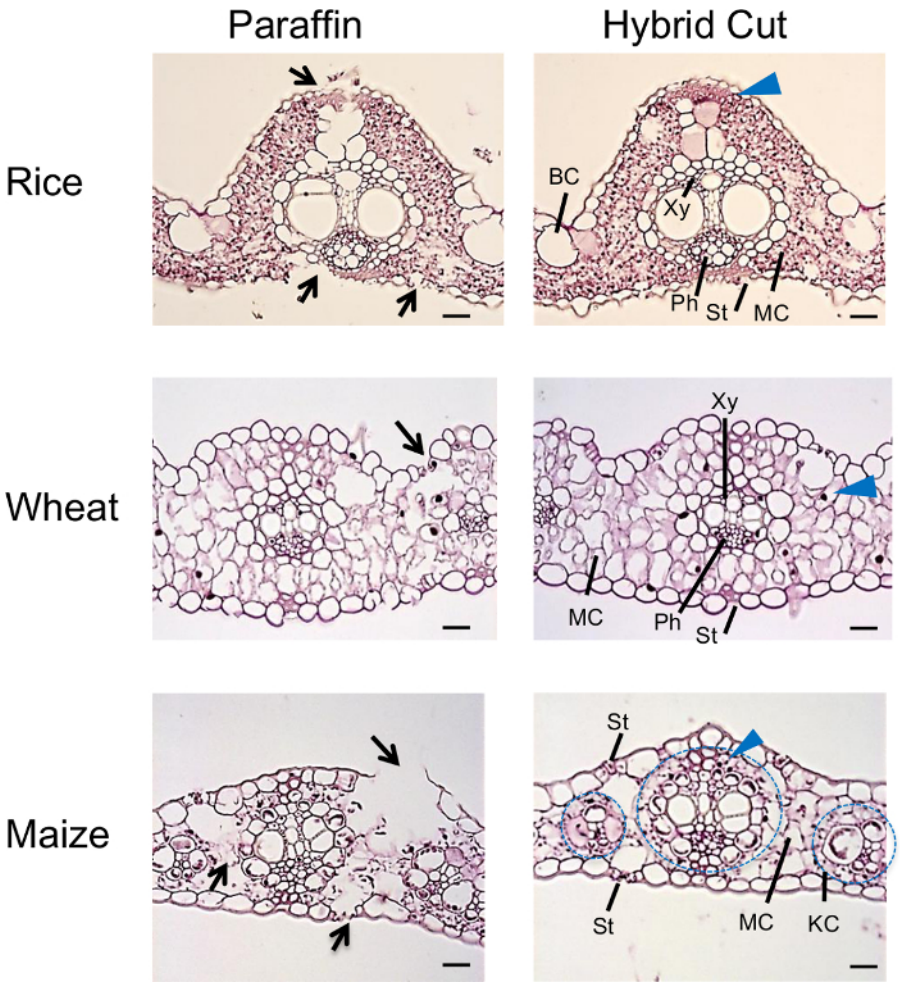

Figure 5: Hybrid-Cut Preserves Leaf Tissue Integrity in Several Cereal Crops. Comparison of leaf tissue using the traditional paraffin method (left-hand panel) and the Hybrid-Cut technique developed in this study (right-hand panel). Images show leaf transverse sections of rice, wheat, and maize. MC, mesophyll cell; Ph, phloem; St, stomata; BC, bulliform cell. Arrows show the tearing of tissue slice. Arrowheads show silica bodies. The blue dashed circles indicate Kranz anatomy in C4 maize. Scale bars 20 μm. Please click here to view a larger version of this figure.

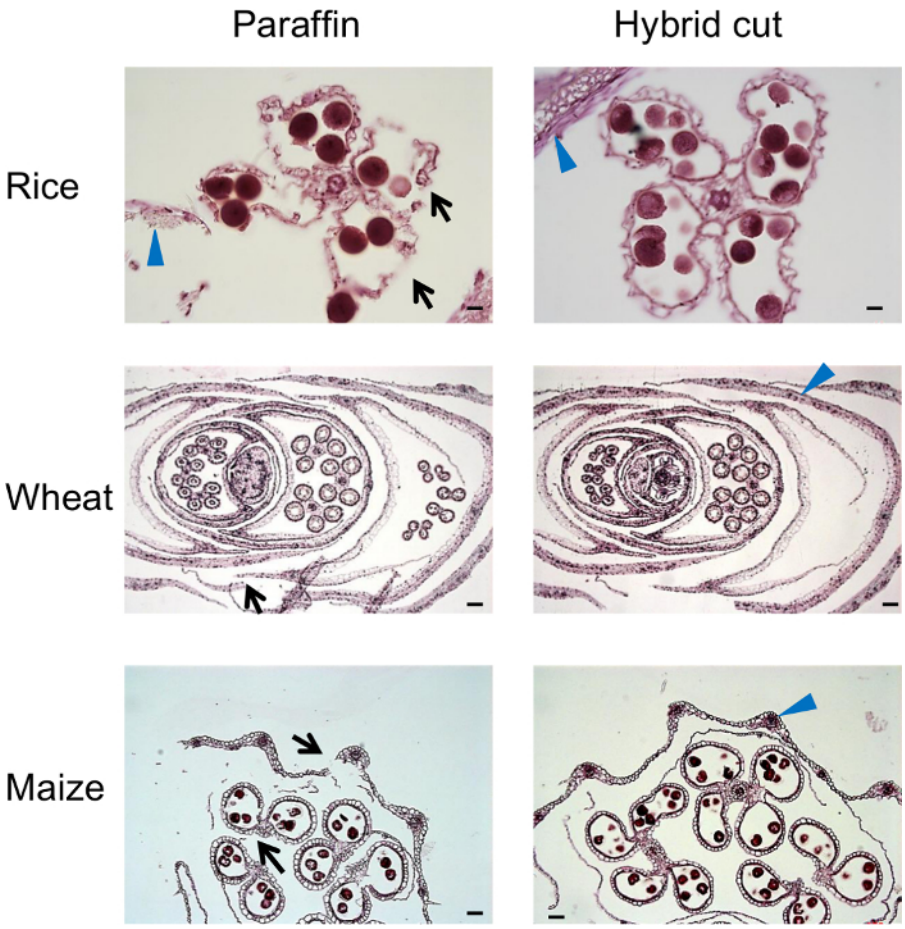

Figure 6: Hybrid-Cut Preserves Spikelet Tissue Integrity in Several Cereal Crops. Comparison of tissue integrity of spikelet sections between traditional paraffin and Hybrid-Cut methods. Arrows show the tearing of tissue slice. Arrowheads indicate silica bodies. Scale bars 20 μm (rice), and 200 μm (wheat and maize). Please click here to view a larger version of this figure.

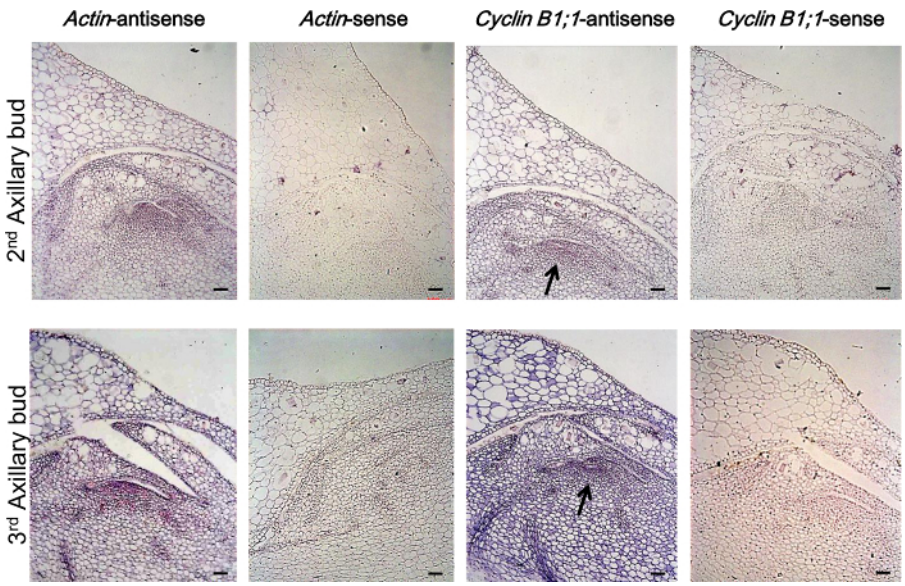

Figure 7: *In Situ* Hybridization of *Actin* and *CyclinB1;1* Expression Patterns in the Axillary Bud of *Phalaenopsis* orchid. Tissue slices of the 2nd axillary buds and 3rd axillary bud were prepared using the Hybrid-Cut method. A total of 40 ng of *Actin* and *CyclinB1;1* dig-labeled probe were used for hybridization. The sense probe was used as a negative control. Arrows indicate the reproductive meristem of axillary bud. Scale bars 100 μm. Please click here to view a larger version of this figure.

Discussion

Plant cells have rigid cell walls, tough fibers, crystals, and high water content that cause tissue tearing problems during plant tissue sectioning. Even though paraffin-based sectioning is frequently used for plant tissues, the endogenous crystals often lacerate the plant tissue during sectioning (**Figure 1**). Because of the inherently high water content within the plant cells, cryostat-based sectioning often causes broken cells and cracked tissue sections.

In the present study, a combined paraffin-embedding and cryosection protocol named Hybrid-Cut was developed and good quality tissue sections were obtained. This protocol resolves the problem associated with high water content by introducing paraffin embedding, and reduces the tearing effect by hardening paraffin wax under low temperature during sectioning (**Figure 3**). Therefore, this modified protocol is advantageous over either paraffin-based sectioning or cryosectioning for preserving plant tissue integrity.

This manuscript demonstrates that the Hybrid-Cut method preserves tissue integrity in many tissues of *Phalaenopsis* orchid such as axillary bud, seed, and PLB, *etc.* that contain high levels of crystals (**Figures 3-4**). Moreover, this protocol is amenable to cereal crops such as reproductive organs and leaves of rice, maize, and wheat that contain high silica (**Figures 5-6**). Presumably, this protocol may be applied to woody plants containing high fiber.

In general, fixing tissue thoroughly is very important for Hybrid-Cut. We found that formaldehyde-alcohol-acidic acid (FAA) fixative is better than PFA to preserve the tissue integrity of some recalcitrant tissues such as axially buds, roots, *etc.* However, PFA works better than FAA in preserving the RNA integrity. Therefore PFA is recommended to fix sample for *in situ* hybridization (ISH) work. The protocol described here is designed for RNA ISH experiment. Therefore, all reagents were prepared to avoid RNA degradation by eliminating RNase contamination by DEPC treatment. If Hybrid-Cut section is for anatomical studies, regular reverse osmosis (RO) water and its derived buffer or reagents are acceptable.

Reducing specimen size and thickness to less than 3 mm is helpful for infiltration. Moreover, increasing immersion time for dehydration and infiltration are necessary for hard texture tissues. Limitation of this protocol might due to problems caused by inadequate fixation, dehydration, and infiltration of specimen. Therefore, adjustment of processing time for each step is critical to produce good quality paraffin block. Normally, harder tissue needs longer processing time than softer tissue.

In addition, we showed that Hybrid-Cut worked successfully in combination with ISH to provide spatial distribution of the selected transcripts (**Figure 7**). In summary, this protocol is useful for the study of plant anatomy and provides a tissue-specific RNA map of the selected genes. In addition it may be applied to other molecular studies such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, fluorescence *in situ* hybridization *(*FISH), and immunostaining techniques. In conclusion, this improved tissue sectioning protocol is both useful and helpful for researchers in plant communities.

Disclosures

No conflicts of interest declared.

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