The Plant Cell Wall Biology Lab., Tohoku University *28 May 2013*

Indirect Immunofluorescence Assay with Cell Wall Polysaccharide-Directed Monoclonal Antibody for Plant cells and Tissues.

1. Fixation, Embedding, and Sectioning of Plant Tissues

1.1. Fixation

1. Prepare fixative by dissolving 0.4g of paraformaldehyde powder in 10ml of 20mM sodium cacodylate buffer (pH 7.4). Heat this solution in the fume hood on the hotplate/stirrer to approx. 70ºC until the solution clears, and allow to cool. The fixative mix should be prepared immediately before use.
2. Cut the plant tissue into pieces about 2 to 3 mm in length, and immediately immerse in fixative (cut tissue will float in the fixative).
3. Place tissue/fixative in a vacuum for 5 minutes, and release the vacuum very slowly. Pull and release the vacuum again until the tissue sink.
4. Incubate overnight at 4ºC without vacuum.

1.2. Embedding and Sectioning

Material:

Melted 5% agar medium. Dissolve 5g agar in 100ml PBS using a microwave oven. Place the molten agar medium in an oven at 60ºC well in advance.

1. Remove the fixative. Rince the tissue three times with phosphate-buffered saline (PBS).
2. Pour the tissue into a parafin dish.
3. Top off the parafin dish with molten agar medium.
4. Arrange the tissue into a regular array. For example, orient the stem tissue either straight up (Fig1A) or lying on their side. Let harden completely.
5. Glue the agar block on the stage of a Leica VT1200S vibrating blade microtome (Leica Microsystems) (Fig1B).
6. Cut the agar-embedded tissue transversely (50-70 μ m thick) with the vibroslicer (Fig.3a). Collect the transverse sections in PBS.

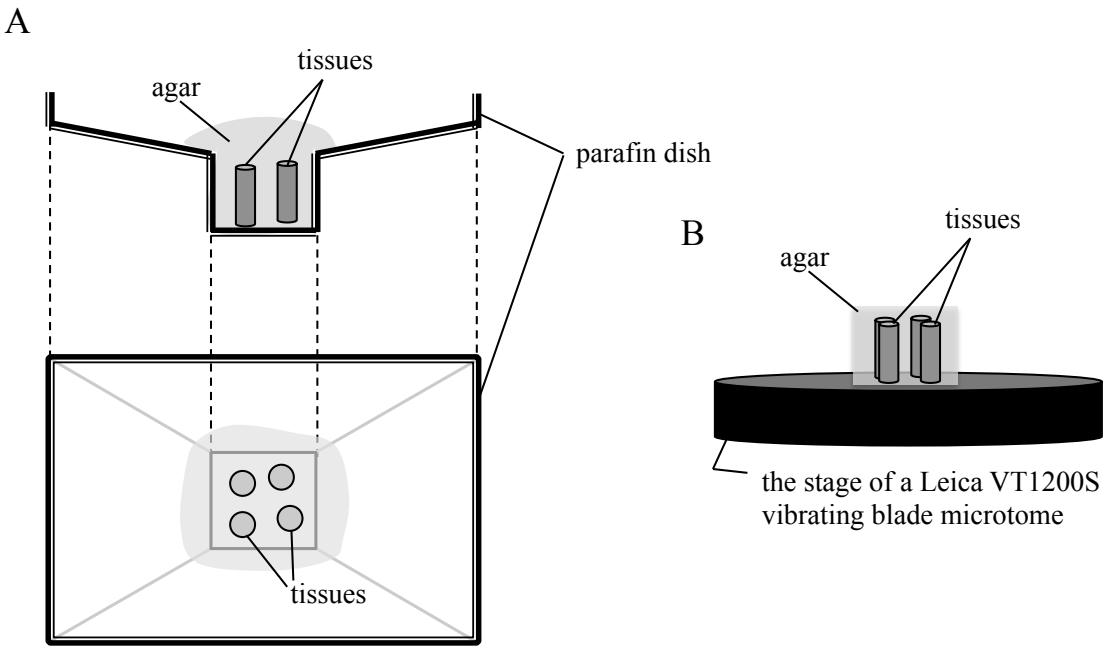

Fig.1 Tissues are embedded in agar (A), and the agar block is glued on the stage of the microtome (B).(Step2~5)

2. Immunohistochemistry

To enable ultrasensitive immunodetection, we used the tyramide signal amplification method based on the peroxidation of Alexa Fluor 488 tyramide accoding to the manufacturer's protocol (Tyramide Signal Amplification kits; Invitrogen-Molecular Probes) (Fig.2). In the course of the peroxidation reaction, the highly reactive fluorescent radicals covalently couple to nucleophilic residues near the binding sites of a horseradish peroxidase antibody conjugate used as a secondary antibody.

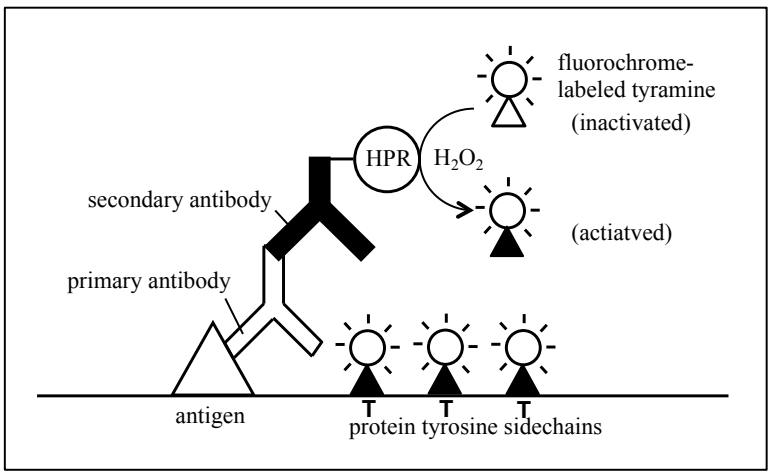

Fig.2 Schematic representation of tyramide signal amplification

2.1. Preparation

1. Prepare tyramide stock solution by dissolving the solid material provided in 150 μ l of DMSO.
2. Prepare a 1% (10mg/ml) solution of blocking reagent in PBS.
3. Prepare the HPR conjugate stock solution by reconstituting the material provided in 200 μ l of PBS
4. Prepare amplification buffer/0.0015% H_2O_2 by adding 30% hydrogen peroxide to amplification buffer to obtain a final concentration of 0.0015% H_2O_2.
5. Prepare peroxidase quenching buffer (PBS/1-3% H_2O_2) by adding 30% hydrogen peroxide to PBS to obtain a final concentration of 1-3% H_2O_2.

2.2 Peroxidase Labeling

Materials:

Cell wall polysaccharide-directed monoclonal antibodies: approximately 200 plant cell wall-directed monoclonal antibodies are available to the cell wall research community from CarboSource (http://www.CarboSource.net) and from PlantProbes (http://www.PlantProbes.net). The antibodies are annotated in a database accessible on the Internet (http://www.WallMabdb.net).

1. Incubate the sections with peroxidase quenching buffer (prepared in 2.1.5) for 60 minutes at room temperature (for quenching endogenous peroxidase activity).
2. Remove the peroxidase quenching buffer and replace 1% blocking solution (prepared in 2.1.2). Incubate for 60 minutes at room temperature.
3. Remove blocking reagent and label the sections with primary antibody diluted* in blocking solution for 60 minutes at room temperature.
4. Rinse the sections three times with PBS.
5. Prepare a working solution of the HRP conjugate by the HPR conjugate stock solution (prepared in 2.1.3) 1:100 in blocking reagent.
6. Incubate the sections with the HRP conjugate working solution for 30-60 minutes at room temperature.
7. Rinse the sections three times with PBS.

* The extent of dilution depends on antibody characteristics. Dilutions of the primary

antibody between 1:5 and 1:100 are generally used.

2.3 Tyramide Labeling

1. Prepare a tyramide working solution by diluting the tyramide stock solution (prepared in 2.1.1) 1:100 in amplification buffer/0.0015% H_2O_2 (prepared in 2.1.4) just prior to labeling.
2. Incubate the sections with the tyramide working solution for 5-10 minutes at room temperature.
3. Rinse the sections three times with PBS.
4. After final washings with PBS, mount the specimen on microscope slides using PBS/50% glycerol medium or ProLong®Gold antifade reagent/mounting medium (Invitrogen-Molecular Probes)*.
5. Examine the specimen with fluorescence microscopy or confocal microscopy using an appropriate filter set (Fig. 3b). Fluorescence excitation and emission maxima of Alexa Fluor® 488 dye are 495nm and 519nm, respectively.

*PBS/50% glycerol medium is suitable for mounting speciments for immediate examination. For longer-term storage and observation, ProLong®Gold antifade reagent/mounting medium has been recommended.

3. Comparison of Monoclonal Antibody and Calcofluor White Stains

If necessary, the immunolabeled sections can be additionally stained with Calcofluor White. Calcofluor White readily binds to cellulose and other β-linked glucans, and often is used for visualization of whole cell wall.

3.1 Calcofluor White Stain

1. After final washings of Tyramide labeling (Step2.3.4), the sections were incubated with 0.001-0.01% calcofluor white (Sigma; Fluorescent Brightener 28 F6259) in PBS for 15 min.
2. Rinse the sections three times with PBS.
3. Mount the specimen on microscope slides using PBS/50% glycerol medium.
4. Examine the specimen with fluorescence microscopy or confocal microscopy using an appropriate filter set (Fig.3c). Fluorescence excitation and emission maxima of

calcofluor white are 350nm and 430nm, respectively.

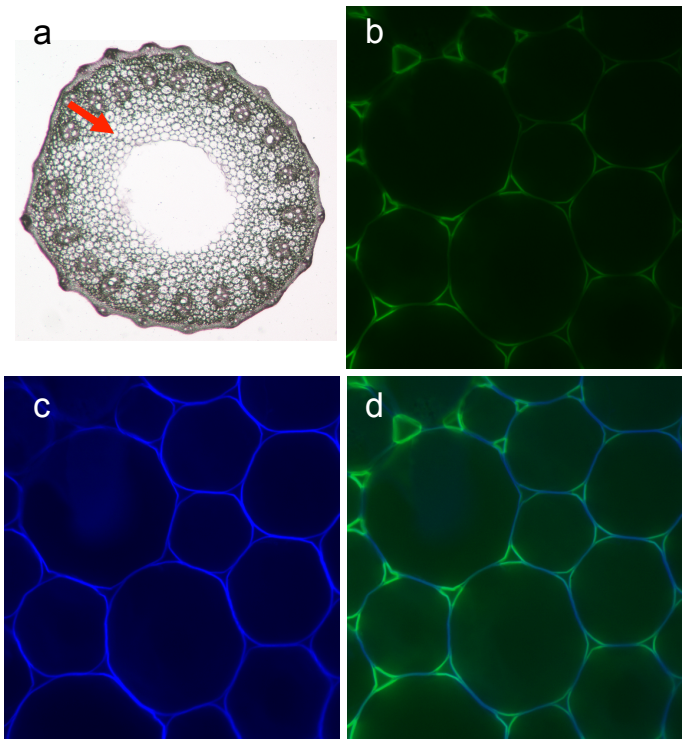

Fig. 3 Distribution of partially methyl-esterified or un-esterified homogalacturonan in pith of rice. Rice stem sliced transversely with the vibroslicer (a). The arrow shows pith tissue. A transverse stem section of rice was incubated JIM5, a monoclonal antibody to partially methyl-esterified or un-esterified homogalacturonan. The signal was amplified with TSA Kit, which includes HRP–goat anti-mouse IgG and Alexa Fluor 488 tyramide (b). The sample was then stained with Calcofluor White (c). Merged image is shown in (d).