

An Optical Clearing Technique to Visualize Internal Root Structure

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Methodology

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Abstract

Background

Roots play an important role in the foraging and uptake of nutrients and water from soil to support sessile plant growth. Research on root growth and development in plants is very limited due to the thickness and opacity of roots. We developed a tissue clearing technique that enables visualization of crop internal root structure.

Results

The application of methyl salicylate reduced the time taken for root clearing and accelerated the dye permeation into the tissue; the whole procedure for root clearing was performed within 3 days. We applied our technique on the roots of monocotyledonous plants, such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.), and dicotyledonous plants, such as rape (*Brassica napus* L. var *oleifera*), tomato (*Solanum lycopersicum*), and soybean (*Glycine max* (Linn.) Merr.), and obtained clear root structure. It not only shortens the time for root tissue clearing but also keeps the cell structure of intact roots. The transparent sample can be preserved for more than one year without any structural deformation.

Conclusions

Our technique can create a 3-D reconstruction of the entire root structure. In summary, this method is a useful tool for visualizing the structures of thick root tissues and will be a valuable tool for research on root growth and development in plants.

1. Introduction

Roots are an important organ responsible for the foraging and uptake of nutrient and water from the soil, which are then transported to the overground plant parts for growth and development (McCully, 1995). It is, therefore, important to study the structure, development, and functions of roots (Knapp et al., 2012; Barrada et al., 2015). However, visualizing the internal or 3D structure of roots is challenging in relatively thick specimens—e.g., rice, wheat, maize, soybean, and rape—because the internal root structure of only thin and semitransparent roots can be visualized by using confocal laser scanning microscopy (Nelson et al., 2007; Coudert et al., 2010).

Currently, visualizing internal root structures involves manually or mechanically sectioning samples, then processing the sections before imaging. However, sectioning is laborious and time-consuming, and does not allow for 3D reconstruction. In addition, sectioning may damage the fine structures of the roots, obscuring any representation of a specimen (Knapp et al., 2012), thus impeding efforts to visualize cellular and intracellular structures in detail. In 2018, Bureau et al. reported a protocol for the deep 3D imaging of rice roots by combining multiphoton microscopy and propidium iodide (PI) staining (Bureau et

al., 2018), but this instrument is not accessible for most researchers, and the experiment itself is costly. Hence, better plant tissue clearing techniques are urgently needed to image and investigate thick roots (Lamont, 2003; Netuschil et al., 2014).

Tissue clearing techniques facilitate microscopic analyses of tissues and intact organisms (Treweek et al., 2015). In recent years, optical clearing techniques have developed rapidly for animal tissues, as researchers have learned to better preserve tissue structure using improved chemical mixtures such as PEGASOS, CLARITY, and SHIELD (Chung et al., 2013; Warner et al., 2014). These clearing methods have successfully been applied in the mouse brain to show intact tissue images of long-range projections, local circuit wiring, cellular relationships, subcellular structure, protein complexes, nucleic acids, and neurotransmitters (Hama et al., 2011; Chung et al., 2013; Soderblom et al., 2015; Jensen and Berg, 2017). In plants, light penetration has significantly limited the investigation of roots using fluorescence-based microscopy (Wymer et al., 1999).

Several plant tissue cleaning methods have recently been reported, such as an optical clearing technique, which has cleared maize leaves, pea root nodules, *Medicago truncatula*, pea leaves, and others, and allows for deep imaging. However, it is inconvenient because different plant materials require different recipes, and the entire clearing process takes 1–3 weeks (Warner et al., 2014). Herr and Crane invented a "4½" composite transparent agent and used methyl salicylate to observe the embryo of the whole ovule (Herr, 1971; Crane, 1978). Based on their study, Yang developed a new stain-clearing procedure that successfully observed embryos, embryo sacs before fertilization, and embryos and endosperms after fertilization (Hongyuan Yang, 1986; Jianhua Hao, 2007). This method highlights the effectiveness of methyl salicylate for clearing plant tissues; however, the process of staining in this technique is difficult to control, and this method can exclusively be used on ovules and pollen.

To shorten the clearing time and simplify the clearing procedure, we developed a method to facilitate deep-tissue imaging of plant roots using methyl salicylate, a volatile liquid with a strong Holly oil aroma that is produced in a number of plants. This is the first report on the application of methyl salicylate for plant root clearing. We found that the use of methyl salicylate greatly reduced the time for root opacity without damaging the root cell structure. Furthermore, the cleared root samples can be stored for more than one year. To expedite understanding of root growth and development, we selected the root or shoot tips after culturing for 7 days. Monocotyledonous plants such as rice (*Oryza sativa L.*), wheat (*Triticum aestivum L.*), and maize (*Zea mays L.*) and dicotyledonous plants such as rape (*Brassica napus L. var oleifera*), tomato (*Solanum lycopersicum*), and soybean (*Glycine max (Linn.) Merr.*) were used for this study. Additionally, we cleared the root and shoot tip samples for seedlings under salt stress (i.e., after 24 h of 500 mM NaCl treatment) and observed their clear internal structure. Furthermore, we observed that this method was not suitable for very small and thin root samples, which could get damaged or shrunk, and no significant difference in co-localization analysis could be observed.

2. Materials And Equipment

3.1 Materials

Rice, wheat, maize, rape, tomato and soybean seedlings were investigated in our study. The rice UBI: EGFP transgenic line was used to observe green fluorescent protein (GFP) signals in roots (Matsushita et al., 2013).

RNaseA (100 µg/mL), PI (5 µg/mL) (Molecular Probes/Invitrogen/Thermo, P21493), methanol, and methyl salicylate were applied for clearing the tissue.

3.2 Equipment

The cleared root tissues were observed under a Leica M205FA stereomicroscope. The detailed root cell images were obtained by using a 40×, NA 1.30 oil objective on a Leica TCS-SP8 STED 3X spectral confocal laser scanning microscope (Leica Microsystems). The excitation wavelength for the PI-stained samples was 561 nm, and emissions were recorded at 571–660 nm. The excitation wavelength for GFP samples was 488 nm, and emissions were recorded at 498–550 nm. An incubator (SANYO) was used for growing plants. Data were processed for some two-dimensional orthogonal sections, and three-dimensional rendering and movie exports were conducted using Imaris software on an Intel Core i7-2820QM 2.30 GHz, 16 GB RAM DELL workstation. For cell length measurements, we used the Leica Confocal Software version 2.0.1.

3. Methods

Plant growth conditions

Soybean, rice, maize, tomato, and rape seedlings were grown on a dish with pure water under room conditions (25°C, 70% humidity, 12 h light/12 h dark). Seedlings of *Arabidopsis thaliana* were grown on Murashige and Skoog medium supplemented with 1% sucrose in a growth chamber (25°C, 70% humidity, 12 h light/12 h dark). All species were cultured for 7 d, then the roots or shoot tips were cut for the experiments. The UBI: EGFP transgenic line was used to image GFP signals in rice roots (Matsushita et al., 2013). For the salt stress assay, seedlings were cultivated 7 d after germinating for 24 h in 500 mM NaCl solution, and then the rice roots were observed.

Tissue Clearing and PI staining

The process of clearing has been described step by step, as follows:

1. Plant roots were fixed for 60 min in PEX buffer [5 mM MgCl₂, 100 mM PIPES (pH 6.9), and 5 mM EGTA] containing 4% paraformaldehyde and 5% DMSO at room temperature (Jung et al., 2011).
2. The plant tissues were transferred to 70% ethanol and incubated at room temperature for 12 h, then transferred to 70, 50, and 30% ethanol in sequence for 30 min each. It took 13.5 h for this step.
3. Tissues were rinsed twice in PBS solution (50 mM phosphate buffer, pH 7.4, and 0.9% NaCl) for 30 min each. It took 1 h for this step.

4. Tissues were incubated in RNaseA (100 µg/mL) at 37°C for 12 h. Tips: this step was overnight, the time was around 12 hours but not exactly.
5. The tissues were then stained with PI (5 µg/mL) (Molecular Probes/Invitrogen/Thermo, P21493) at 4°C for 12 h. Tips: this step was overnight, the time was around 12 hours but not exactly.
6. Next, tissues were rinsed in PBS solution three times, each for 3 h. It took 9 h for this step. Pause Point: this step could be prolonged.
7. The tissues were transferred to 30, 50, 70, 80, 90, and 100% ethanol in sequence for 30 min each, then rinsed in 100% ethanol twice, for 30 min each time. It took 4 h for this step.
8. The samples were immersed in a solution of methanol and methyl salicylate as a volume ratio at 2:1 for 1 h, then transferred to a volume ratio at 1:1 and 1:2 solutions in sequence for 1 h each. It took 3 h for this step.
9. The sample was rinsed in methyl salicylate three times each for 1 h and kept in this solution overnight. It took approximately 15 h for this step.
10. The samples could then be transferred onto microscope slides with methyl salicylate for observing under a confocal microscope. Alternatively, they could be stored in methyl salicylate for over one year. The overall time taken during this method is about 3 days.

4.3 Image capture and data processing

Cells were imaged with a 40×, NA 1.30 oil objective on a Leica TCS-SP8 STED 3X spectral confocal laser scanning microscope (Leica Microsystems). The excitation wavelength for the PI-stained samples was 561 nm, and emissions were collected at 571–660 nm. The excitation wavelength for GFP samples was 488 nm, and emission was collected at 498–550 nm. Then, images were processed for some two-dimensional orthogonal sections, three-dimensional rendering, and movie exports using Imaris 9.0 software (Oxford Instrument) on an Intel Core i7-2820QM 2.30 GHz, 16 GB RAM DELL workstation. For cell length measurements, we used the Leica Confocal Software version 2.0.1.

4. Results

Tissue clearing techniques have been widely developed and used in animals to observe the internal structures in depth but clearing methods for plant samples are very limited. To facilitate cellular observation of the internal structure of plant roots, we developed a clearing method to specifically observe the thick root tissues using methyl salicylate. First, we found that the 3-day clearing time is enough to make fresh rice root samples transparent (Fig. 1E and 1F), and significantly reduced the tissue clearing time compared to previous methods. Next, we further compared rice root structure by confocal microscopy with or without clearing treatment and could easily visualize the interval structures of PI-stained roots after clearing (Fig. 1C), but not for roots without clearing (Fig. 1B). This method is not applicable for small and thin materials—e.g., young *Arabidopsis* roots—because it is very easy to observe the root internal structures of *Arabidopsis* without clearing (Fig. 1A), and thin *Arabidopsis* roots treated with methyl salicylate were easily damaged and caused shrinkage, which made it difficult to obtain the appropriate images under confocal microscopy.

To investigate whether root samples treated with our method were for visualizing observations after long-term storage, we compared the rice root structures of long-term storage and freshly prepared samples. Figure 1C shows that the structure of freshly prepared rice root tips is very easy to observe after tissue clearing. Similarly, the rice root tip structures after long-term storage for thirteen months were very easy to observe (Fig. 1D), and there was no significant difference between the stored and fresh samples (Fig. 1.C, D). The above analysis indicated that the samples treated with our method can be perfectly preserved for over one year without their root structure or morphology being damaged.

To determine whether our method can be widely applied to the tissues of other plant species, the roots of monocotyledonous rice, wheat, maize, and dicotyledonous rape, tomato, and soybean were further tested. Our method clearly visualized the internal structure of these species' root tips because root tips are very important for plant development, so we focused on this part and made it easy for PI dye to enter the tissue and stain the cell wall and nucleus. A high-resolution image of all observed parts of the roots was obtained to show the internal structure (Fig. 2 and Fig. 3). These assays revealed that our method is advantageous for completely observing the roots of different plants including rice, wheat, maize, rape, tomato, and soybean.

Based on the above observations, we hypothesized that, after clearing, the plant root is also optimal for root 3D reconstruction, from which the entire root structure is easily presented in detail. To verify our hypothesis, the root structure was automatically captured by XYZ stack scan without having to cut the root by hand or machine, and the 3D structure of the root was constructed using Leica software. Using this method, the cleared root was suitable for observing and capturing the cell structure in depth through confocal microscopy. All cell types and internal structures in the root were clearly visible, and a high-resolution 3D structure of the root is presented in Fig. 4. Currently, the only limitation for capturing the whole root structure is the working distance of the objective lens, but this is not related to the quality or state of the cleared root.

Next, the transverse section of the root tip and elongation region was reconstructed using Imaris software (Oxford Instrument) based on the observation of the whole root. The distinct transverse sections of rice, tomato, and wheat root are shown in Fig. 3, and we found that the root structure in our assay was consistent with those from a previous study (Bhandari et al., 2015). For example, the numbers of cell layers were not distinct between the root tip and elongation zone of the same species, but the tip region had smaller cells and a higher cell density than did the elongation zone. The wheat root structures were similar to rice at both the tip and elongation parts (Fig. 3C–C'). Similarly, except for the difference in cell size and density, the dicotyledonous plant tomato does not show much difference between tip and elongation parts by observation of root transverse section (Fig. 3B–B'), but the cell size between the stele and cortex is not as obvious as that in the monocotyledon plants rice (Fig. 3A–A') and wheat (Fig. 3C–C').

The root structure was easily affected by complicated soil environments. To test whether our method is useful for comparing the difference in permeability between tissue treated with and without stress, we analyzed the rice root structures after treatment with 500 mM salt stress for 24 h. Figure 5 shows that the

nuclei in salt-treated rice roots were easily stained by PI (Fig. 5A). Conversely, cells inhibited the PI entrance, and it was not easy to visualize the nuclei in rice roots without salt treatment (Fig. 5B). As a control, only with the salt treatment for roots, the shoot tip did not show significant differences in cell structure and permeability before and after salt stress (Fig. 5C and 5D), and the nuclei staining by PI was difficult to observe in the shoot. This indicates that our clearing method is suitable for research on salt stress in plant roots.

5. Discussion

The plant root is an important organ for plant growth and development, and clearing methods are becoming increasingly crucial for understanding the internal structure of plants. Many methods—e.g., PEGASOS (Jing et al., 2018), CLARITY (Chung et al., 2013), and SHIELD (Park et al., 2018)—have been reported to be suitable for animal tissue clearing. PEGASOS can clear the hard and soft tissue, CLARITY was applied on soft tissue, and SHIELD finely protects the signal of fluorescent protein. Ehrlich's hematoxylin-methyl salicylate technique has been applied for ovule and pollen clearing (Warner et al., 2014). In this study, we established a new clearing method to observe plant roots that is suitable not only for plant root research, but possibly other tissues or organs. Previous research has shown that the root structures are different in different crops (Lincoln Taiz, 2009), but further investigation is still needed to identify the detailed differences. Therefore, this study tested the roots of six species: rice, wheat, maize, rape, tomato, and soybean.

From the results, we found that our method is effective for observing the structures of thick root tissue (Fig. 1A). This method gave reproducible results, and the cleared tissue could be stored for more than one year in the dark at 4°C for staining without the root structure being destroyed. Moreover, the root tissue treated using our method was easy for dye entrance and staining, and the image scanning and capture were not affected by photo bleaching (Fig. 1E–F). However, in some thick materials, such as soybean roots, the working distance of the objective greatly limits the depth of image collection. If the material is more than 200 µm thick, it is better to observe the cell structure using a two-photon microscope (Feijo and Moreno, 2004; Chung et al., 2013; Littlejohn et al., 2014).

It is currently very difficult for researchers to perform colocalization assays between target protein localization and marker dye within root cells using confocal microscopy, such as GFP-labeled nuclei protein colocalization with DAPI-stained nuclei. To test whether our clearing method is suitable for maintaining GFP-fused protein localization for this assay, a rice transgenic line with GFP driven by a 35S promoter was used. Compared to the GFP signaling in the root without clearing, GFP signaling in the root with clearing was widely distributed throughout the nuclei, cytosol, and plasma membrane (PM). The fluorescence distribution in the nuclei disappeared after the clearing treatment (Fig. 6). Hence, our method still needs to be improved by stabilizing protein localization *in vivo* in cleared root cells.

This method allowed us to clearly observe interval structures and the overall structure of plant roots (Fig. 2 and Fig. 3). Based on this, we could also further analyze the 3D exhibition by software (Fig. 4).

This method could help researchers investigate the internal root structure of different plant species.

Our method also helped illustrate how root cells acclimate to a complex environment in soil. For instance, after 24 h of salt stress in rice root tissue, the nuclei of the root tip were dyed clearly, but those of the shoot tip were not. However, the cell structure of the root and shoot tips did not differ with or without treatment (Fig. 5A–D). This implies that the permeability of the membrane was affected more under salt stress than it was in the control (Fig. 5A–B).

In summary, our method makes the cell structure of thick and opaque roots permeable for staining and easy to visualize. This method will help further research on root structure and function, especially for important crops. However, if the root is too thin such as that of *Arabidopsis thaliana*, after clearing with this method, it will be damaged and undergo shrinkage. Therefore, this method is not suitable for very thin roots, and it did not allow sharp illustration for colocalization analyses. Nevertheless, the advantage of the study is obvious; the overall clearing time is about 3 days, and the samples could be preserved for more than one year. Moreover, the chemical used is easily available in laboratories, and its cost is very low.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions: Yunxiao He designed and conducted the experiment. Qingqing Yan, Hui Li and Yuda Fang given useful advice to the experiment. Dongyong Yang and Min Li take part in the experiment. Yunxiao He organized all data and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

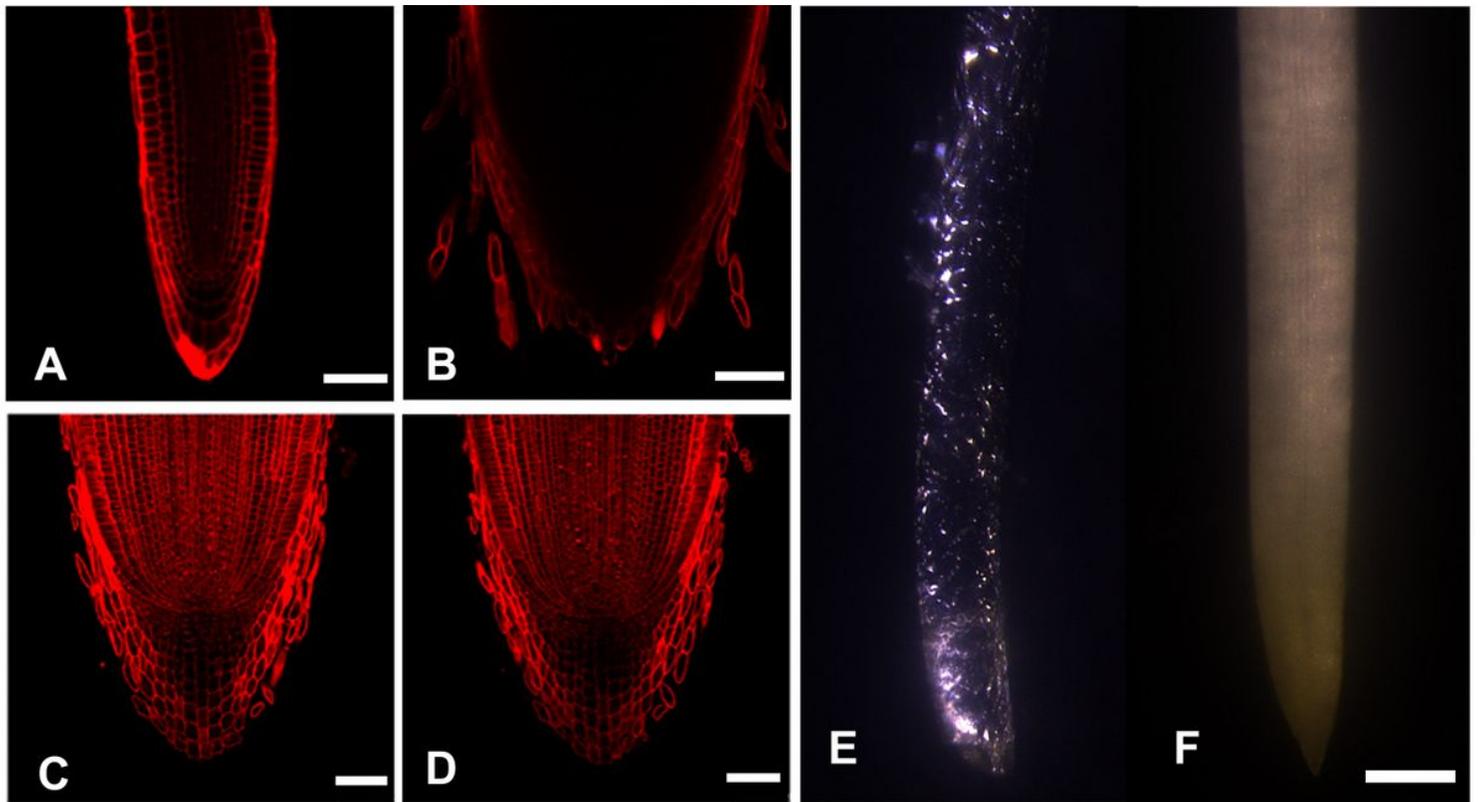


Figure 1

The cellular structures of rice and *Arabidopsis* root tips. (A) *Arabidopsis* root tip without clearing, after PI staining. (B) Rice root tip without clearing, after PI staining. (C) Rice root tip after clearing, with PI staining. (D) Rice root tip after clearing, with PI staining, followed by storage for over one year. (E) Rice root tip after clearing under stereo microscope. (F) Rice root tip without clearing under stereo microscope. Scale bars=50 μm in A, B, C, D. Scale bar=0.5 mm in E, F.

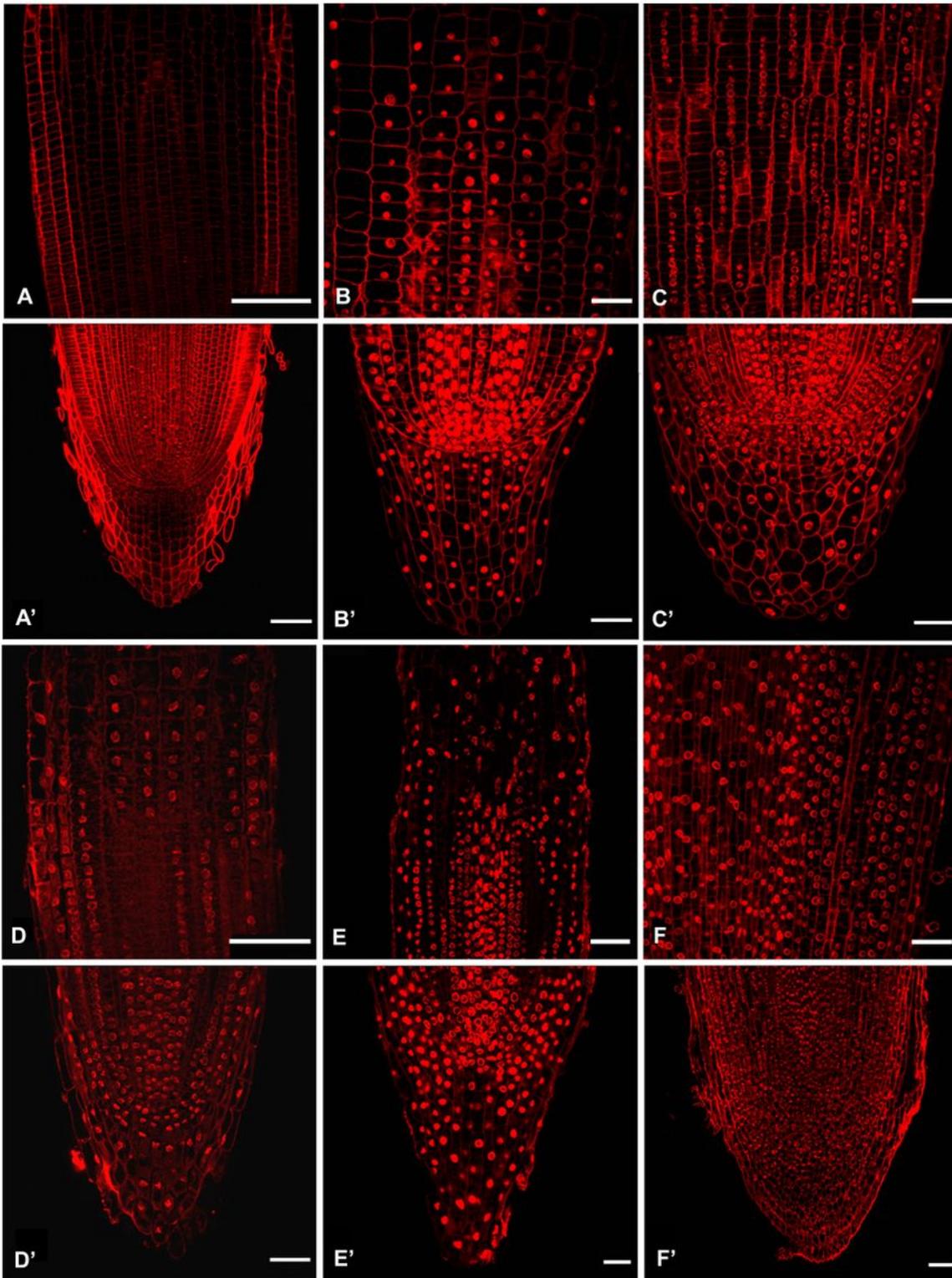


Figure 2

Root tip and elongation zone structures of different plants. (A) Rice root tip after clearing, with PI staining. (A') Rice elongation zone after clearing, with PI staining. (B) Wheat root tip after clearing, with PI staining. (B') Wheat elongation zone after clearing, with PI staining. (C) Maize root tip after clearing, with PI staining. (C') Maize elongation zone after clearing, with PI staining. (D) Rape root tip after clearing, with PI staining. (D') Rape elongation zone after clearing, with PI staining. (E) Tomato root tips after clearing,

with PI staining. (E') Tomato elongation zone after clearing, with PI staining. (F) Soybean root tip after clearing, with PI staining. (F') Soybean elongation zone after clearing, with PI staining. Scale bar=50 μm in A, B, C, D, B', C', D', E', F'. Scale bar=100 μm in A'. Scale bar=25 μm in e. Scale bar=75 μm in F.

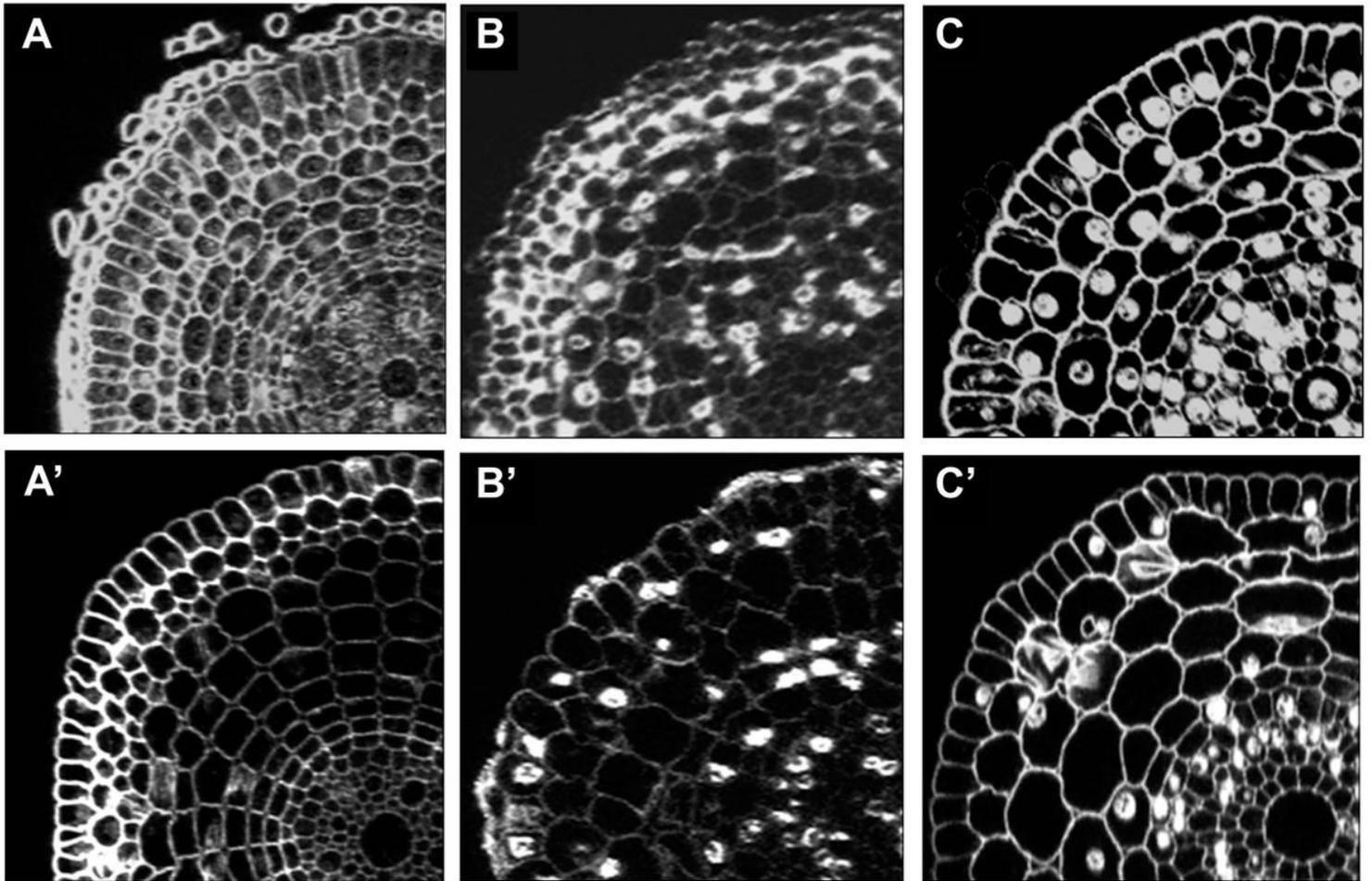


Figure 3

Transverse section view after three-dimensional reconstruction in different parts of the plant root. (A) Cross-section of the rice root tip. (A') Cross-section of rice root elongation zone. (B) Cross section of tomato root tip. (B') The cross section of the tomato root elongation zone. (C) Cross section of the wheat root tip. (C') The cross section of wheat root elongation zone. All the figures are cleared and stained with PI.

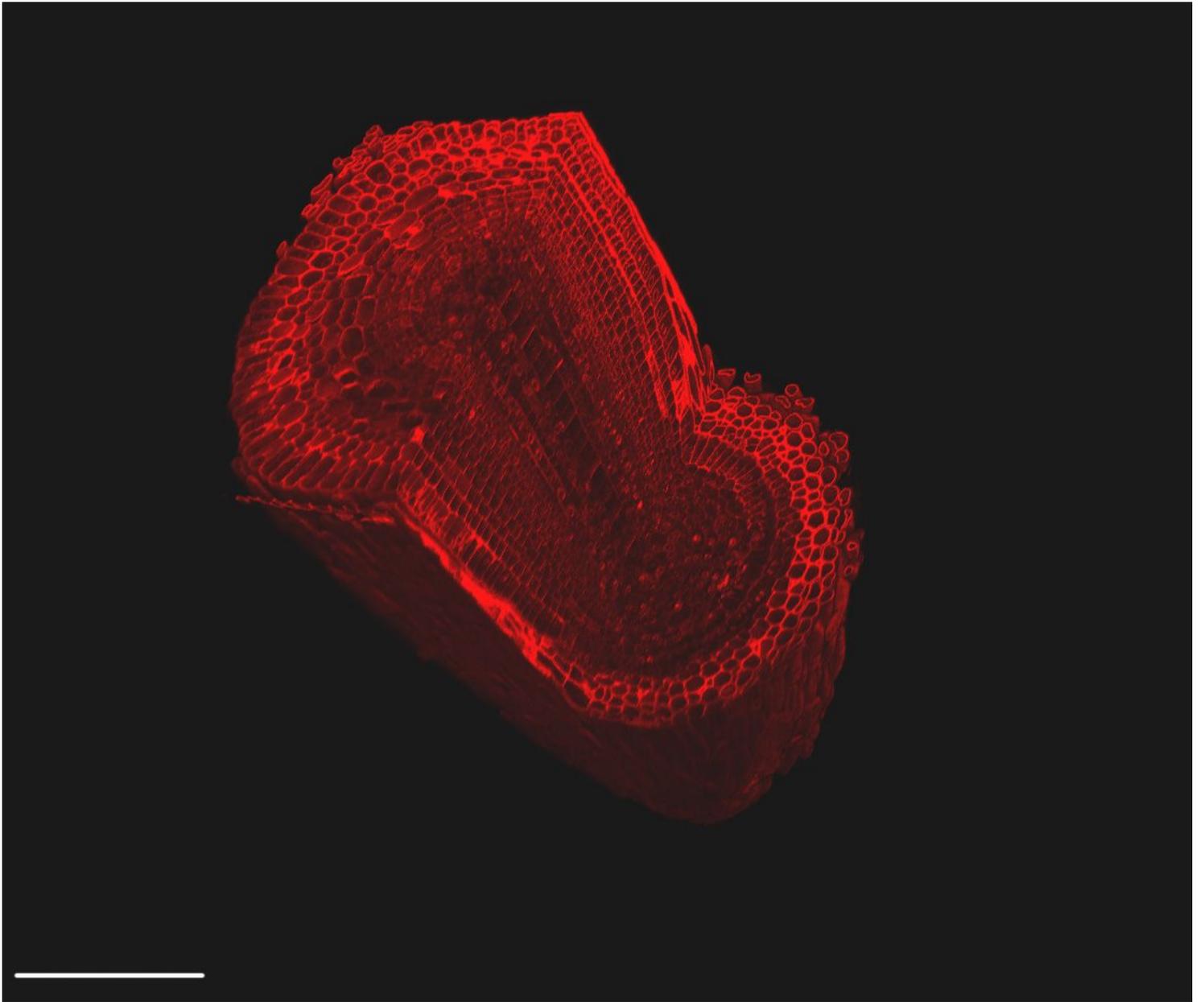


Figure 4

Three-dimensional reconstructions of rice root tip; rendered from multiple z stacks. Scale bar 100 μm .

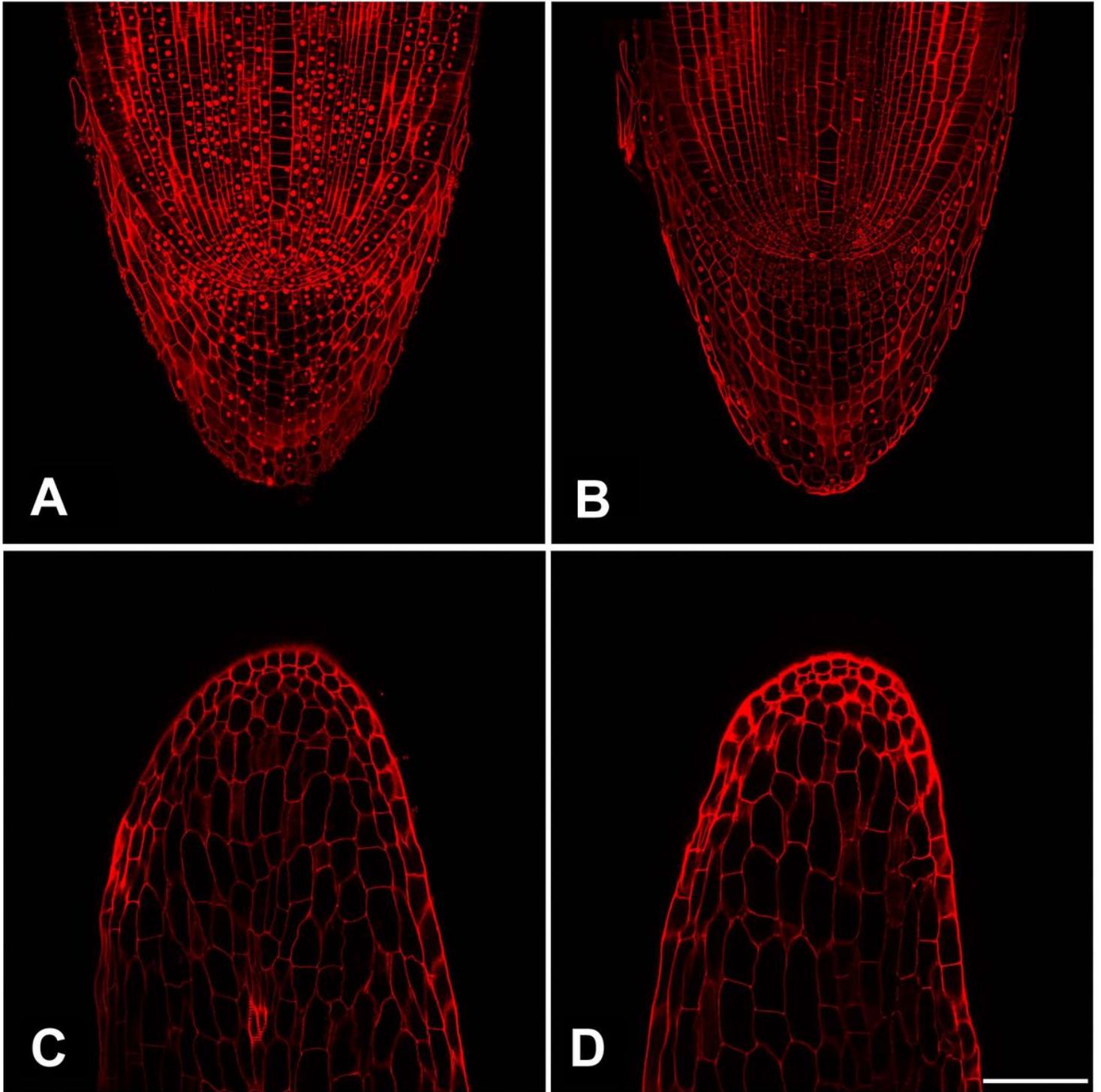


Figure 5

Rice root tip after 24 h of 500 mM salt stress (A). Rice root tip without stress (B). Rice shoot tip after 24 h of 500 mM salt stress (C). Rice shoot tip without stress (D). Scale bars: 75 μ m. All the figures are cleared and stained with PI.

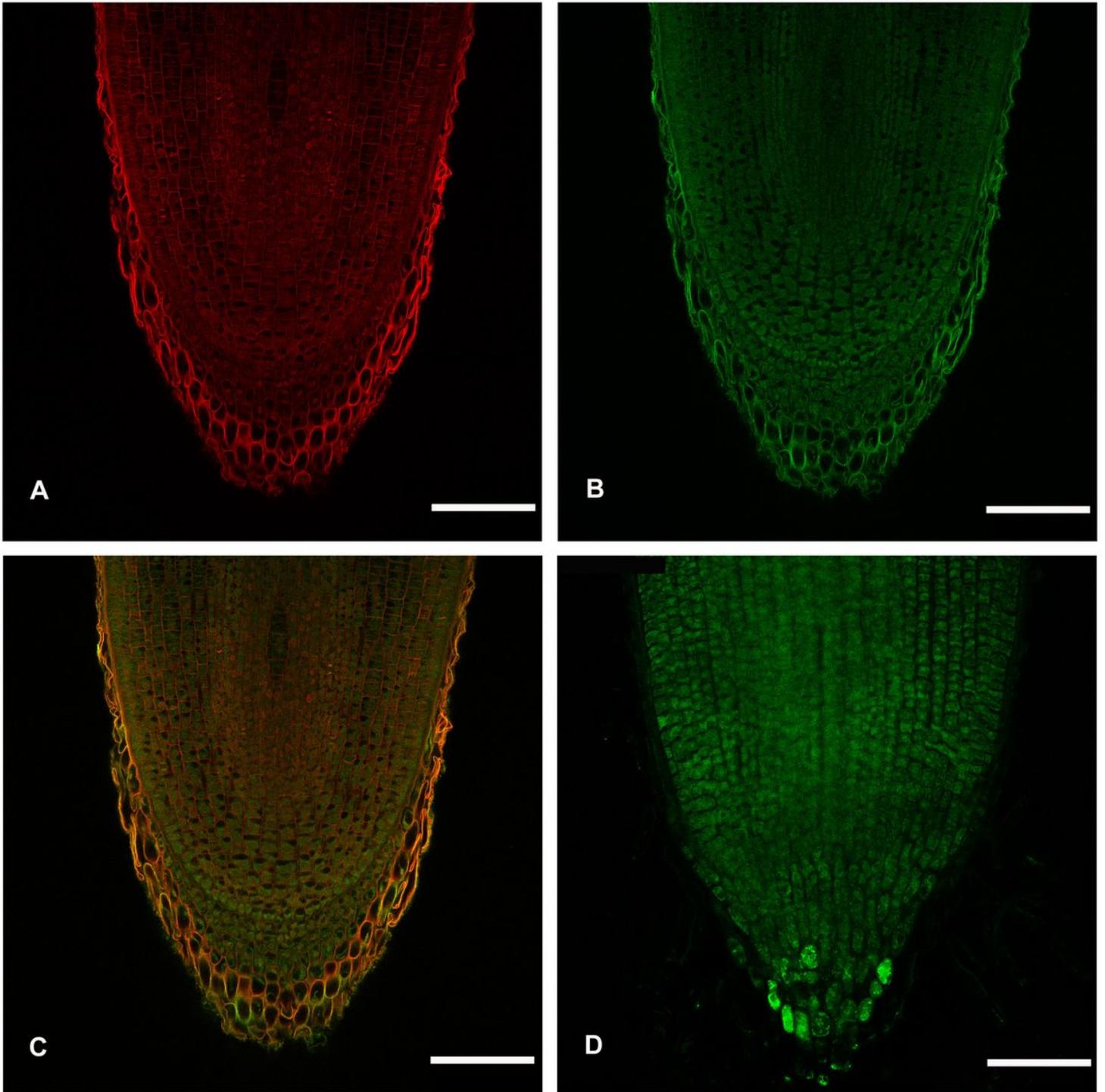


Figure 6

Rice root tip after clearing, with PI staining (A). GFP localization in rice root tip, after clearing (B). The merged image of A and B (C). The GFP localization in rice root tip, without clearing.

Supplementary Files

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