Research Article

Development and Applications of Plant Tissue-Clearing Technology

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Abstract

Plants harbor various pigments, which are serious barriers to fluorescence observation since they absorb and scatter light. Plant tissue-clearing technologies have been developed to remove these pigments and improve optical conditions; however, they require a long incubation time to enhance the transparency of specimens. We have newly developed iTOMEI in which a decolorizing solution, including caprylyl sulfobetaine, excludes chlorophylls efficiently and reactivates GFP quenched by fixation. iTOMEI achieved clearing within 27 hours while maintaining GFP fluorescence. This technology can be adapted to *Arabidopsis thaliana*, *Oryza sativa*, and *Marchantia polymorpha*.

Keywords: plant tissue-clearing technology, deep imaging, microscope

Introduction

Many biological tissues do not allow light to pass through. Biologists have embedded the specimens in resin and paraffin and cut thin sections to observe the inner structure of tissues and organs in detail. Although imaging using thin sections is sufficient for observing local structures, it is not suitable for obtaining a whole image of the tissue due to the loss of positional information of the sections. Therefore, biological tissue-clearing technologies have been developed for microscopic observation of the inner structure through transparent outer tissues. Because the history, basics, and applications to animals of tissue clearing technologies were explained by Dr. Suzaki in a previous TCIMAIL,[1] this paper focuses on plant tissue-clearing technologies and their applications.

Development of plant tissue-clearing technology

Plant tissues contain various pigments, such as chlorophylls, carotenoids, and flavonoids. Among them, chlorophylls, photosynthetic pigments, are the major obstacles to fluorescence observation because they are abundantly contained in chloroplasts and emit strong autofluorescence. To resolve this problem, plant tissue-clearing technologies were developed in which chlorophylls were excluded from cells, and tissues were made transparent without inactivation of fluorescent proteins (FPs).

Littlejohn *et al*. successfully increased the transparency of *A. thaliana* leaves while maintaining FP fluorescence by infiltrating fluorocarbon, which has a refractive index (RI) similar to living cells and can unify the RI throughout the leaves.[2,3] This method differs from other clearing methods in that it can treat

living cells but does not remove chlorophylls from chloroplasts; hence the transparency is not high.

Warner *et al.*'s method is based on Sca*l*eA2, an animal brain-clearing technique.[4,5] They fixed *Nicotiana benthamiana* leaves expressing FPs in paraformaldehyde (PFA) solution and treated them with a clearing solution containing urea, glycerol, and Triton X-100 for 3 weeks. They succeeded in chlorophyll removal and high transparency while maintaining the fluorescence of FPs.

In PEA-CLARITY, *A. thaliana* and *N. benthamiana* leaves were fixed in a hydrogel solution containing PFA and acrylamide. After polymerization of acrylamide, leaves embedded in the acrylamide gel were treated with a detergent solution containing sodium dodecyl sulfate for 4-6 weeks to achieve high transparency while maintaining the fluorescence of FPs.[6] Subsequent cell wall digestion and antibody treatment enabled immunofluorescent staining.

In ClearSee®, *A. thaliana* shoots, leaves, and pistils were fixed with PFA solution and incubated in ClearSee solution containing xylitol, urea, and sodium deoxycholate for 4 days to several weeks to achieve high transparency while maintaining fluorescence of FPs.[7] In 2021, it was found that ClearSee solution oxidizes polyphenols such as proanthocyanidins in plant tissues, leading to the deposition of brown pigments.[8] Since the brown pigments reduce light transmission, the addition of a reducing agent suppressed tissue browning and elevated transparency in the new method ClearSee alpha.[8]

Development and applications of iTOMEI

We have attempted to develop a novel plant tissue-clearing technology with an emphasis on the time required. Transparent plant Organ Methods for Imaging (TOMEI), in which high RI reagent 2,2'-thiodiethanol is used, was developed in 2016.[9] There are two types of TOMEI, TOMEI-I and TOMEI-II. In TOMEI-I, *A. thaliana* leaves were fixed and decolorized with a solution containing ethanol and acetic acid and then incubated in 2,2'-thiodiethanol solution. TOMEI-I provides high transparency within only several hours but FPs are quenched completely hence TOMEI-I is not used for observation of FPs. In TOMEI-II, *A. thaliana* leaves were fixed with PFA solution and then replaced with 2,2'-thiodiethanol solution in a stepwise manner to alleviate FP quenching. TOMEI-II also completes the clearing within several hours, however, the transparency is not high because chlorophylls partly remained. In addition, it was reported that high concentration 2,2'-thiodiethanol negatively affected FP fluorescence,[10–12] although TOMEI-II can be used for observation of FPs. In other words, TOMEI-II left room for improvement. Therefore, we have developed improved TOMEI (iTOMEI) which greatly ameliorated transparency and suppressed FP quenching compared with TOMEI-II.[13] In iTOMEI, *A. thaliana* seedlings were fixed in PBS containing formaldehyde (FA) and then treated with a decolorizing solution including caprylyl sulfobetaine which elutes chlorophylls effectively. Additionally, bright GFP fluorescence was maintained in iTOMEI because the decolorization solution was prepared at pH 8.0 to reactivate GFP inactivated by FA fixation. After decolorization, the samples were treated with a high RI mounting solution containing iohexol to unify the RI in the samples. iTOMEI made *A. thaliana* seedlings transparent within 27 hours (**Fig. 1**). Histone H2B-GFP expressing *A. thaliana* seedlings were treated with iTOMEI, and the cells at a depth of 20 μm from the adaxial surface of the cotyledons were observed by confocal microscopy. Compared with control plants, much brighter GFP was detected in iTOMEI-treated plants. The thallus of *M. polymorpha* was also cleared by iTOMEI. Histone H2B-tdTomato expressing thallus was cleared and stained with calcofluor white, a cell wall staining reagent, and observed by confocal microscopy (**Fig. 2**). Subsequently, the cleared gemma cup of *M. polymorpha* was observed using two-photon excitation microscopy (**Fig. 2c**). The gemmae of various sizes can be observed through the wall of the gemma cup. In addition, it was verified that iTOMEI was suitable for observation of young *O. sativa* leaves, roots, and floral meristems.[13,14] It can also be applied to the clearing of mouse brains by modifying the procedure. The reagents used for iTOMEI are manufactured and sold by TCI.

Conclusions

Although several plant tissue-clearing technologies have been developed, each has its own advantages and disadvantages. The most appropriate clearing technique should be selected according to the plant species, the type of tissues or organs, the organelles or proteins to be observed, the required tissue transparency, and the type of microscope used for observation. In some cases, it is necessary to try several clearing techniques, which may seem complicated. However, the advantage of these technologies is that they are much cheaper and easier to do than installing an expensive multiphoton excitation microscope system for deep tissue imaging (of course, additive effects can be expected when combining the technologies with the microscope system). We hope that this paper will provide you with an opportunity to try deep tissue imaging using the clearing technologies.

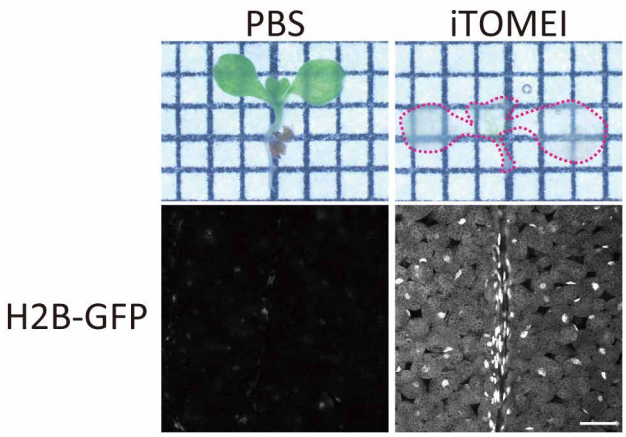

Figure 1. Clearing of Arabidopsis thaliana seedling using iTOMEI
Images of PBS- and iTOMEI-treated seedlings (upper panels). Cotyledons, expressing histone H2B-GFP, were fixed with 4% FA and treated with PBS or iTOMEI. Fluorescence images were taken at a depth of 20 µm from the adaxial surface of cotyledons using a confocal laser microscope (lower panels). Dotted lines indicate outlines of a seedling. The scale bar is 50 µm. The images were partially modified from Ref. 12 (CC-BY-4.0).

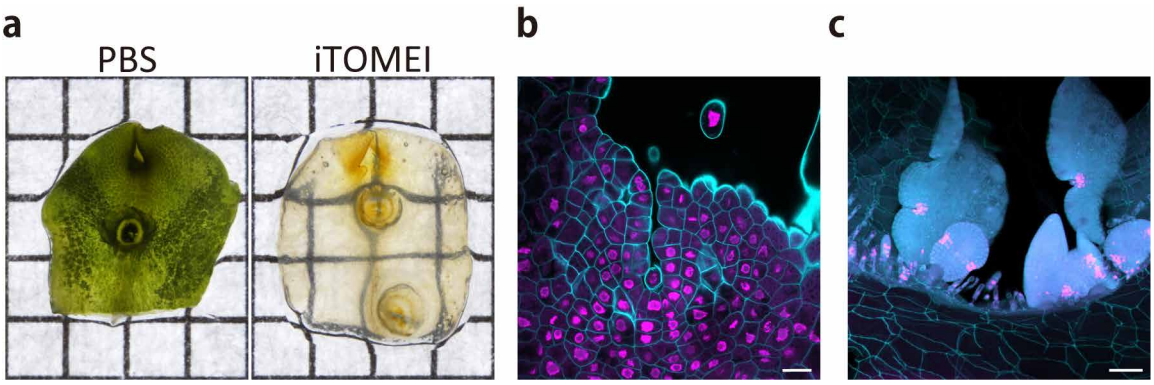

Figure 2. Clearing of Marchantia polymorpha using iTOMEI
a Images of PBS- and iTOMEI-treated thallus. **b** Thalli, expressing histone H2B-tdTomato (magenta), were treated with iTOMEI, stained with calcofluor white (cyan), and observed using a confocal laser microscope. The scale bar is 20 µm. **c** Gemmae in the gemma cup were observed through the wall of the gemma cup using the two-photon excitation microscope. pMp*RSL1*::tdTomato-NLS is magenta and calcofluor white is cyan. The scale bar is 50 µm. The images were modified from Ref. 12 (CC-BY-4.0).

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