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ClearSeeAlpha: Advanced Optical Clearing for Whole-Plant Imaging

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ABSTRACT

To understand how the body of plants is made, it is essential to observe the morphology, structure, and arrangement of constituent cells. However, the opaque nature of the plant body makes it difficult to observe the internal structures directly under a microscope. To overcome this problem, we developed a reagent, ClearSee, that makes plants transparent (Kurihara et al. 2015), allowing direct observation of the inside of a plant body without inflicting damage on it, for example through physical cutting. However, because ClearSee is not effective in making some plant species and tissues transparent, in this study, we further improved its composition to prevent oxidation and have developed ClearSeeAlpha, which can be applied to a broader range of plant species and tissues. Sodium sulphite, one of the reductants, prevented brown pigmentation due to oxidation during clearing treatment. Using ClearSeeAlpha, we show that it is possible to obtain clear chrysanthemum leaves, tobacco and Torenia pistils, and fertilized Arabidopsis thaliana fruits—tissues which have hitherto been challenging to clear. Moreover, we show that the fluorescence intensity of purified fluorescent proteins emitting light of various colours was unaffected in the ClearSeeAlpha solution; only the fluorescence intensity of TagRFP was reduced by about half. ClearSeeAlpha should be useful in the discovery and analysis of biological phenomena occurring deep inside the plant tissues.

KEYWORDS

Clearing, Fluorescent proteins, Fluorescent microscopy, Oxidation, Reductant
INTRODUCTION

Biological processes occur inside and between cells in tissues. It is important to understand their roles and functions *in vivo* by analysing the structure of the plant body. Many researchers have used microscopy to observe the inside of the body. Recently, several clearing reagents applicable to fluorescent microscopy using fluorescent proteins in plants have been developed. Littlejohn et al. used perfluorocarbon to infiltrate the leaves and make them transparent to detect fluorescent proteins (Littlejohn et al. 2010; Littlejohn and Love 2012; Littlejohn et al. 2014). In this reagent, leaves become clear in 5 min but there is no reduction in the content of chlorophyll, which interferes with the visualisation of fluorescent proteins. Warner et al. (2014) performed clearing of plant tissues using Scale-based clearing reagents (urea, glycerol, Triton X-100). It took 1–3 weeks for leaves and root nodules to become transparent using these reagents. Hasegawa et al. (2016) and Musielak et al. (2016) used 2,2’-thiodiethanol to rapidly (1 h to 1 day) reduce the refractive index mismatch in plant tissues. These reagents can make plant tissues transparent, but the transparency is not sufficient to perform imaging of deep-seated structures in plants because they cannot remove chlorophyll (which causes autofluorescence). We also developed a clearing reagent, ClearSee, composed of urea, xylitol, and sodium deoxycholate, to perform whole tissue imaging using fluorescent proteins, in tissues, such as roots, leaves, seedlings, and pistils (Kurihara et al. 2015). Although this is a useful method for deep imaging of plants by removing chlorophyll, 3 days to 1 month are required to achieve transparency. ClearSee is applicable to a wide range of plant species, such as *Arabidopsis thaliana*, *Physcomitrella patens* (Kurihara et al. 2015), *Astragalus sinicus* (Ohtsu et al. 2017), avocado (Duman et al. 2020), barley (Ho et al. 2020), *Brassica rapa* (Arsovski 2020), *Eucalyptus* (Eliyahu et al. 2020), maize (Kelliher et al. 2017), *Marchantia polymorpha* (Aki et al. 2019), *Monophyllae glabra* (Kinoshita et al. 2020), petunia (Chen et al. 2020), rice (Chu et al. 2018), soybean (Okuda et al. 2017), strawberry (Kim et al. 2019), *Uromyces japonicus* (Tanaka and Ono 2018), wheat (Wu et al. 2019), and *Wolffiella hyalina* (Isoda and Oyama 2018), it is not applicable to all tissues and species.

In this study, we found that oxidation causes brown pigmentation to form in the tissue during ClearSee treatment. Some reductants prevented the formation of this brown pigment while
retaining the stability of the fluorescent proteins used for imaging. We developed a modified clearing reagent, named ClearSeeAlpha, to prevent oxidation during clearing treatment. Moreover, we analysed the stability of several fluorescent proteins, emitting fluorescence from blue to red, in ClearSee solutions and found that TagRFP was not suitable for imaging using this clearing treatment. These findings demonstrate that ClearSeeAlpha allows deep imaging of a broad range of plant species and tissues using selected fluorescent proteins.

RESULTS AND DISCUSSION

**ClearSee treatment induced proanthocyanadin derivative accumulation in some tissues**

Previously, we developed a clearing reagent, ClearSee, to make transparent plant specimens for fluorescent microscopic observation (Kurihara et al. 2015). In *A. thaliana*, ClearSee could clear the roots, leaves, seedlings, and pistils to maintain the stability of signals from fluorescent proteins. However, we found that the ClearSee-treated siliques contained brown pigment on the seed coat (Fig. 1A, Bright Field). The fluorescent signals were not detected in the regions of blown colouration, which instead appeared dark (Fig. 1A, H2B–mClover, Autofluorescence). Thus, this brown pigment interfered with the fluorescence imaging. The brown pigment on the seed coat was similar to that obtained with vanillin staining (Debeaujon et al. 2003). Vanillin staining can result in the visualisation of proanthocyanindins as brown pigment owing to its oxidation. Proanthocyanidin derivatives accumulate on the seed coat during seed maturation after fertilization (Debeaujon et al. 2003). This brown pigment was not detected in the unfertilized ovules.

To identify plant species in which brown pigment appeared upon ClearSee-treatment, we treated paraformaledehyde (PFA)-fixed leaves of different plants species with ClearSee. Like *A. thaliana*, leaves from most of the plants including *Torenia fournieri*, *Petunia x hybrida*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Cucumis sativus*, and *Oryza sativa* were cleared upon ClearSee-treatment (Fig. 1B). Thus, various plant species, including horticultural plants and crops, both dicot and monocot, were compatible with ClearSee. However, ClearSee could not clear the leaves of *Chamaecyparis obtusa* and *Cyrtomium fortunei* (Fig. 1C). Because the leaves of plants such as...
conifers and pteridophytes, are less permeable, the ClearSee solution could not penetrate inside the tissue. Moreover, leaves of Chrysanthemum morifolium and Perilla frutescens were not cleared even after 8 days of ClearSee treatment (Fig. 1C). The leaves retained green colour in PBS, whereas ClearSee-treated leaves displayed a brown pigment like in A. thaliana seeds. In tobacco, polyphenol oxidation by peroxidase causes brown pigmentation during leaf senescence (Sheen 1974). These results suggest that polyphenol, such as proanthocyanidin, oxidation induced the brown pigmentation observed in ClearSee-treated samples.

ClearSee consists of urea, xylitol, and sodium deoxycholate (Kurihara et al. 2015). Torenia and tobacco pistils were very prone to brown pigmentation (Fig. 2A). To determine which of these ClearSee components are involved in oxidation, we incubated Torenia and tobacco pistils in urea, xylitol, and sodium deoxycholate solutions. When incubated for 2 days, both Torenia and tobacco pistils turned brown in all the solutions (Fig. 2A). This indicates that oxidation occurred in each of the component of ClearSee. Moreover, precipitation was observed in the sodium deoxycholate solution (Fig. 2A). The absence of this precipitation in ClearSee suggests that sodium deoxycholate is homogenously dissolved in ClearSee.

Reductants prevent proanthocyanidin derivative accumulation in the leaf and ovary

Proanthocyanidins are colourless compounds which are converted into brown derivatives by oxidation during seed maturation (Debeaujon et al., 2003; Pourcel et al., 2006). We speculated that if reductants suppress oxidation, it could prevent brown pigmentation. To search for reductants that prevent oxidation in ClearSee solution, we incubated chrysanthemum leaves and tobacco ovaries in ClearSee solution containing some reductants (Fig. 2B,C). We selected seven reductants commonly used in biochemical experiments for our analysis (Table 1). Reduced glutathione was rejected because of its insolubility; TCEP-HCl was rejected because of its high cost. Tobacco ovaries were incubated with various concentrations of 1-thioglycerol (CS1), 2-mercaptoethanol (CS2), DTT (CS3), 2-aminoethanethiol hydrochloride (CS4), and sodium sulphite (CS5) (Table 1). At 1 and 10 mM, brown pigment was seen 2 days after treatment (2 DAT) of leaves in all the reductant solutions. In tobacco ovaries incubated with 50 mM
reductants, no brown pigment was observed at 2 DAT, and transparency was seen at 13 DAT. At 78 DAT, there was no brown pigment in ClearSee containing reductants, and the transparency was increased.

Similarly, when chrysanthemum leaves were incubated for 1 day, CS2 and CS5 solutions resulted in the elution of more chlorophyll in the solution; however, the leaves remained green (Fig. 2B; 1DAT). Further incubation resulted in the brown pigmentation of the leaves treated with ClearSee, but other leaves were pale green with reduced chlorophyll in CS1 to CS5 (Fig. 2B; 10DAT). These results indicate that each reductant’s addition can suppress the oxidation in ClearSee and prevent brown pigmentation of tissues.

**Modified ClearSee stabilizes the fluorescent proteins**

Next, to analyse the effect of reductants on the fluorescent proteins, purified fluorescent proteins were incubated in the modified ClearSee, and their fluorescence intensity was measured over time for 7 days (Fig. 3A). For CFP and sGFP, there was no significant change in fluorescence intensity in any of the modified ClearSee solutions (Fig. 3A; CFP, sGFP). In contrast, the fluorescence intensity of mApple and mCherry was decreased in some of the modified ClearSee solutions (Fig. 3A; mApple, mCherry). In CS2, the fluorescence was almost quenched after 1 day, and in CS1, the intensity was halved after 1–2 days. No significant differences were found for CS4 and CS5, while the fluorescence of mApple slightly decreased (Fig. 3A; mApple, mCherry). These results indicate that red fluorescent proteins are affected by reducing agents depending on their type.

To determine whether there is a difference in the effect of reductants on different types of fluorescent proteins in more detail, the effects of CS4 and CS5 were analysed (Fig. 3B). Among the 10 fluorescent proteins that emitted fluorescence of different colours from blue to red, only TagRFP emitted fluorescence with half the intensity in both CS4 and CS5 (Fig. 3B). However, no decrease in fluorescence intensity was observed for other fluorescent proteins, suggesting that CS4 and CS5 are useful for fluorescence imaging.

To determine whether there is a difference between CS4 and CS5, we treated *N. benthamiana* ovaries, *N. tabacum* pistils, and *A. thaliana* siliques (Fig. 4). In ClearSee solution, all the ovaries
showed brown pigmentation at 24 DAT. Although the surface of the ovary accumulated proanthocyanidin in \textit{N. benthamiana}, the placenta or the seeds inside the ovary accumulated proanthocyanidin in \textit{Torania}, and \textit{A. thaliana}, respectively (Fig. 4). In the case of CS4 and CS5, proanthocyanidin accumulation was inhibited in the treated samples (Fig. 4). There was no difference in transparency between the CS4 and CS5 treatments. The cost of sodium sulphate (CS5) compared with that of 2-aminoethanethiol hydrochloride (CS4) is 1/100; therefore, we preferred the use of CS5 as the new ClearSee reagent, which was named as ClearSeeAlpha.

\textbf{ClearSeeAlpha enables deep imaging}

It is challenging to observe the pollen tubes elongating within the pistils of \textit{N. benthamiana}. The style of \textit{N. benthamiana} is very long and brittle, making it difficult to handle (Fig. 5A). Although the pollen tube elongation was observed with aniline blue staining (Isogai et al. 2020), it is still difficult to follow how the pollen tubes elongate within intact pistils without dissecting the style. Therefore, to confirm whether pollen tubes in intact pistils can be observed using ClearSeeAlpha, we prepared \textit{AtUBQ10pro::sGFP} of \textit{N. benthamiana} to label the pollen tubes. At 24 h after pollinating with \textit{AtUBQ10pro::sGFP} pollen, the pistils were treated with ClearSeeAlpha for 3.5 months. Because \textit{N. benthamiana} turns brown as soon as it is injured, we handled it gently. In the case of ovaries, ClearSeeAlpha could not make the ovary wall perfectly clear even after 3.5 months (Fig. 5A). We obtained the merged images from 31 z-stacks with 10 \(\mu\)m intervals using 800 nm excitation. As shown in Fig. 5B, we could detect the elongated pollen tubes on the ovules by removing the ovary wall. However, autofluorescence for green (519–549 nm) and red (560–630 nm) channels was also detected in the ovary (white signals in Fig. 5B). The elongated pollen tubes were detected without dissecting the style (Fig. 5C). The \(yz\)-cross-section also showed elongated pollen tubes within the style (Fig. 5D).

In the case of the stigma, the nuclei and the pollen tube were clearly detected in a ClearSeeAlpha-treated, \textit{AtUBQ10pro::H2B–mClover} pistil (Fig. 6). We obtained the merged images from 88 z-stacks with 5 \(\mu\)m intervals using 920 nm excitation. As shown in Fig. 6A, we could detect the nuclei in the pollen grain, stigmatic papilla, and style. The nuclei at the opposite
side of the style from the objective lens were also detected in the ClearSeeAlpha-treated pistil (Fig. 6B). As shown in Fig. 6C, we were also able to detect the elongated pollen tube within the style in the ClearSeeAlpha-treated \( \text{LAT52pro::mTFP1} \) pistil, in which the cytosol is labeled (Nagahara et al., 2021). We obtained the merged images from 26 z-stacks with 12 \( \mu \text{m} \) intervals using 930 nm excitation. In \( \text{yz} \) view, the position of the pollen tubes in the transmitting tract could be observed (Fig. 6D). The pollen tube position at the top of the style was broader than that of the middle of the style (Fig. 5D, 6D). Thus, ClearSeeAlpha is useful for observing the pollen tube elongation within pistils.

**Toward further whole-plant imaging**

Because the style of \( \text{N. benthamiana} \) is easily broken and is difficult to use for semi-\textit{in vitro} assay, we had not been able to observe the elongation of the pollen tube towards the ovule. We could observe the pollen tube elongation within the style and on the ovule \textit{in vivo} by ClearSeeAlpha treatment. However, the ovary wall was not completely transparent and emitted red and green autofluorescence upon excitation at 800 nm; therefore, we should select the optimal fluorescent protein for labelling pollen tubes. Two-photon excitation microscopy of orange fluorescent proteins (FPs) at the near-infrared wavelength (1,000 nm) enables us to reduce the autofluorescence and to detect the labelled target proteins in \textit{Arabidopsis} tissues (Mizuta et al. 2015). TagRFP was not suitable for ClearSeeAlpha treatment (Fig. 3B). Other orange FPs, such as tdTomato and mApple, should be good candidates to label the protein of interest.

In \textit{Torenia}, ClearSeeAlpha could make the whole flower transparent. As shown in Supplementary Fig. S1, the petals and sepals were cleared after 3 months of ClearSeeAlpha treatment. The ovary remained slightly coloured, but most of the floral tissues were transparent. Although it takes a long time for the clearing treatment, ClearSeeAlpha was compatible for the clearing of large samples.

In this study, we developed a modified clearing solution, ClearSeeAlpha, to reduce the formation of brown pigment by preventing oxidation. The fluorescent proteins in the ClearSeeAlpha-treated samples are stable for more than a month (Kurihara et al. 2015). However, because
ClearSeeAlpha has a reducing agent, it is necessary to pay attention to the following points:

1. Reducing agent activity

Because the reducing agent is easily deactivated, it is better to change the ClearSeeAlpha solution every week. ClearSeeAlpha can be made quickly by adding a reducing agent to ClearSee.

2. The quality of sodium deoxycholate

One notable thing is that the performance of clearing with ClearSee and ClearSeeAlpha depends on the quality of sodium deoxycholate. Sodium deoxycholate purchased from some manufacturers has a pale yellow colour when dissolved, and interferes with optical clearing and fluorescence imaging.

3. The time required for clearing with ClearSeeAlpha

Because reducing agents suppress brown pigment, but do not accelerate chlorophyll removal, the time required for clearing with ClearSeeAlpha is the same as that with ClearSee. In *A. thaliana*, the required clearing times with ClearSee are four days for leaves and roots, seven days for seedlings, and four weeks for pistils (Kurihara et al., 2015). Tobacco and *Torenia* pistils also require one to two months to become transparent with ClearSeeAlpha. Note that larger tissues, such as *Chrysanthemum* leaves, require more time and more reagents (Fig. 2B).

Further optical clearing for whole-plant fluorescence imaging with ClearSeeAlpha will contribute to providing novel insights into the molecular mechanisms underlying various phenomena associated with plant development.

**MATERIALS AND METHODS**

**Plant materials and plant growth conditions**

The leaves of the following plants were used for leaf clearing: *Chamaecyparis obtusa*, *Chrysanthemum morifolium*, *Cucumis sativus*, *Cyrtomium fortunei*, *Nicotiana benthamiana*, *Nicotiana tabacum*, *Petunia x hybrida*, *Perilla frutescens*, and *Solanum lycopersicum* leaves were kindly provided by Dr. M. Notaguchi (Nagoya University). *Oryza sativa* leaf was kindly provided by Dr. R.D. Kasahara (Fujian Agriculture and Forestry University). We used *Arabidopsis*
thaliana accession Columbia (Col-0) and *Torenia fournieri* cv. ‘Blue and White’. The following transgenic lines were also used: *UBQ10p::H2B–mClover* of *A. thaliana* (Kurihara et al., 2015), *UBQ10p::sGFP, UBQ10p::H2B–mClover*, and *LAT52pro::mTFP1* of *N. benthamiana* (Nagahara et al., 2021).

*A. thaliana* seeds were sown on plates containing half-strength Murashige and Skoog salts (Duchefa Biochemie B.V., Haarlem, The Netherlands), 0.05% MES-KOH (pH 5.8), 1× Gamborg’s vitamin solution (Sigma, St. Louis, MO, USA), and 1% agar. The plates were incubated in a growth chamber at 22°C under continuous lighting after cold treatment at 4°C for 2–3 days. Two-week-old seedlings were transferred to the soil (Sakata no Tane; Sakata Seed, Yokohama, Japan) and grown at 21–25°C under long-day conditions (16-h light/8-h dark).

**ClearSeeAlpha protocol**

Tissue clearing was performed as described by Kurihara et al. (2015) with slightly modifications. ClearSee solutions were prepared by mixing xylitol powder [10% (w/v) final concentration; Wako, Osaka, Japan, 248-00545], sodium deoxycholate [15% (w/v) final concentration; TokyoChemical Industry, Tokyo, Japan, C0316], and urea [25% (w/v) final concentration; Wako, 211-01213] in water. The following reductants (50 mM final concentration) were used for screening: reduced glutathione (Wako, 071-02014); 1-thioglycerol (Nacalai Tesque, Kyoto, Japan, 33709-62); 2-mercaptoethanol (Wako, 131-14572); 1,4-dithiothreitol (Wako, 048-29224); 2-aminoethanethiol hydrochloride (Nacalai Tesque, 21419-32); sodium sulphite (Wako, 190-03411). The leaves were fixed in 4% (w/v) PFA (Wako, 162-16065) for 120 min in PBS under vacuum (690 mmHg) at room temperature. Fixed tissues were washed twice for 1 min in PBS and cleared with modified ClearSee at 4°C until clearing.

**Fluorescence stability**

The fluorescence stability of fluorescent proteins in modified ClearSee was measured with a
microplate reader (EnSpire; PerkinElmer, Waltham, MA, USA) as described previously (Kurihara et al. 2015). To prepare the recombinant fluorescent proteins, the full-length coding regions of fluorescent proteins (CFP, SECFP, mTFP1, sGFP, Venus, tdTomato, TagRFP, mApple, mRFP, mCherry) were cloned into the pCold I expression vector (Takara, Kyoto, Japan). The recombinant proteins were expressed in *Escherichia coli* strain Rosetta-gami2 (DE3) pLysS (Novegen, Darmstadt, Germany). After induction with 1 mM isopropyl-β-D-thiogalactopyranoside at 15°C overnight, cells were harvested and lysed in 20 mM phosphate buffer (pH 7.3) containing 500 mM NaCl (Wako, 191-01665), 5 mM imidazole (Wako, 097-05391), 1 mM 2-mercaptoethanol, 0.5% Tween-20 (Tokyo Chemical Industry, T0543), DNase (TURBO DNase, Invitrogen, Carlsbad, CA, USA), and cOmplete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). After sonication and centrifugation, the supernatants were collected and applied on TALON resin (Clontech, Mountain View, CA, USA). After washing with 20 mM phosphate buffer (pH 7.3) containing 500 mM NaCl, 5 mM imidazole, the recombinant proteins were eluted with 20 mM phosphate buffer (pH 7.3) containing 500 mM NaCl, 50 mM imidazole, 1 mM 2-mercaptoethanol. Purified fluorescent proteins were incubated in modified ClearSee solutions for 7 day and the fluorescence intensities were measured at excitation and emission wavelengths of 485 and 515 nm, respectively, at every hour.

**Microscopy settings**

For deep imaging, we used a laser scanning inverted microscope (A1R MP; Nikon, Tokyo, Japan) equipped with a Ti:sapphire femtosecond pulse laser (Mai Tai DeepSee; Spectra-Physics, Mountain View, CA, USA). The Z-stack images were acquired using a 25× water-immersion objective lens (CFI Apo LWD 25× WI, NA = 1.10, WD = 2.00 mm; Nikon). A Ti:sapphire laser was used for excitation of the mTFP1, sGFP, and mClover. Fluorescence signals were detected by the external non-descanned GaAsP PMT detectors. We used dichroic mirrors, DM495LP and DM560LP, and the following band-pass filters, 479/40 nm for mTFP1, 534/30 nm for mClover and sGFP, and 578/105 nm for autofluorescence. Images were processed with the NIS-Elements AR software 5.20.02 (Nikon) to create maximum-intensity projection images and to
add colour.

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**DISCLOSURES**

Conflicts of interest: No conflicts of interest declared.

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**TABLE 1.** List of reductants used for screening

| Reductant                          | Name | ¥/50ml for 50 mM |
|------------------------------------|------|------------------|
| 1-thioglycerol                     | CS1  | 147              |
| 2-mercaptoethanol                  | CS2  | 18               |
| DTT                                | CS3  | 879              |
| 2-aminoethanethiol hydrochloride   | CS4  | 106              |
| Sodium sulphite                    | CS5  | 1                |
| Reduced glutathione                | Not dissolved | 1106          |
| TCEP-HCl                           | Expensive | 7052          |
Legends to figures

**Fig. 1.** Optical clearing of tissues using ClearSee. (A) Fixed *UBQ10p::H2B–mClover* expressing pistil of *Arabidopsis thaliana* was incubated in ClearSee for 6 days. Arrowheads indicate brown pigment regions in the bright field image. (B,C) Fixed leaves of various species were incubated in phosphate-buffered saline or ClearSee for 8 days. The leaves were cleared (B) or not cleared in ClearSee. Scale bars: 500 \( \mu \)m (A), 1 mm (B, C).

**Fig. 2.** Effect of reductants on clearing using ClearSee. (A) The pistils of *Torenia fournieri* and *Nicotiana benthamiana* were incubated in ClearSee, 10% (w/v) xylitol, 25% (w/v) urea, or 15% (w/v) sodium deoxycholate for 2 days. (B) The leaves of *Chrysanthemum morifolium* were incubated in modified ClearSee series for 1 and 10 days. (C) The ovaries of *N. benthamiana* were incubated in modified ClearSee series for 2, 13, and 78 days. Scale bars: 5 mm (A,C), 1 cm (B).

**Fig. 3.** Stability of fluorescent proteins (FPs) in modified ClearSee series. Recombinant fluorescent proteins were incubated with modified ClearSee series. The fluorescent signal intensities were measured every hour over 7-day incubation. Each plot shows the fluorescent intensities relative to that in the case of PBS incubation (mean, n = 3). (A) CFP, sGFP, mApple, and mCherry were incubated in modified ClearSee series. The fluorescent intensities of mApple and mCherry were decreased in CS2 and CS3. (B) Different FPs were incubated in CS4 and CS5. The fluorescent intensity of TagRFP was decreased in CS4 and CS5.

**Fig. 4.** ClearSeeAlpha prevents the formation of brown pigment during the clearing process. The ovaries of *Nicotiana benthamiana*, the pistils of *Torenia fournieri*, and the siliques of *Arabidopsis thaliana* were incubated in modified ClearSee series for 24 days. Scale bars: 5 mm.

**Fig. 5.** Growth of a pollen tube in a pistil of *Nicotiana benthamiana*. (A) *N. benthamiana* pistil pollinated with *UBQ10pro::sGFP* pollen and treated with ClearSeeAlpha for 3.5 months. Cyan and orange dotted regions are magnified in (B) and (C, D), respectively. Reconstituted 3D images (B,D) or maximum intensity projection for the xy view (C) were generated from 31 z-stack images with 10 \( \mu \)m intervals by 2PEM with 800 nm excitation. Yellow arrowheads indicate the elongated pollen tubes on the ovaries (B) or in the style (C). Yellow dotted region indicates the transmitting tract (D). Scale bars: 2 mm (A), 100 \( \mu \)m (B,C,D).

**Fig. 6.** Cleared stigma of *Nicotiana benthamiana*. (A–D) *N. benthamiana* stigma of *UBQ10pro::H2B–mClover* (A,B) *LAT52pro::mTFP1* (C,D) treated with ClearSeeAlpha for one month. Cyan shows the signal of mTFP1 (C,D), green shows that of H2B–mClover (A,B) and magenta shows autofluorescence signals at 560–630 nm (A–D).
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