



A rapid and effective optical-clearing technique for deep tissue fluorescence imaging in trees

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Abstract

Key message This non-invasive clearing technique allows for deep clear imaging of plant tissue and 3D structural analysis of leaf cells.

Abstract Optical-clearing technology allows the reduction of light scattering and absorption in internal biological tissues. This technology allows microscopy techniques, such as confocal laser scanning microscopy, to obtain structural information deep within the internal tissue and also allows for three-dimensional (3D) tissue analysis. However, the transparent method in plants remains to be further elucidated. In this study, we first report on a non-invasive, rapid and effective clearing technique that increases the transmission of light through plants. Importantly, we found that the leaf terminal veins of four species had different characteristics based on fluorescence imaging observations. Moreover, through the combination of transparent technology and microscopic observation, we clearly observed the three-dimensional structure of plant leaf cells. Our simple and rapid clearing method is useful for fluorescence imaging the intact internal structure. Taken together, the results of this study will provide valuable information for further microscopic experimental investigations in trees.

Keywords Tissue optical clearing · Deep fluorescence imaging · Three-dimensional structure · Terminal veins

Introduction

Although some cellular and subcellular structures *in vivo* can be genetically labeled with fluorescent proteins (Knapp et al. 2012; Kost et al. 1998), sample opacity usually limits deep imaging of the internal tissue (Feijo et al. 2004; Paddock et al. 2014). For example, three-dimensional imaging of plants by confocal microscopy has been limited to semi-transparent tissue types such as root tips or meristems, but the resolution becomes limiting in cells deeper within

tissues. Light scattering and light absorption are the main causes of sample opacity (Ke et al. 2013). To overcome this problem, tissue impregnated with biocompatible molecular reagents can reduce the scattering coefficient and absorption coefficient (Tuchin 2005, 2009; Zhu et al. 2013). Reports of many investigations on the optical-clearing technology of different types of biological tissues and clearing agents are available in the literature (Bashkatov et al. 2009; Genina et al. 2005, 2006, 2008; Tuchin 2005, 2009; Tuchina et al. 2016; Wen et al. 2009; Zhu et al. 2013). Optical-clearing techniques, first proposed by Tuchin for tissues (Tuchin et al. 1997), can effectively reduce scattering by immersion of tissues in optical-clearing agents with high refractive indices and hyperosmolarity and can make tissue more transparent (McNichols et al. 2005; Tuchin et al. 1997, 2016, Tuchin 2005).

Typical optical-clearing agents usually have a high refractive index, so the penetration of optical-clearing reagents into the extracellular space matches the refractive index of the tissue, which, in turn, leads to a reduction in light scattering (Sdobnov et al. 2018; Zhu et al. 2013). This process leads to a significantly enhanced imaging depth as light penetrates deeper into the tissues (Tuchina

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et al. 2016). Various optical-clearing methods have been developed to achieve refractive index uniformity of the entire sample by either replacing water with a higher refractive index chemical agent or changing the structure of tissue components (Wen et al. 2009). In addition to this, it is important to select the working distance of the objective lens and the correct coverslip thickness to achieve refractive index uniformity of the entire sample.

Tissue optical clearing has become one of the key studies in biomedical optical imaging and microscopic observation. Application of this powerful optical-clearing technology had been demonstrated in mouse embryo, mouse head, and humans (human cranial bone) (Bashkatov et al. 2009; Choi et al. 2005; Genina et al. 2008; Sudheendran et al. 2010; Vargas et al. 2008). The application of tissue optical clearing mainly depends on a variety of biocompatible chemical reagents (Genina et al. 2008; Tuchin et al. 1997; Tuchin 2005). The biocompatible chemical agents come in many forms, such as ethanol (Gardner 1975), chloral hydrate (Lersten 1986), glycerol (Cicchi et al. 2005; Genina et al. 2008; Wen et al. 2009), 1, 4-butanediol (Wen et al. 2009), xylitol, polyethylene glycol (Cicchi et al. 2005; Tuchin 2005), fructose (Feng et al. 2017; Ito et al. 2014), and glucose (Genina et al. 2006; Sudheendran et al. 2010; Tuchin et al. 1997). These sugar–alcohol optical reagents have been widely used to demonstrate different efficiencies in tissue optical clearing. In addition, organic acid (oleic acid) (Helmchen et al. 2005; Jahrling et al. 2010) and other organic solvents (dimethyl sulfoxide, DMSO) (Economo et al. 2016; Helmchen et al. 2005; Jahrling et al. 2010; Sudheendran et al. 2010) have been widely used for tissue optical clearing. In recent years, biocompatible clearing agents have been continuously studied and developed. Recently, an optical clearing agent, named Scale, was reported to be based on urea (Hama et al. 2011). Scale is capable of preserving fluorescent proteins, so this method enables deep imaging of mouse tissues (Hama et al. 2011). Nevertheless, Scale requires a long-term cleaning process, ranging from weeks to months (Ke et al. 2013). In addition, Scale-treated samples are very fragile, especially with increasing processing time (Hama et al. 2011). Many clearing agents have similar disadvantages.

In this paper, we developed a simple and rapid optical-clearing agent, namely 1% NaClO, which overcomes some limitations seen with other reported reagents, such as a long treatment time and tissue damage. Combined with optical microscopy, this method is useful for whole imaging of intact morphology. We further examined the characteristics of leaf terminal vein, as well as the 3D structure of plant cells. This will help to accelerate the discovery of this new field and will promote the application of transparent techniques in plant biological research.

Materials and methods

Plant materials and plant growth conditions

In this study, the species used were *Acer truncatum*, *Platanus occidentalis*, and *Robinia pseudoacacia*, on the campus of Beijing Forestry University, and *Arabidopsis thaliana*, planted in the greenhouse of Beijing Forestry University. The leaves of *Acer truncatum*, *Platanus occidentalis* and *Robinia pseudoacacia* were collected during spring, approximately between April and July. For all of the experiments, *Arabidopsis thaliana* accession Columbia (Col-0) was used as the wild type. *Arabidopsis thaliana* plants were grown in a glasshouse at Beijing Forestry University.

Reagent preparation

The clearing agent SeeDB (See Deep Brain) was a saturated solution of fructose (80.2% w/w) in water with 0.5% α -thioglycerol (Ke et al. 2013). The OCR (optical clearing reagent) was prepared by mixing 6 M urea, 30% (v/v) glycerol, and 0.1% (v/v) Triton X-100 in water (Warner et al. 2014). The concentration of NaOH was 10% (w/v) (Morley 1968). The original concentration of NaClO was 8% (w/v), and the 8% NaClO was diluted to 1%, 3% and 5% NaClO. The concentration of PI (propidium iodide) stain was 0.1 mg/mL. The concentration of Calcofluor white was 0.01% (w/v). A 0.1 M PBS (phosphate-buffered saline, pH 7.2) solution was prepared from 0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 and water in a ratio of 72:28:100 (Kurihara et al. 2015). A 0.1 M PBS (phosphate-buffered saline, pH 7.4) solution was prepared from 0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 and water in a ratio of 81:19:100 (Kurihara et al. 2015). FAA (formalin–acetic acid–alcohol) was composed of 5 mL 38% formaldehyde, 5 mL acetic acid and 90 mL 70% alcohol.

Sample preparation and tissue fixation

The four kinds of leaves were cleaned with tap water. The leaf discs were excised using a hole punch with a diameter of 1 cm. Discs were then immediately drop fixed in FAA and rinsed with PBS (pH 7.2) 3–4 times (Kurihara et al. 2015). The cleaned discs were washed successively with 75%, 85%, and 95% ethanol for 1 h, and they were dehydrated overnight with anhydrous ethanol. These processes increase disc strength.

Chemical screening and clearing

The first screening used four kinds of clearing agents: SeeDB (Ke et al. 2013), OCR (Warner et al. 2014), NaClO,

and NaOH (Morley 1968). The previously prepared four disc types were placed in new Petri dishes, and four kinds of clearing agents were added to different Petri dishes to completely immerse the discs. In addition to clearing the tissue, the treatment also removed chlorophyll, which has high autofluorescence. When PI stain was used, it could avoid the same fluorescence as chlorophyll. The discs were treated until clear in the dark for 1–3 days.

Staining

After the clearing treatment, the leaves of *Robinia pseudoacacia*, *Platanus occidentalis*, *Acer truncatum* and *Arabidopsis thaliana* were placed in a new 1.5-mL centrifuge tube, and 0.1 mg/mL of PI stain was added to submerge the leaves. The leaves were stained in the dark for 3–5 min and were washed with PBS (pH 7.4) (Palmer et al. 2015). The transparently treated *Robinia pseudoacacia* leaves were stained with 0.01% Calcofluor white for 2–4 min to view 3D structures (Palmer et al. 2015).

Imaging of cleared tissue

Four kinds of leaf discs were imaged against a patterned background before clearing. Samples were then imaged against the same background after 1 day of clearing. Leaves of the three tree species were fixed in PBS (pH 7.4), and were imaged with a Leica SP8 CLSM (Tsinghua University, Beijing, China) using a Leica 20 \times water immersion objective, laser (excitation wavelength: 488–493; emission wavelength: 620–660) and hybrid detectors with a resolution of 1024 \times 1024 pixels. All images shown in this article are original images taken from the Leica SP8. Unprocessed 3D reconstruction was performed using Bitplane's Imaris 9.3.1 software.

Results

Comparison of clearing agents for plant leaves

To evaluate the effectiveness of the clearing agents, including SeeDB, OCR, NaClO, and the strong base-clearing agent NaOH, we selected four kinds of plant leaves for experiments from *Acer truncatum*, *Platanus occidentalis*, *Robinia pseudoacacia*, and *Arabidopsis* (Fig. 1a). After 1 day of treatment with the four clearing agents, leaves subjected to SeeDB, OCR, and 10% NaOH reagents were still green (Fig. 1a), whereas 8% NaClO-treated leaves did not contain green pigmentation and were transparent (Fig. 1a). After 5 days of treatment, 8% NaClO-treated leaves were still transparent, but the 10% NaOH reagent contained a small amount of pigment, and the leaves became yellow and were still

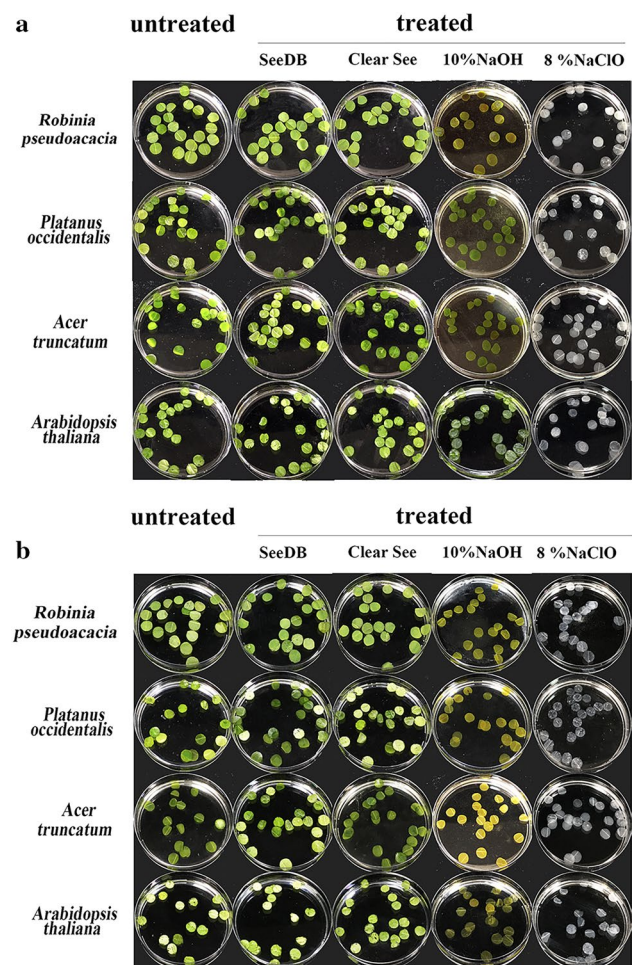


Fig. 1 Comparison of clearing effects of different clearing agents for four kinds of plant tissues. **a** Photos of four kinds of plant leaves taken before and 1 day after treatment with different clearing reagents. **b** Contrasting photos of four kinds of plant leaves taken before and after 5 days of treatment with four clearing agents

opaque (Fig. 1b). Surprisingly, the SeeDB and OCR-treated leaves were still green after 4 days, and the condition of the leaves was similar to that of the untreated state (Fig. 1b). Obviously, SeeDB and OCR had the poorest clearing effect under the same treatment time. The results showed that compared with the three other transparent reagents, 8% NaClO could rapidly clear the leaf tissues, significantly shortening the clearing time, and with an improved clearing effect.

Screening of NaClO concentration

According to the preliminary results of the clearing effects of the different clearing reagents, 8% NaClO had the best clearing effect and the shortest treatment time. Although 8% NaClO quickly cleared the materials, it caused a certain degree of damage to the materials with an increase in the processing time (Fig. 2a).

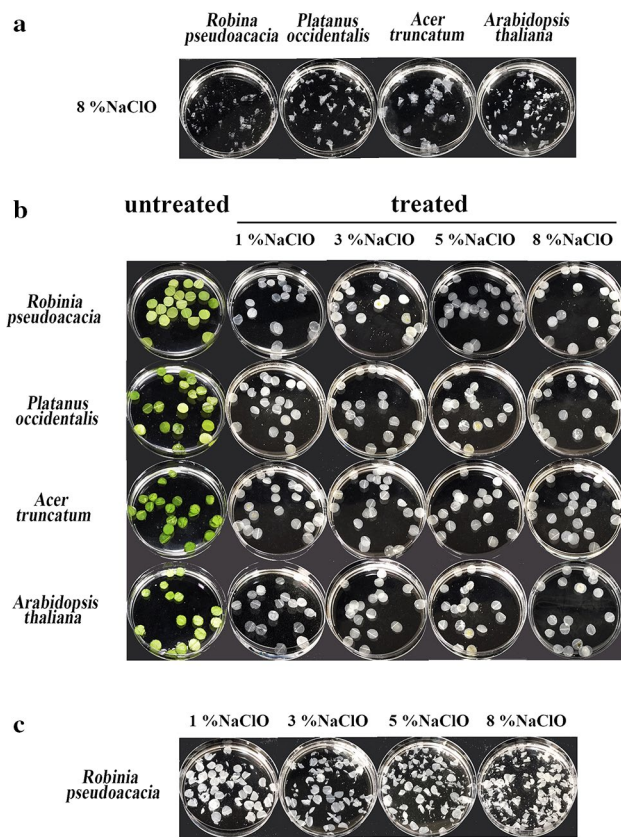


Fig. 2 Screening of NaClO concentration and comparison of damage levels. **a** The damage degree of four kinds of leaves treated with 8% NaClO for 20 days. **b** Results of four kinds of leaves treated with four different concentrations of NaClO for 1 day. **c** Comparison of the damage degree of *Robinia pseudoacacia* leaves treated with four concentrations of NaClO for 20 days

To screen the concentration of NaClO required for the optimal clearing effect, we set up a concentration series of 1%, 3%, 5%, and 8% (Fig. 2b). Similarly, we evaluated the clearing effect and integrity of the leaves from plant species treated with different concentrations of NaClO. After 1 day of treatment with the different concentrations of NaClO, the four leaf types were transparent (Fig. 2b). The four concentration gradients of NaClO had similar clearing effects on the four leaf types (Fig. 2b) indicating that NaClO is an effective clearing reagent. However, the degree of damage caused to the plant tissue also increased with the concentration and the processing time with NaClO treatment. The 8% NaClO treatment caused the greatest degree of damage to the *Robinia pseudoacacia* leaf tissue (Fig. 2c). Second, 5.0% NaClO caused a greater degree of damage to the leaves than 3.0% NaClO and 1% NaClO (Fig. 2c). Leaves treated with 3.0% NaClO were partially intact and partially destroyed (Fig. 2c). We found that 1% NaClO had an effective clearing effect, and the material had the highest integrity compared with other materials treated with other three concentrations

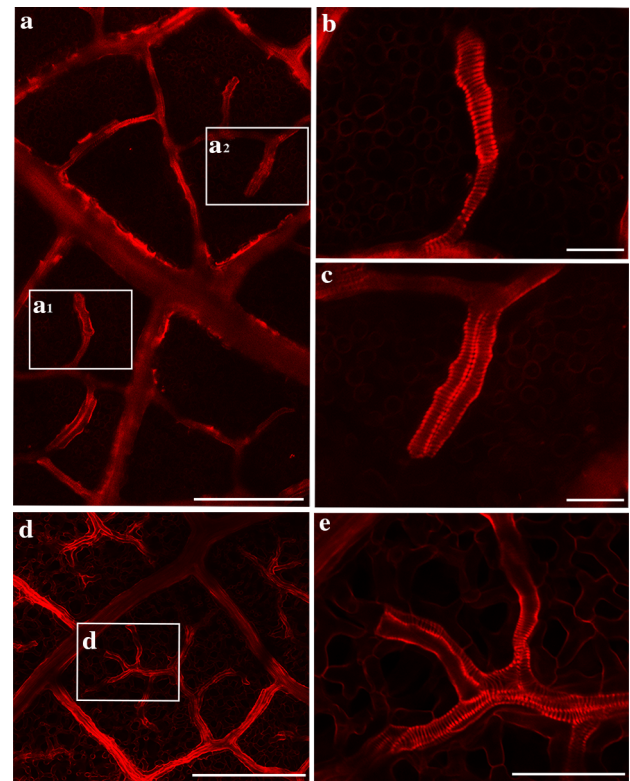


Fig. 3 Performance and imaging of terminal veins in the leaves of transparent *Robinia pseudoacacia* and *Platanus occidentalis*. **a** Imaging of terminal veins in leaves of *Robinia pseudoacacia* under $\times 20$ magnification. **b**, **c** The zoomed-in views of the white rectangular regions in **a**, represented as a_1 and a_2 . **d** Imaging of terminal veins in leaves of *Platanus occidentalis* under $\times 20$ magnification. **e** The zoomed-in views of the white rectangular regions in **d**, represented as d_1 . Scale bar: 150 μm (**a**, **d** and **e**); 40 μm (**b**, **c**)

of NaClO. The experimental results showed that the lower the NaClO concentration, the less the damage caused to the leaves.

Characteristics of terminal veins in leaves

After clearing, the leaf cells of *Robinia pseudoacacia*, *Platanus occidentalis*, *Acer truncatum* and *Arabidopsis thaliana* were stained with propidium iodide (PI) and were then observed under the laser scanning confocal microscope (LSCM). Interestingly, we clearly observed the vascular structure of the cleared leaves by PI staining, while the vascular structure was barely visible in untreated leaves (Fig. S1). Most importantly, we further found that the structure of terminal veins was different (Figs. 3, 4 and S2), and we clearly observed the annular thickenings in cell walls in veins (Fig. 3 and S2). The terminal veins in the leaves of *Robinia pseudoacacia* may be classified into two types (Fig. 3a): unbranched (Fig. 3b); the other has two terminal veins side by side (Fig. 3c). Compared

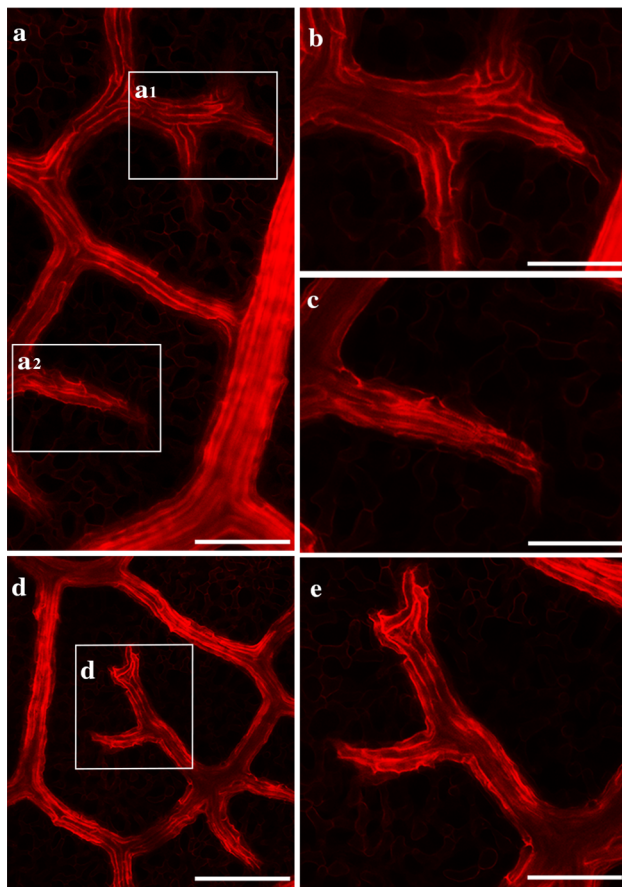


Fig. 4 Imaging and characteristics of terminal veins in the leaves of transparent *Acer truncatum*. **a, d** imaging of terminal veins in leaves of *Acer truncatum* under $\times 20$ magnification. **b, c** The zoomed-in views of the white rectangular regions in **a**, represented as a_1 and a_2 . **e** The zoomed-in views of the white rectangular regions in **d**, represented as d_1 . Scale bar: $75\ \mu\text{m}$ (**a, d**); $40\ \mu\text{m}$ (**b, c, e**)

to the leaf terminal veins of *Robinia pseudoacacia*, the terminal veins in the leaves of *Platanus occidentalis* were branched, and the number of terminal vein branches was also different (Fig. 3d). The branches of the terminal veins included one branch (Fig. 3e), two branches (Fig. 3e), and many branches (Fig. 3e), resembling an umbrella-like distribution. It is worth mentioning that the terminal veins in the leaves of *Acer truncatum* were different from those in *Robinia pseudoacacia* and *Platanus occidentalis* (Fig. 4a, d). The terminal veins included three types: the terminal veins had many branches (Fig. 4b); the terminal veins had no branches and existed alone (Fig. 4c); the terminal veins had two branches, and the two branches were similar in size (Fig. 4e). Using LSCM, we observed that the leaf terminal veins of *Arabidopsis thaliana* were elongated and had no branches (Fig. S2). The results showed that the terminal veins of different species were different, and they all had their own unique characteristics.

3D structure of *Robinia pseudoacacia* leaf cells

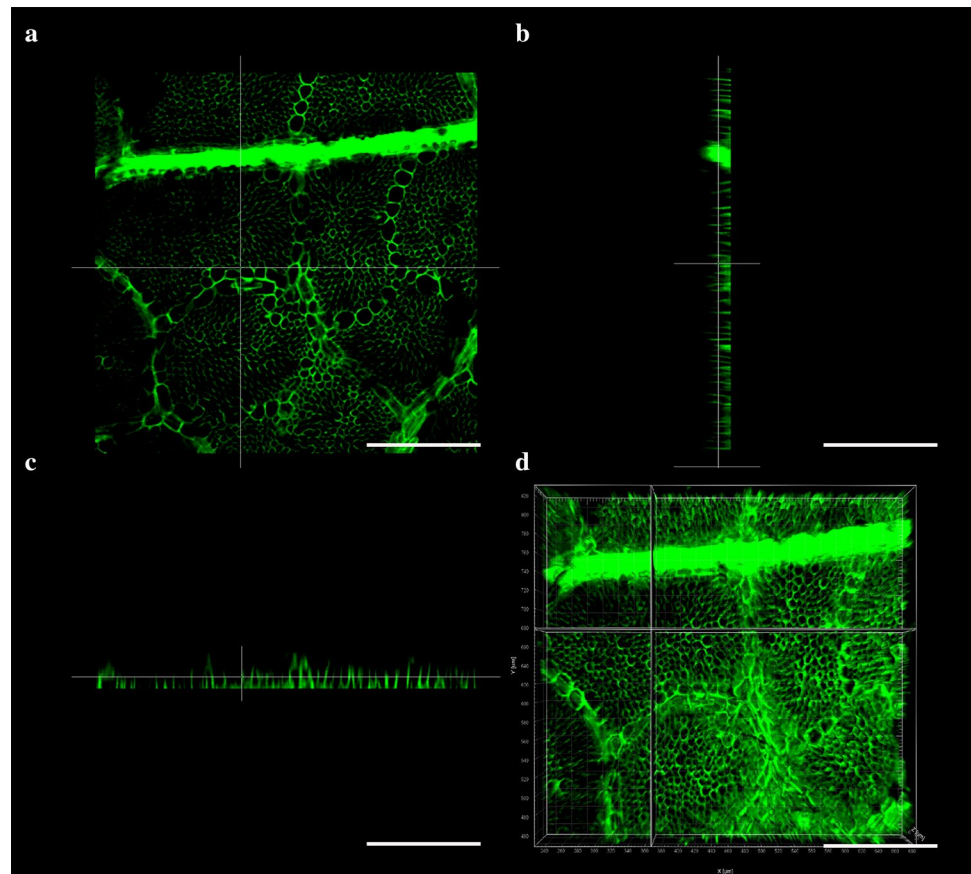
To better understand the cellular structure of *Robinia pseudoacacia* leaves, we stained the transparently treated leaves with Calcofluor white and observed them using CLSM. As shown in Fig. 5, we can not only observe the morphology of leaf mesophyll cells but also clearly observe the arrangement of vascular tissue in the leaf (Fig. 5a–c). Most importantly, the intact internal structures of the leaf were clearly observed using 3D slice reconstruction (Fig. 5d). In general, the 3D fluorescence imaging of leaf cells demonstrated a more comprehensive, complete and visual three-dimensional representation, and 3D structural analysis can help us to better understand the leaf cellular structure and anatomy investigation in trees.

Discussion

Clearing technology has a wide range of uses in the teaching and research of plant biology. As a means of rapid sample preparation and observation, it not only greatly reduces the man power and material resources required for slicing technology (Warner et al. 2014), but it also facilitates the acquisition of three-dimensional images during observation (Kurihara et al. 2015). The most common approach to imaging is to make thin sections (Miyamichi et al. 2011; Zingg et al. 2014), which are valuable but often labor-intensive, time-consuming, and prone to misinterpretation due to the loss or deformation of sections. Thin sections also make image registration and volumetric reconstruction sophisticated and difficult (Steward et al. 2003). However, the clearing technique can be used to investigate plant specimens without destroying the overall structure. Most importantly, the clearing technique could allow deep and complete imaging of plant samples using an accessible formulation combined with fluorescent dyes. Moreover, the clearing technique allows a better visualization of details that can help reaching a more comprehensive understanding of the relationship between structure and function (Amitrano et al. 2019).

The development of clearing technology mainly depends on the range of clearing reagents. There are many types of clearing reagents (Supplementary Table. 1), such as "4 1/2" complex clearing agent, lactic acid–phenol (Grabe et al. 1998), chloral hydrate (Kurihara et al. 2015), xylene (Alwahaibi et al. 2018; Sermadi et al. 2014), clove oil (Adeniyi et al. 2016), cedar oil (Adeniyi et al. 2016), and aniline oil (Popham 1950). Chloral hydrate has been used for the preservation of specimens since the late-nineteenth century and has a high refractive index, which allows high penetration of light without scattering for a wide variety of plant tissues (Hoyer 1882; Villani et al. 2013). In the study, we selected four kinds of clearing reagents to study the clearing effects of

Fig. 5 Complete 3D projection of a cleared *Robinia pseudoacacia* leaf showing retention of the cellular structure. **d** CLSM 3D projection of a passively cleared leaf showing cell walls stained with Calcofluor white (green) and the 3D projection with the epidermal layer cut away. **a** The *x* slice in the 3D projection. **b** The *Y* slice in the 3D projection. **c** The *Y* slice in the 3D projection. Scale bars: 120 μm (**a–c**); 90 μm (**d**)



different materials, including SeeDB (Ke et al. 2013), OCR (Warner et al. 2014), NaOH (Morley 1968), and NaClO. A previous study showed that hypochlorite and phenol could be used to determine ammonia (Weatherburn 1967). Recently, hypochlorite was also used to remove DNA contamination from ancient bones and teeth (Petra et al. 2019). However, we have not found a report on sodium hypochlorite as a clearing agent. Based on the treatment results of different materials, we found that NaClO had an obvious clearing effect and took less time than the other clearing agents. However, the original concentration of NaClO damaged the plant materials. Therefore, selecting a suitable clearing agent and treatment time should be explored according to the characteristics of plant organs and the structure to be observed after clearing.

In previous studies, tissue treated with clearing agents had a longer clearing time. Even a short clearing time took 1–2 weeks, while longer ones took a month or more. Scale required 2 weeks for clearing fixed mouse brain samples (Hama et al. 2011). A nondestructive technique cleared specimens optically in as little as 1–3 weeks. ClearSee took 1 week to render whole *Arabidopsis* seedling optically transparent (Morley 1968). In addition, some clearing technologies are complex to operate (Kurihara et al. 2015). For example, SeeDB requires samples with long incubation periods in

fructose solution (> 7 days) (Ke et al. 2013). Samples treated with PEA-CLARITY (plant enzyme-assisted-CLARITY) were passively cleared at 37 °C with gentle agitation for 4–6 weeks (or until clear), and this method requires changing the clearing solution daily (Palmer et al. 2015). In this study, by comparing the clearing effect of different concentrations of NaClO, we found that 1% NaClO had the best clearing effect. The 1% NaClO not only meets the requirement of a short clearing time but also causes less damage to tissue compared with the original concentration.

The development of microscopy has greatly promoted the application of clearing technology (Warner et al. 2014). The combination of laser scanning confocal microscopy and a clearing technique was used to study 3D molecular imaging of a cleared *N. tabacum* leaf (Palmer et al. 2015). Moreover, the combination was helpful for clear imaging and 3D reconstruction. In this study, we can clearly observe the terminal veins of plant leaves using fluorescence imaging and can distinguish the characteristics of terminal veins in different plants, providing a reference for plant taxonomy studies. Our clearing technique combined with fluorescence imaging allows for a more complete 3D imaging of cleared leaves.

In conclusion, we developed 1% NaClO as a clearing agent, which quickly removed colored substances in plant tissues and made them transparent. More importantly, the

clearing method is applicable to various types of plant leaves. It is believed that in the future, non-damaging clearing agents with better clearing effects will be discovered, which will lay a foundation for the wide application of clearing techniques. Moreover, the combination of new clearing techniques and higher resolution microscopy will provide more flexible and diverse methods for biology research in trees.

Author contribution statement LL and YC were the co-equal chief investigators of this study. QH participated in the experiments and modified the picture in the article. WX standardized the format of the references. YZ and LL offered some suggestions for the results. RL designed the experiments and revised the manuscript for submission.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Amitrano C, Arena C, Roupael Y, Pascale SD, Micco VD (2019) Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. *Ann Appl Biol* 175:313–325
- Adeniyi IM, Adejoba OR, Akinlabi FM, Alao OJ (2016) Vegetable oils as clearing agents. *Ach Life Sci* 10:1–4
- Alwahaibi N, Aljaradi S, Alazri H (2018) Alternative to xylene as a clearing agent in histopathology. *J Lab Phys* 10:189–193
- Bashkatov AN, Genina EA, Tuchin VV, Altshuler GB (2009) Skin optical clearing for improvement of laser tattoo removal. *Laser Phys* 19:1312–1322
- Choi B, Tsu L, Chen E, Ishak TS, Iskandar SM, Chess S, Nelson JS (2005) Determination of chemical agent optical clearing potential using in vitro human skin. *Laser Surg Med* 36:72–75
- Cicchini R, Sampson D, Massi D, Pavone F (2005) Contrast and depth enhancement in two-photon microscopy of human skin ex vivo by use of optical clearing agents. *Opt Express* 13:2337–2344
- Economu MN, Clack NG, Lavis LD, Gerfen CR, Svoboda K, Myers EW, Chandrashekar J (2016) A platform for brain-wide imaging and reconstruction of individual neurons. *Elife* 5:e10566
- Feijo JA, Moreno N (2004) Imaging plant cells by two-photon excitation. *Protoplasma* 223:1–32
- Feng W, Shi R, Zhang C, Zhu D (2017) In vivo skin optical clearing efficiency of sucrose and fructose. *J Opt Soc Am B*. <https://doi.org/10.1364/PIBM.2017.W3A.22>
- Gardner RO (1975) An overview of botanical clearing technique. *Stain Tech* 50:99–105
- Genina EA, Bashkatov AN, Kochubey VI, Tuchin VV (2005) Optical clearing of human dura mater. *Opt Spectrosc* 98:470–476
- Genina EA, Bashkatov AN, Sinichkin YP, Tuchin VV (2006) Optical clearing of the eye sclera in vivo caused by glucose. *Quantum Electron* 36:1119–1124
- Genina EA, Bashkatov AN, Korobko AA, Zubkova EA, Tuchin VV, Yaroslavsky I, Altshuler GB (2008) Optical clearing of human skin: comparative study of permeability and dehydration of intact and photothermally perforated skin. *J Biomed Opt* 13:1–8
- Grabe DF, Peters JA (1998) Lactic acid clearing of grass seeds in tetrazolium tests. *Seed Sci Technol* 20:106–108
- Hama H, Kurokawa H, Kawano H, Ando R, Shimogori T, Noda H, Fukami K, Sakaue-Sawano A, Miyawaki A (2011) Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. *Nat Neurosci* 14:1481–1488
- Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. *Nat Methods* 2:932–940
- Hoyer H (1882) Beiträge zur histologischen Technik. *Biol Centralbl* 2:23–24
- Ito M, Shirakawa R, Arita R, Karasawa Y, Imaki J, Amano S, Kobayashi Y, Takeuchi M (2014) Observation of whole-mount meibomian glands from cadaveric eyelids using a fructose-based optical clearing agent. *IVOS* 55:4442
- Jahrling N, Becker K, Schonbauer C, Schnorrer F, Dodt HU (2010) Three-dimensional reconstruction and segmentation of intact *Drosophila* by ultramicroscopy. *Front Neuroendocrin* 4:1
- Ke MT, Fujimoto S, Imai T (2013) SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. *Nat Neurosci* 16:1154–1161
- Knapp E, Flores R, Scheiblin D, Scheiblin D, Modla S, Czymmek K, Czymmek K, Yusibov V (2012) A cryohistological protocol for preparation of large plant tissue sections for screening intracellular fluorescent protein expression. *BioTechniques* 52:31–37
- Kost B, Spielhofer P, Chua NH (1998) A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J* 16:393–401
- Kurihara D, Mizuta Y, Sato Y, Higashiyama T (2015) ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development* 142:4168–4179
- Lersten NR (1986) Modified clearing method to show sieve tubes in minor veins of leaves. *Stain Tech* 61:231–234
- McNichols RJ, Fox MA, Gowda A, Tuya S, Bell B, Motamedi M (2005) Temporary dermal scatter reduction: quantitative assessment and implications for improved laser tattoo removal. *Laser Surg Med* 36:289–296
- Miyamichi K, Amat F, Moussavi F, Wang C, Wickersham I, Wall NR, Taniguchi H, Tasic B, Huang ZJ, He Z, Callaway EM, Horowitz MA, Luo L (2011) Cortical representations of olfactory input by trans-synaptic tracing. *Nature* 472:191–196
- Morley T (1968) Accelerated clearing of plant leaves by NaOH in association with oxygen. *Stain Technol* 433:15–19
- Paddock SW, Eliceiri KW (2014) Laser scanning confocal microscopy: history, applications, and related optical sectioning techniques. *Methods Mol Biol* 1075:9–47
- Palmer WM, Martin AP, Flynn JR, Reed SL, White RG, Furbank RT, Grof CPL (2015) PEA-CLARITY: 3D molecular imaging of whole plant organs. *Sci Rep* 5:13492
- Petra K, Matthias M (2019) Pretreatment: removing DNA contamination from ancient bones and teeth using sodium hypochlorite and phosphate. *Ancient DNA*. https://doi.org/10.1007/978-1-4939-9176-1_2
- Popham RE (1950) Cedarwood oil as a clearing agent with acetic alcohol fixatives. *Biotech Histochem* 25:112–113
- Sermadi W, Prabhu S, Acharya S, Javali SB (2014) Comparing the efficacy of coconut oil and xylene as a clearing agent in the histopathology laboratory. *J Oral Maxillofac Pathol* 18:S49–S53
- Sdobnov AY, Darvin ME, Geninab EA, Bashkatov AN, Lademann J, Tuchin VV (2018) Recent progress in tissue optical clearing

- for spectroscopic application. *Spectrochim Acta A Mol Biomol Spectrosc* 197:216–229
- Steward O, Zheng B, Tessier-Lavigne M (2003) False resurrections: distinguishing regenerated from spared axons in the injured central nervous system. *J Comp Neurol* 459:1–8
- Sudheendran N, Mohamed M, Ghosn MG, Tuchin VV, Larin KV (2010) Assessment of tissue optical clearing as a function of glucose concentration using optical coherence tomography. *J Innov Opt Heal Sci* 3:169–176
- Tuchina DK, Genin VD, Bashkatov AN, Genina EA, Tuchin VV (2016) Optical clearing of skin tissue ex vivo with polyethylene glycol. *Opt Spectrosc+* 120:28–37
- Tuchin VV, Maksimova IL, Zimnyakov DA, Kon IL, Mavlyutov AH, Mishin AA (1997) Light propagation in tissues with controlled optical properties. *J Biomed Opt* 2:401–417
- Tuchin VV (2005) Optical clearing of tissues and blood using the immersion method. *J Phys D Appl Phys* 38:2497–2518
- Tuchin VV (2009) *Hand book of optical sensing of glucose in biological fluids and tissues*. CRC Press, Boca Raton, p 709
- Vargas G, Barton JK, Welch AJ (2008) Use of hyperosmotic chemical agent to improve the laser treatment of cutaneous vascular lesions. *J Biomed Opt* 13:021114
- Villani TS, Koroch AR, Simon JE (2013) An improved clearing and mounting solution to replace chloral hydrate in microscopic applications. *Appl Plant Sci* 1:1300016
- Warner CA, Biedrzycki ML, Jacobs SS, Wisser RJ, Caplan JL, Sherrier DJ (2014) An optical clearing technique for plant tissues allowing deep imaging and compatible with fluorescence microscopy. *Plant Physiol* 166:1684–1687
- Weatherburn MW (1967) Phenol-hypochlorite reaction for determination of ammonia. *J Infect Dis* 39:971–974
- Wen X, Tuchin VV, Luo QM, Zhu D (2009) Controlling the scattering of Intralipid by using optical clearing agents. *Phys Med Biol* 54:6917–6930
- Zhu D, Larin KV, Luo Q, Tuchin VV (2013) Recent progress in tissue optical clearing. *Laser Photon Rev* 7:732–757
- Zingg B, Hintiryan H, Gou L, Song MY, Bay M, Bienkowski MS, Foster NN, Yamashita S, Bowman I, Toga AW, Dong HW (2014) Neural networks of the mouse neocortex. *Cell* 156:1096–1111

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