

Original Research

Establishment of an Efficient Protoplast Isolation and Transfection Method for *Eucommia ulmoides* Oliver

Bin Hu^{1,2}, Mingyang Dong^{1,2}, Ruonan Liu², Wen Shan², Yi Wang², Yang Ding², Jingyi Peng², Luyang Meng², Chaoyong Wang³, Qiang Zhou^{1,2,3,*}

¹Key Laboratory of Plant Resources Conservation and Utilization, College of Biological Resources and Environmental Sciences, Jishou University, 416000 Jishou, Hunan, China

²College of Biology and Environmental Sciences, Jishou University, 416000 Jishou, Hunan, China

³National and Local United Engineering Laboratory of Integrative Utilization of *Eucommia ulmoides*, 416000 Jishou, Hunan, China

*Correspondence: zhouqiang@jsu.edu.cn (Qiang Zhou)

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Abstract

Background: *Eucommia ulmoides* Oliver is a unique high-quality natural rubber tree species and rare medicinal tree species in China. The rapid characterization of *E. ulmoides* gene function has been severely hampered by the limitations of genetic transformation methods and breeding cycles. The polyethylene glycol (PEG)-mediated protoplast transformation system is a multifunctional and rapid tool for the analysis of functional genes *in vivo*, but it has not been established in *E. ulmoides*. **Methods:** In this study, a large number of highly active protoplasts were isolated from the stems of *E. ulmoides* seedlings by enzymatic digestion, and green fluorescent protein expression was facilitated using a PEG-mediated method. **Results:** Optimal enzymatic digestion occurred when the enzyme was digested for 10 h in an enzymatic solution containing 2.5% Cellulase R-10 (w/v), 0.6% Macerozyme R-10 (w/v), 2.5% pectinase (w/v), 0.5% hemicellulase (w/v), and 0.6 mol/L mannitol. The active protoplast yield under this condition was 1.13×10^6 protoplasts/g fresh weight, and the protoplast activity was as high as 94.84%. **Conclusions:** This study established the first protoplast isolation and transient transformation system in hard rubber wood, which lays the foundation for subsequent functional studies of *E. ulmoides* genes to achieve high-throughput analysis, and provides a reference for future gene function studies of medicinal and woody plants.

Keywords: *E. ulmoides*; stem; protoplast isolation; transient transformation

1. Introduction

Eucommia ulmoides Oliver is in the family Eucommiaceae and is a relict plant of the tertiary period [1]. It is distributed in the subtropical and temperate areas of China (24°50'N–41°50'N, 76°00'E–126°00'E), and is also a high-quality natural rubber tree species and a rare medicinal tree species unique to China [2]. As a national strategic reverse resource, *E. ulmoides* gum in *E. ulmoides* has the characteristics of radar penetration, energy storage, energy absorption, energy conversion, shock absorption, and shape memory, etc. [3–5], and has potential value in the fields of national defense and military, aviation and aerospace, transportation, precision instruments, and medicine. In addition, *E. ulmoides* contain lignins, iridoids, phenylpropanoids, flavonoids, and other active ingredients [6], and has medicinal value in regulating blood pressure, lowering blood sugar, and enhancing immunity. Therefore, *E. ulmoides* has great development prospects in the international market.

Historically, due to technological limitations, researchers have primarily concentrated on pharmacology [7,8] and resource collection [9,10] when studying and utilizing *E. ulmoides*, but have not searched for functional genes [11], which has severely limited the research of molecular breeding and related biological characteristics of

E. ulmoides. With the advancement of omics technology and the availability of *E. ulmoides* genome and transcriptome resources, there is a growing interest in identifying *E. ulmoides* functional genes.

However, genetic transformation technology still suffers from limitations of the cost of time, labor, and economy. For instance, a particle bombardment method and *Agrobacterium*-mediated method can establish a stable genetic transformation system in *E. ulmoides*, but they are limited by expensive equipment, low transformation efficiency, difficult genetic transformation, challenging *in vitro* regeneration, the long time required, and low functional expression efficiency of genes. These factors hinder the high-throughput analyses of functional genes.

Transient expression of protoplasts is a general and convenient technique for functional gene analysis. Protoplasts can be obtained by mechanical isolation or enzymolysis. Protoplasts can be used for high-throughput analysis of subcellular localization, gene function, promoter activity, protein interactions, hybridization, and gene expression. Currently, protoplast transformation systems have been established in a variety of commercial crops, including rice [12], sugarcane [13], perennial ryegrass [14], freesia [15], orchid [16], and soybean [17], of which some re-



sults have been achieved in the protoplast isolation of a few woody economic plants such as cassava [18], Magnolia [19], camellia oleifera [20], and poplar [21], indicating that the establishment of an efficient protoplast isolation method is feasible in the study of gene function of woody plants.

To the best of our knowledge, establishment of a protoplast transient expression system of *E. ulmoides* has not been reported. As the model plant of natural rubber and woody medicinal plants, it is necessary to establish a transient expression system, which is of great significance for the study of functional genes of *E. ulmoides*.

The aim of this study was to establish an efficient and simple protoplast isolation and transient transformation system for *E. ulmoides*. Efficient protoplasts were obtained by optimizing the factors that affect the yield and activity of protoplasts including the concentration of mannitol, enzymolysis time, and combination of enzymes. Furthermore, using green fluorescent protein (GFP) as the reporter gene, we verified the feasibility of the polyethylene glycol (PEG)-mediated protoplast transient expression system of *E. ulmoides*.

The results showed that the system is efficient and convenient, and can be used as a reference for future gene function studies of medicinal plants and hard rubber plants.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

The stems of *E. ulmoides* seedlings were selected for protoplast isolation in this study. Whole *E. ulmoides* seeds were soaked in 400 mg/L gibberellic acid 3 solution overnight. Then the seeds were transferred to a petri dish covered with wet absorbent paper, and grown in a dark incubator at 25 °C for 3 days. The budding *E. ulmoides* seeds were transferred to loose soil for 1 week of seedling cultivation. The *E. ulmoides* seedlings grew in a growth cabinet (RTOP-310Y; Zhejiang TOP Cloud-Agri Technology Co., Ltd., Zhejiang, China) at a temperature of 26/22 °C (day/night) and a 16/8 h light-dark cycle.

2.2 Plasmid Preparation

The *Escherichia coli* DH5 α was donated by the Beijing Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) and contained plasmid pCambia1303-mGFP5. The plasmid was extracted using the E.Z.N.A. Endofree Plasmid Mini Kit D6943-01B (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions.

2.3 Protoplast Isolation

One-week-old *E. ulmoides* seedlings collected were rinsed in pure water until the surface was clean, and only the stems of seedlings were used as material for protoplast isolation. Approximately 0.3 g stems were weighed and cut into 0.2–0.4 mm stem segments using a sharp razor. Then all stem segments were immediately transferred to a

3 mL enzyme solution (10 mmol/L MES [Macklin, Shanghai, China] KOH, pH 5.7, 0.22 mmol/L KH₂PO₄ [Macklin, Shanghai, China], 1 mmol/L KNO₃ [Macklin, Shanghai, China], 11.5 mmol/L CaCl₂ [Sigma, St. Louis, MO, USA], 1 mmol/L MgSO₄·7H₂O [Macklin, Shanghai, China], 1 μ mol/L KI [Macklin, Shanghai, China], 0.8 μ mol/L CuSO₄·5H₂O [Macklin, Shanghai, China], 1.5–3.5% [w/v] Cellulase R-10 [Yakult U.S.A. Inc., Fountain Valley, CA, USA], 0.3–0.9% [w/v] Macerozyme R-10 [Yakult Honsha Co., Ltd, Tokyo, Japan], 1.5–3.5% [w/v] pectinase [Shanghai Regal Biotech Co., Ltd, Shanghai, China], 0.5–2.5% [w/v] hemicellulases [Shanghai Ryon Biotech Co., Ltd, Shanghai, China], 0.1% [w/v] bovine serum albumin [Shanghai Ryon Biotech Co., Ltd, Shanghai, China], and 0.4–0.8 mol/L mannitol [Hefei BASF Biotech Co., Ltd, Hefei, China]). After 30 min of vacuum treatment (–0.85 MPa) in the dark, materials were enzymatically digested at 40–50 rpm at a constant temperature (25 °C) for 12 h in the dark. During enzymatic digestion, the stem segments were gently tapped 2–3 times with the back of a Pasteur pipette to accelerate the release of protoplasts. Then precooled W5 buffer (154 mmol/L NaCl, 125 mmol/L CaCl₂, 5 mmol/L KCl, 2 mmol/L MES KOH, pH 5.7) was added to stop the digestion and fix the volume. The digestion mixture was filtered through a clean 70 μ m nylon mesh sieve into a 10 mL round bottom centrifuge tube, and the volume was fixed to maximum scale. The mixture was centrifuged at 600 rpm for 5 min in the Benchtop Low Speed Centrifuge (TDZ5-WS; Cence, Hunan, China) and the supernatant was discarded. Next, the protoplasts were resuspended in 6 mL precooled W5 buffer, and centrifuged again at 600 rpm for 4 min. The supernatant was discarded to obtain the final purified protoplasts.

2.4 Protoplast Counting and Viability Assessment

The concentration of protoplasts was calculated with a hemocytometer (XB.K.25; QiuJing, Shanghai, China). Protoplast viability was measured by staining with Evans blue. Specifically, protoplasts and 0.25% Evans blue staining solution (Phygene, Fuzhou, China) were mixed in a 1:1 ratio and then incubated for 5 min at room temperature in the dark. Living protoplasts (unstained) and dead protoplasts (blue) were visualized with an optical microscope. Protoplast viability (%) = number of unstained protoplasts in view/total number of protoplasts in view $\times$ 100%. Each experiment had at least five replicate samples.

2.5 PEG-Mediated Protoplast Transfection

The harvested protoplasts were resuspended in MMG solution (0.6 mol/L mannitol, 15 mmol/L MgCl₂, 4 mmol/L MES KOH, pH 5.7) and diluted to $1.0\text{--}1.2 \times 10^6$ protoplasts/mL for PEG-mediated transfection. Then 30 μ L (0.9–1.1 μ g/ μ L) plasmid pCAM-BIA1303-mGFP5 was mixed with 120 μ L MMG protoplast resuspension solution in a round bottom centrifuge tube, and an equal vol-

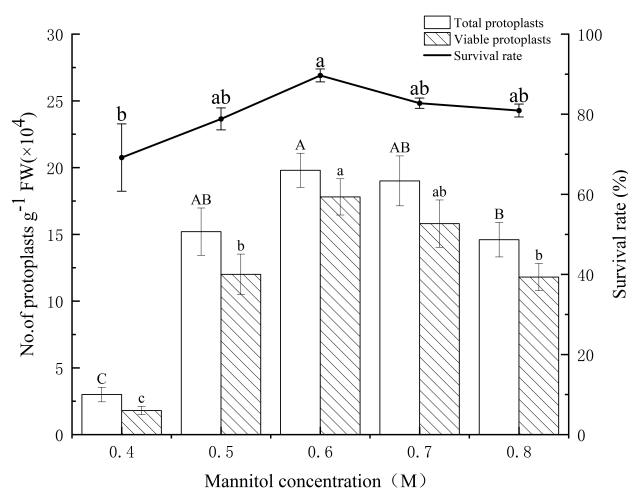


Fig. 1. Yield and survival rate of isolated protoplasts of *E. ulmoides* at different mannitol concentrations. The white and patterned bars indicate total yield of protoplasts and yield of viable protoplasts, respectively. The black solid line indicates the survival rate. Values indicate the mean $\pm$ standard error of the mean ($n = 5$). Different letters indicate significant differences at $p < 0.05$. FW, Fresh Weight.

ume (150 μ L) of PEG4000- Ca^{2+} solution (35% PEG4000; Sigma), 0.2 mol/L mannitol, and 0.1 mol/L CaCl_2 (Sigma) was added. After mixing, the solution was incubated for 5–20 min at room temperature in the dark. W5 was added to stop the transfection and the volume set to 3 mL. Protoplasts were harvested by centrifuging at 600 rpm for 3 min, followed by resuspending in 1 mL W5 buffer. After incubating for 15 h at 25 $^{\circ}\text{C}$ in the dark, GFP signals were detected using a confocal laser scanning microscope (LSM980; Zeiss, Oberkochen, Germany). The excitation wavelengths and emission filter sets were as follows: mGFP, 488 nm (Ex)/530 nm (Em).

3. Results

3.1 High-Efficiency Isolation of Protoplasts from *E. ulmoides* Seedlings

To establish a transient transformation system for high-throughput functional gene analysis in *E. ulmoides*, the selection of appropriate materials to obtain high-yielding and high-viability protoplasts is a crucial factor. Since woody plants contain high lignin, we selected stems of 1-week-old birth *E. ulmoides* seedlings as materials to assess the effect of protoplast isolation.

To obtain high quality protoplasts in good condition, different concentrations of mannitol were added to the enzyme solution containing 2.5% Cellulase R-10 (w/v), 0.6% Macerozyme R-10 (w/v), 2.5% pectinase (w/v), and 1.5% hemicellulase (w/v) to explore the effect of osmotic pressure on the quantity and quality of protoplasts. After 4 h of enzymatic digestion, the total number of protoplasts, number of viable protoplasts, and the survival rate were

counted (Fig. 1). Significantly, total protoplast yield (19.80×10^4 protoplasts g^{-1} Fresh Weight (FW)) and viable protoplast yield (17.80×10^4 protoplasts g^{-1} FW) peaked at 0.6 mol/L mannitol concentration, accompanied by the highest survival rate (89.68%). In addition, when observing the protoplast states (Fig. 2), the protoplasts at mannitol concentrations of 0.4 mol/L (Fig. 2A,B) and 0.5 mol/L (Fig. 2C,D) swelled and ruptured, and the enzymatic digest contained a large amount of broken protoplasts residue inclusions, while the protoplasts at 0.8 mol/L (Fig. 2I,J) mannitol concentration were significantly crumpled. The protoplasts can only maintain their normal morphology when the concentration of mannitol is 0.6 mol/L (Fig. 2E,F) and 0.7 mol/L (Fig. 2G,H). The production of total protoplasts and viable protoplasts at 0.6 mol/L mannitol concentration was more than the production at 0.7 mol/L mannitol concentration (Fig. 1). Thus, 0.6 mol/L was considered the most suitable concentration of mannitol for isolating protoplasts from the stems of *E. ulmoides* seedlings.

Next, we analyzed the effect of enzymatic digestion time (1–14 h) on the isolation efficiency of protoplasts from the stems of *E. ulmoides* seedlings. The experimental data (Fig. 3) showed that the total yield of protoplasts began to significantly increase in the 4th hour of enzymatic digestion and peaked at 10 h (92.80×10^4 protoplasts g^{-1} FW). Then, as the enzymatic digestion time increased, there was a slow downward trend. For the yield of viable protoplasts, the peak was also reached at 10 h (85.60×10^4 protoplasts g^{-1} FW), which was similar to the trend of total protoplast yield. However, there was no significant difference in protoplast survival rate (84.17–92.25%) within 14 h of enzymatic digestion. The results indicated that 10 h was the optimal time of enzymatic digestion for isolated protoplasts from the stems of *E. ulmoides* seedlings.

For the isolation of protoplasts, it is often not the most appropriate approach to obtain the optimal level of each factor separately by simply superimposing one-way analysis of variance. Therefore, we selected four enzymes, each of which had three concentration levels (Table 1), and performed a total of nine treatments with an orthogonal experimental design, as shown in Table 2. It can be seen that the highest yield of protoplasts (12.00×10^5 protoplasts/g FW) was obtained by enzymatic digestion in treatment No. 5 (Table 2), and the yield of this treatment was significantly higher than that of the other treatments. Although protoplast viability was the highest in treatment No. 4 (96.30%) (Table 2), there was no significant difference between this treatment and the other eight treatments, including treatment No. 5 (94.84%). Considering that the subsequent transient transfection required a large number of live protoplasts, we determined the yield of viable protoplasts in a final concentration of 0.125% Evans blue staining solution. The results showed that the yield reached a maximum of 11.33×10^5 protoplasts/g FW (Table 2) in treatment No. 5. Therefore, protoplasts were most suitable

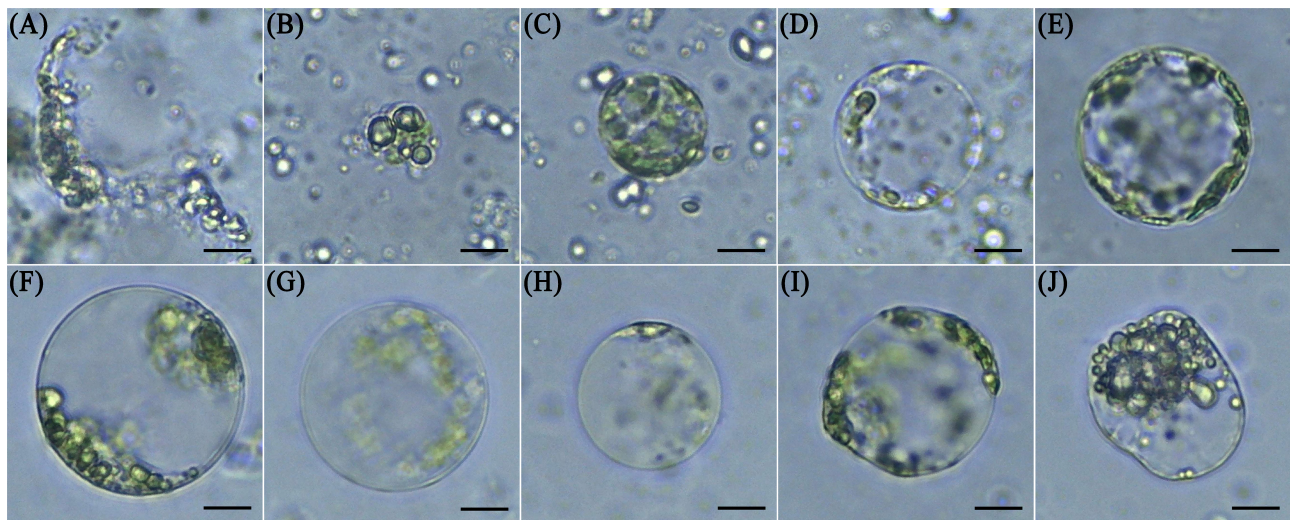


Fig. 2. Characterization of protoplasts in mannitol concentrations of 0.4 mol/L to 0.8 mol/L. Protoplast states in 0.4 mol/L mannitol (A,B). Protoplast states in 0.5 mol/L mannitol (C,D). Protoplast states in 0.6 mol/L mannitol (E,F). Protoplast states in 0.7 mol/L mannitol (G,H). Protoplast states in 0.8 mol/L mannitol (I,J). Scale bar, 15 µm.

Table 1. Enzyme concentration and enzyme ratios probed in protoplast isolation.

Combination	Cellulase R-10 (%)	Pectinase (%)	Macerozyme R-10 (%)	Hemicellulase (%)
1	1.5	1.5	0.3	0.5
2	2.5	2.5	0.6	1.5
3	3.5	3.5	0.9	2.5

for isolation from stems of *E. ulmoides* seedlings when the enzymatic solution contained 2.5% Cellulase R-10 (w/v), 0.6% Macerozyme R-10 (w/v), 2.5% pectinase (w/v), and 0.5% hemicellulase (w/v). Furthermore, the results of the range analysis showed that the four enzymes had different effects on the total yield and active yield of isolated protoplasts. The main factor influencing the yield of protoplasts was Macerozyme R-10, followed by Cellulase R-10, pectinase, and hemicellulases (Table 2). For active protoplast, the order of influence of these factors was Macerozyme R-10 > Cellulase R-10 > hemicellulase > pectinase (Table 2). The concentration of Macerozyme R-10 exerted the greatest effects on both total and active protoplast yield. In addition, the correlation analysis showed that the yield of live protoplasts was determined by the yield of protoplasts (correlation coefficient of 0.998; Table 3).

Overall, combined with the above optimized parameters, a large amount of protoplasts could be efficiently isolated from the stems of *E. ulmoides* seedlings. That is, the concentrations of Cellulase R-10, Macerozyme R-10, pectinase, hemicellulase, and mannitol in the enzymatic digestion solution were 2.5%, 0.6%, 2.5%, 0.5%, and 0.6 mol/L, respectively. The enzymatic digestion time was 10 h. The vast majority of protoplasts harvested by centrifugal precipitation were round (Fig. 4A), and Evans blue staining showed a high viability of protoplasts (Fig. 4B).

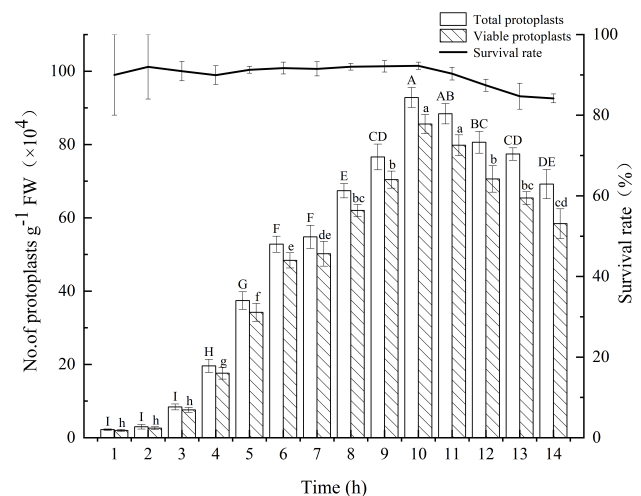


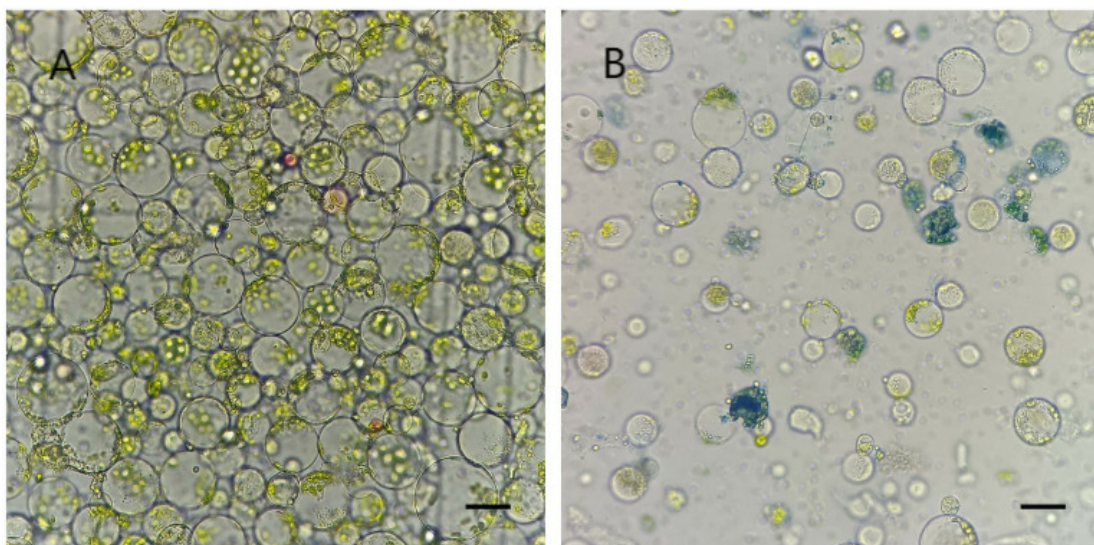
Fig. 3. Yield and survival rate of isolated protoplasts from *E. ulmoides* for different enzymatic digestion times. The white and patterned bars indicate total yield of protoplasts and yield of viable protoplasts, respectively. The black solid line indicates the survival rate. Values indicate the mean ± standard error of the mean (n = 5). Different letters indicate significant differences at $p < 0.05$. Differences in Total protoplasts are indicated by uppercase letters, differences in Viable protoplasts are indicated by lowercase letters, and there is no correlation between the two.

Table 2. Orthogonal experiment L9 (3⁴) affecting isolation efficiency of protoplasts from the stems of *E. ulmoides* seedlings.

Treatment No.	Cellulase R-10 (%)	Pectinase (%)	Macerozyme R-10 (%)	Hemicellulase (%)	Protoplast yield ($\times 10^5$ protoplasts·g FW ⁻¹)	Active protoplast yield ($\times 10^5$ protoplasts·g FW ⁻¹)	Protoplast viability (%)
1	1	1	1	1	4.00	3.33	85.00%
2	1	2	3	3	7.00	6.67	95.83%
3	1	3	2	2	8.33	7.67	92.13%
4	2	1	3	2	9.00	8.67	96.30%
5	2	2	2	1	12.00	11.33	94.84%
6	2	3	1	3	5.33	4.67	87.78%
7	3	1	2	3	5.33	4.67	87.78%
8	3	2	1	2	4.00	3.67	93.33%
9	3	3	3	1	5.67	5.00	87.78%
Protoplast yield		K1	19.33	18.33	13.33	21.67	
		K2	26.33	23.00	25.67	21.33	
		K3	15.00	19.33	21.67	17.67	
		Range	11.33	4.67	12.33	4.00	
		Rank	Macerozyme R-10>Cellulase R-10>Pectinase>Hemicellulase				
Active protoplast yield		K1	17.67	16.67	11.67	19.67	
		K2	24.67	21.67	23.67	20.00	
		K3	13.33	17.33	20.33	16.00	
		Range	11.33	5.00	12.00	4.00	
		Rank	Macerozyme R-10>Cellulase R-10>Hemicellulase>Pectinase				
Protoplast viability		K1	2.88	2.75	2.60	2.68	
		K2	2.76	2.72	2.83	2.82	
		K3	2.60	2.72	2.76	2.71	
		Range	0.28	0.03	0.23	0.14	
		Rank	Cellulase R-10>Macerozyme R-10>Hemicellulase>Pectinase				

Table 3. Correlation coefficients of various indicators for protoplasts isolation from *E. ulmoides*.

Category	Protoplasts yield	Active protoplasts yield	Protoplast viability
Protoplast yield	1		
Active protoplast yield	0.998	1	
Protoplast viability	0.711	0.752	1

**Fig. 4. Isolated protoplasts from the stems of *E. ulmoides* seedlings. (A) Morphological features of protoplasts observed under a light microscope. (B) Evans blue-stained inactive protoplasts under a light microscope. Scale bar, 20 μ m.**

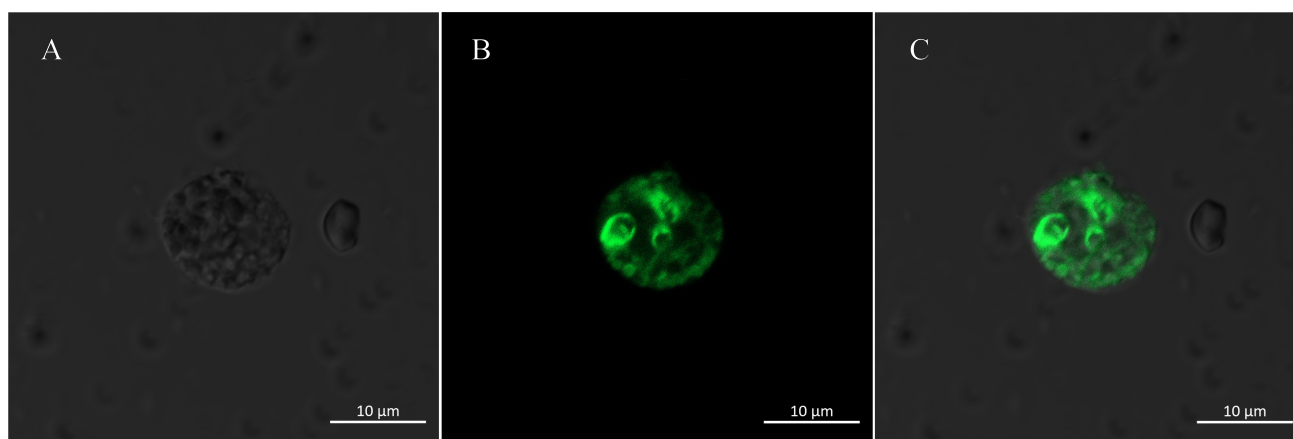


Fig. 5. pCambia1303-mGFP5 distribution in the protoplasts of *E. ulmoides*. (A) mGFP fluorescence image. (B) Bright field image. (C) Merged field image. Scale bar, 10 μ m.

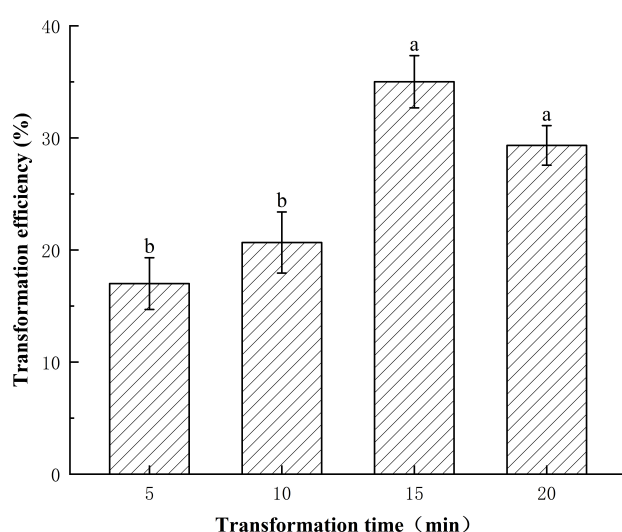


Fig. 6. Transfection efficiency of protoplasts of *E. ulmoides* at different transfection times. Transfection efficiency was calculated after 15 h of cultivation. Values indicate the mean $\pm$ standard error of the mean ($n = 3$), and different letters indicate significant differences at $p < 0.05$.

3.2 PEG Mediates Protoplast Transformation of *E. ulmoides* Seedlings

The feasibility of PEG-mediated transfection of the pCambia1303-mGFP5 plasmid into *E. ulmoides* was initially explored. The plasmid encoded mGFP under the control of CaMV35S promoter. The pCambia1303-mGFP5 was transfected at 25 $^{\circ}$ C in the dark in 35% PEG4000. GFP was detected in the cytoplasm with an obvious distribution (Fig. 5). Meanwhile, the effects of different transfection times on transfection efficiency were explored, and the results showed that the transformation efficiency was as high as 35.01% after 15 min of transfection (Fig. 6).

4. Discussion

Plant protoplasts can be isolated from plants' specific tissues or organs and can efficiently provide multiple independent single cell systems to solve specific problems because they retain their cellular properties [16]. It was once thought that the source tissues suitable for protoplast isolation were derived from non-seedling plants [22]. However, several studies in recent years have indicated that protoplasts can be isolated from young plant organs such as hypocotyls, leaves, roots, and root hairs [23], which provides a reference idea for the isolation of protoplasts from plants of different species.

The establishment of a stable protoplast isolation system is a prerequisite for generating a large number of protoplasts with high viability. The isolation of protoplasts is typically influenced by several factors including material selection, enzyme type and concentration, osmotic pressure, and enzymatic digestion time. Although efficient protoplast isolation systems have been reported in several herbaceous plants in recent years, isolation of sufficient amounts of live protoplasts in woody plants is still a challenge. This is especially true for *Eucommia*, which has great potential for exploitation because the whole plant, except the xylem, is rich in *Eucommia*-rubber (hard rubber), which is a major challenge for the isolation of *E. ulmoides* protoplasts.

Experiments have been reported on the selection of materials from plants from the outdoors that can affect the efficiency of protoplast isolation due to their thick cuticle and thick cell walls [24], but the preparation and supply of test tube seedlings often require skillful aseptic manipulation, economic cost, and time cost. With the aim of convenience, economy, and efficiency, we used stems from 1-week-old live seedlings of *E. ulmoides* cultured at a simulated room temperature, which were not lignified and contained less *Eucommia* rubber to conduct the study.

Free protoplasts are fragile when they have no cell wall to maintain their shape, and this fragility is reflected in the inability of protoplasts to remain isotonic with the external environment, resulting in swell, burst, or shrinkage of protoplasts. The addition of an appropriate concentration of osmotic stabilizer to the enzymatic solution can maintain the equilibrium of osmotic pressure in and out of the protoplasts. Most current protoplast isolation systems add mannitol and sorbitol to maintain the osmotic pressure of free protoplasts. The appropriate concentration of the osmotic stabilizer depends on the properties of the material itself. The results of several studies showed that appropriate addition of mannitol in the range of 0.4 mol/L to –0.8 mol/L can maintain the shape of protoplasts for a long time, depending on the material. Our results indicated that both hypoosmolarity and hyperosmolarity were detrimental to isolation of protoplasts from the stems of *E. ulmoides* seedlings. When the mannitol concentration was 0.4 mol/L to –0.5 mol/L, most of the protoplasts swelled and ruptured. When the mannitol concentration was greater than 0.6 mol/L, some of the protoplasts shrunk, which was not conducive to the progress of transient transformation. Therefore, the addition of 0.6 mol/L mannitol was determined to be the optimum concentration for protoplast isolation from the stems of *E. ulmoides* seedlings.

Enzymatic digestion time is one of the important factors in obtaining protoplast yield and viability, which varies according to different tissues and organs. When protoplasts were isolated from the leaf mesophyll of *Catalpa bungei*, the enzymatic digestion time was too short, resulting in inadequate enzymatic digestion of protoplasts, and the yield could not meet the need of instantaneous transformation, whereas excessive enzymatic digestion time could lead to a decrease of protoplast activity [23]. The experimental results suggested that the protoplast total yield and live protoplast yield showed a stepwise increase within 10 h, and peaked at 10 h. Therefore, enzymatic digestion time of 10 h is appropriate. Unexpectedly, we found that even though there was a decreasing trend in protoplast total yield and live protoplast yield after 10 h, the decrease in viability was not significant. Based on the comparison of previous methods with this study, it was speculated that the survival rate of protoplasts might be mainly influenced by the following factors: (i) the oscillation rate during enzymatic digestion; (ii) the state of the experimental material; and (iii) the composition of inorganic salts in the enzymatic solution.

The type and concentration of enzymes directly affect the preparation of protoplasts. Cellulases, pectinases, macerozymes, and hemicellulases are commonly used for the degradation of cell walls, and the chemical composition of cell walls varies depending on the species and material sources, so the appropriate enzyme combinations and enzyme concentrations for efficient preparation of protoplasts is not uniform. Protoplast yields of 6.5×10^5 protoplasts/g FW and 1.0×10^5 protoplasts/g FW were obtained

in the preparation of Pineapple leaves and Skunk cabbage leaves protoplasts, respectively [25,26]. Isolated different amounts of protoplasts from different organs of *Cymbidium* orchids tender leaf base, young leave, flower pedicels, root tips, and flower petals were 2.50×10^7 /g FW, 3.22×10^6 /g FW, 5.26×10^6 /g FW, 7.66×10^5 /g FW, and 3.3×10^7 /g FW, respectively [16]. These previous contributions indicate that increasing enzyme concentration resulted in increased protoplast production but reduced protoplast viability [24]. It was thought that this might be due to high concentrations of enzymes that disrupted the integrity of cell membrane, thus affecting the physiological activity of protoplasts. This study showed that the viability of prepared protoplasts from the stems of *E. ulmoides* seedlings did not significantly change after reaching a certain concentration, but the total protoplast yield and live protoplast yield showed a decreasing trend. We obtained the highest total protoplast yield (12.00×10^5 /g FW) and also the highest live protoplast yield (11.33×10^5 /g FW) using medium concentrations of enzymes, that is, 2.5% Cellulase R-10, 2.5% pectinase, 0.5% hemicellulase, and 0.6% Macerozyme R-10.

Establishing a stable genetic transformation system is an important constraint for genetic improvement of woody plants. Protoplast-based transient expression is a rapid, economical, and efficient alternative system compared to the traditional stable expression of transgenes, and is important for the analysis of plant gene function. This system has proven to be a powerful experimental tool in molecular biology for a wide range of applications such as subcellular localization [27], promoter activity analysis [28], protein–protein interactions [29], protein–DNA interactions [30], protein-based biochemical assays [31] and quantitative real-time polymerase chain reaction (QRT-PCR) for gene expression analysis.

In this study, a PEG-mediated transient protoplast transformation method based on the protoplast isolation system was established for stems of *E. ulmoides* seedlings, using *mGFP* as a reporter gene, and the feasibility of the system was verified. At the same time, the effect of transformation time on transformation efficiency was initially investigated by taking the transformation time as an independent variable. Notably, even though 15 min in 35% PEG solution was beneficial for transformation efficiency (up to 35.01%), similar to the results of other studies, there are still optimization factors in our transformation system and the efficiency may be further improved.

5. Conclusions

In this study, we utilized the stems of *E. ulmoides* seedlings as the material and systematically investigated 9 enzyme digestion solutions, 5 mannitol concentrations, and 14 durations, leading to the development of an optimized protocol for efficient isolation of *E. ulmoides* protoplasts. The optimized enzyme solution, composed of 2.5% Cellu-

lase R-10 (w/v), 0.6% Macerozyme R-10 (w/v), 2.5% pectinase (w/v), 0.5% hemicellulase (w/v), and 0.6 mol/L mannitol, enabled the generation of 1.13×10^6 active protoplasts/g FW within a 10 h enzymatic digestion with a viability up to 94.84%. Regarding PEG-mediated expression of GFP, among the four different transfection time periods tested, a 15 min duration yielded optimum transfection efficiency of 35.01%. This study not only established the optimal protocol for *E. ulmoides* protoplasts isolation but also confirmed the feasibility of a PEG-mediated transient transformation system for *E. ulmoides*.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results. The raw data are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization—BH, MD and QZ; Data acquisition—BH, RL, WS and JP; Formal analysis—YW, LM and YD; Methodology—BH, CW and QZ; Writing original draft—BH; Corresponding—QZ. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Eucommia ulmoides (Jishou University, Hunan, China) plants were used in this study. The plant materials were provided by Prof. Qiang Zhou (Jishou University, Hunan, China).

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Conflict of Interest

The authors declare no conflict of interest.

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