Isolation and Purification of Mesophyll Protoplasts from *Ginkgo biloba* L.

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Summary Techniques for protoplast isolation from various plants are widely applied. The purpose of this work is to develop an efficient system for isolating and purifying mesophyll protoplasts from a gymnosperm, *Ginkgo biloba* L. We tried to optimize main factors influencing the isolation of mesophyll protoplasts from *G. biloba*, including the enzyme type and concentration, incubation time, enzyme solution pH, osmotic pressure, nylon mesh size, and centrifugation speed. The suitable enzyme digestion system consisted of digestion by 2% (w/v) cellulase R-10, 0.2% (w/v) pectolyase Y-23 and 1.5% (w/v) macerozyme R-10 for 5 h in the dark at 25°C. After filtration through a 300-mesh nylon membrane and centrifugation at 50 G, the yield and viability of the obtained protoplasts reached approximately 5.39×10⁶ protoplasts g⁻¹ fresh weight (FW) and 80.23%, respectively. Then, the yield and viability of protoplasts isolated via the same method in two different years were compared under the optimal conditions. It proved that the protocol was repeatable and effective.

Keywords *Ginkgo biloba* L., Osmoticum, Protoplast isolation, Protoplast yield.

*Ginkgo biloba* L., the oldest relict plant of the existing seed plants, is the lone member of the family Ginkgoaceae, which has a history of over 170 million years (Uemura 1997). Therefore, it is easy to see why this gymnosperm is called an enigmatic "living fossil" (Shen et al. 2006). This tree has played an indispensable role in food, medicine, culture, and religion and has important scientific research value as a link between primitive nonflowering plants and advanced seed-producing plants. Since the completion of the complete genome sequencing of *G. biloba*, many functional genes have been identified, but due to the lack of a mature genetic transformation system, the research on the function of ginkgo gene can only be carried out indirectly by heterologous transformation of model plants (Yang et al. 2016). As a platform for the rapid analysis and detection of gene function, protoplasts can provide alternative methods for molecular biology research on ginkgo.

The protoplast is a spherical naked plant cell with totipotency, viability and a cell wall that has been enzymatically or mechanically removed; enzymatic hydrolysis is currently the most effective method for this removal and mechanical isolation is still important in history (Navrátilová 2004). The unique single-cell-based experimental system based on protoplasts is a useful tool for gene mapping (Eeckhaut et al. 2013), transgenic expression (Zhao et al. 2015), protein interaction analysis (Jung et al. 2015), light/chloroplast-related activity determination (Augustynowicz et al. 2009) and signaling transduction pathway investigation (Fraiture et al. 2014) in plant cell engineering applications. In previous reports, protocols for protoplast isolation have been very successful with model plant species such as wheat, maize, rice, Arabidopsis, and perennial ryegrass. However, the development of such techniques for woody plants has been much slower, especially for gymnosperms.

Many factors, including the material choice, osmotic strength, digestion period, purification method and others, affect the enzymatic digestion efficiency (Sinha et al. 2003, Reddy et al. 2008). Most researches have shown that the isolation conditions for protoplasts vary greatly among different plant species and even different tissues of the same species (Jones et al. 2012). For example, the protoplast isolation conditions, including the enzyme solution ratio and osmotic pressure, are different among *Solanum tuberosum* leaves and pollen, (Andersson et al. 2016, Wang et al. 2017). Different enzyme concentrations, enzyme types, reaction times and pretreatments were required for protoplast isolation from *Phaseolus vulgaris* flower petal, leaf, root, hypocotyl, and nodule samples (Nanjareddy et al. 2016). Therefore, it is generally necessary to systematically evaluate a protoplast isolation system separately for different plant materials.

In the present paper, we determine the main factors that affect the isolation and purification of mesophyll
protoplasts from the ginkgo, which include enzyme composition, osmotic pressure, digestion time, enzyme solution pH, nylon membrane mesh size, and centrifugation speed. The developed system is reliable, produces repeatable results and provides an experimental platform for research on ginkgo cells and metabolites to study gene function identification.

Materials and methods

Plant materials

Ginkgo seeds were obtained from G. biloba trees growing along a road at Northwest A&F University. The germinated seeds were grown in a 1:1:1 mixture of Hesha, peat soil, and seedling substrate in a greenhouse germinated seeds were grown in a 1:1:1 mixture of Hesha, peat soil, and seedling substrate in a greenhouse with 12 h of light per day under the same conditions (28°C and 65% humidity). Newly and healthily expanded leaves were collected after 3–4 weeks and then preserved at 2°C for 12 h (Dong et al. 2016). Immediately after, the lower epidermis of the preserved leaves (1 g) was removed, and the leaves were cut in longitudinal sections into approximately 1–2 mm strips with fresh sharp razors for protoplast isolation (Lin et al. 2018).

Protoplast isolation and purification

To prepare the enzyme solution, 10 mM KCl, 15 mM 2-(N-morpholino) ethanesulfonic acid (MES), different enzymes and mannitol were mixed and incubated at 55°C for 10 min, the pH of the solution was adjusted to a suitable value with KOH when the enzyme solution was cooled to room temperature, and 8 mM CaCl₂ and 0.1% BSA were added. Immediately after, the enzyme mixture was sterilized by filtration through a 0.45 µm Millex-HP filter (Millipore) into a 6-cm petri plate. The prepared leaves were transferred quickly into the enzyme solution and incubated in the dark at 25°C with gentle orbital shaking (50 rpm). To optimize the key parameters affecting protoplast isolation, the combination of enzyme solution (Table 1) at different digestion time (3, 4, 5, 6, 7, 8 h), osmotic pressures (0.5, 0.6, 0.7, 0.8, 0.9 M mannitol), and pH values (5.5, 5.6, 5.7, 5.8, 5.9) were compared.

After digestion, an equal volume of W5 solution was added to release the protoplasts, the enzyme mixture was immediately filtered through a nylon cell strainer of different sizes (200, 300, 400 and 500 mesh) to remove undigested leaf tissues, and then the filtrate was centrifuged for 3 min at different speeds (30, 40, 50, 60 and 70G). After washing twice with the W5 solution, the collected protoplasts were resuspended in 2 mL MMG solution (0.7 M mannitol, 15 mM MES, and 15 mM MgCl₂), the pH of the W5 solution and MMG solution were both 5.7.

Protoplast yield and viability determination

The protoplast yields were determined using a 0.1 mm hemocytometer, and the protoplast viability was determined via 0.01% (w/v) fluorescein diacetate (FDA) staining (Larkin 1976) under a fluorescence microscope (BX61, Olympus, Tokyo) with B excitation light. Active protoplasts are detected as yellow-green fluorescence. Protoplast yield (g⁻¹)= (total count in 5 medium squares/80×400×10⁴×dilution factor)×total leaf quantity (g). Original biomass viability (%)= number of green fluorescent protoplasts/total number of protoplasts×100%.

Statistical analyses

Three independent repetitions were performed for all treatments, and the results were calculated as the mean and standard error (SE). Statistical analysis of the data consisted of an analysis of variance (ANOVA) in SPSS (version 25.0). The same letter indicates no significant difference. Data with significant differences were determined at p<0.05. Heatmaps were generated using HemI software (version 10).

Results and discussion

Effects of enzymatic composition and digestion time on protoplast isolation

We evaluated the effects of nine enzyme compositions (A–I) with different digestion times (2–7 h) on the protoplast yield and viability, clearly visualizing the quantity and quality of protoplasts in heatmaps (Fig. 1). The protoplast yield varied from 4.1×10⁴ to 5.39×10⁶ g⁻¹ FW, and the viability ranged from 63.76 to 83.28%. The highest yield (5.39×10⁶ protoplasts g⁻¹ FW) was obtained using treatment H and digestion time of 5 h. The highest viability (81.80%) was obtained with treatment G and 6 h, but the viable protoplast yield was much lower than that of treatment H and 5 h. Interestingly, the yield and viability of protoplasts both increased first and then decreased with increasing digestion time for every enzyme composition. When the concentrations of the enzymes in solution were high, after 3 h, protoplasts started to be released, resulting in low yield but high viability. When the time exceeded 4 h, the color of the enzyme solution turned green, and at 6 h, a large

Table 1. Combinations of cellulase R-10, pectolyase Y-23 and macerozyme R-10 used in the enzymolysis solution.

<table>
<thead>
<tr>
<th>Treatment no.</th>
<th>Cellulase R-10 (%)</th>
<th>Pectolyase Y-23 (%)</th>
<th>Macerozyme R-10 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>1.0</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>D</td>
<td>1.5</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>E</td>
<td>1.5</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>F</td>
<td>1.5</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>G</td>
<td>2.0</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>H</td>
<td>2.0</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>I</td>
<td>2.0</td>
<td>0.3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(154 mM NaCl, 125 mM CaCl₂, 15 mM MES, and 5 mM KCl) was added to release the protoplasts, the enzyme mixture was immediately filtered through a nylon cell strainer of different sizes (200, 300, 400 and 500 mesh) to remove undigested leaf tissues, and then the filtrate was centrifuged for 3 min at different speeds (30, 40, 50, 60 and 70G). After washing twice with the W5 solution, the collected protoplasts were resuspended in 2 mL MMG solution (0.7 M mannitol, 15 mM MES, and 15 mM MgCl₂), the pH of the W5 solution and MMG solution were both 5.7.
amount of mesophyll cells became free. When the time exceeded 7 h, the released protoplasts became damaged and were gradually lysed. Therefore, the optimal conditions consisted of treatment H (2% cellulase R-10, 0.2% pectolyase Y-23 and 1.5% macerozyme R-10) with a 5 h digestion time.

The types and concentrations of enzymes best suited for protoplast isolation from ginkgo are very different from those from other plants, and the method should be optimized for different plant materials (Jones et al. 2012). For example, the optimal combination for Magnolia mesophyll protoplasts was 3% cellulase R-10, 0.8% macerozyme R-10, and 0.04% pectolyase Y-23 with a 6 h incubation (Shen et al. 2017). Leaves of Albizia julibrissin need to be digested in 1.5% cellulase Onozuka R-10 and 1% pectolyase Y-23 for 6 h (Rahmani et al. 2016), whereas Platycladus orientalis leaves need 16 h of incubation in 1.5% cellulase R-10, 0.4% macerozyme R-10, 0.4% pectolyase Y-23 and 1.0% ligninase (Zhou et al. 2019). These differences might be due to the differences in cell wall composition and biological activity of cells resulting from differences in the physiological characteristics of different plants and different growth environments.

**Effects of the osmotic stabilizer concentration and enzyme pH on protoplast isolation**

Under the optimal enzyme concentration and time, the effects of osmotic stabilizer concentration and enzyme pH on protoplast isolation were optimized. In this work, we chose mannitol to maintain the osmotic pressure. The protoplast yield and viability decreased as the mannitol concentration increased or decreased from the optimum mannitol concentration of 0.7 M (Fig. 2a) and peaked at 5.11×10⁶ protoplasts g⁻¹ FW and 81.43%, respectively. Below 0.7 M, some cells were round with a uniform cytoplasm distribution. When the concentration exceeded 0.8 M, the intracellular osmotic pressure was lower than the extracellular pressure, the cells dehydrated and shriveled, and protoplast viability was greatly reduced. Then, we investigated the influence of the pH of the enzyme solution on protoplast yield and viability. The variations in yield and viability with pH showed volcano-type relationships (Fig. 2b). pH 5.7 resulted in a significant (p<0.05) increase in protoplast yield (5.18×10⁶ protoplasts g⁻¹ FW) and viability (80.17%) compared to those at other pH values. The protoplast yields were less than 1.00×10⁶ protoplasts g⁻¹ FW in all pH optimization experiments. The pH of the enzyme solution may have less influence than other conditions on the experimental results. Therefore, 0.7 M mannitol and pH 5.7 were employed in the optimized method.

Without the protection of a cell wall, protoplasts are easily ruptured and aggregated, so it is essential to maintain a stable osmotic pressure inside and outside the cell. Sugar derivatives (sucrose, sorbitol, mannitol), inorganic salts (KCl, NaCl, MgSO₄) or combinations thereof are common osmotic pressure regulators. Mannitol is frequently used as an osmotic stabilizer because of its sta-
bility in enzyme solutions (Rezazadeh et al. 2011, Chen et al. 2015). Approximately 0.23–0.9 M mannitol was required to maintain appropriate osmotic pressure in a previous study (Gleddie 1995). The optimum pH of each enzyme is not the same. For example, the optimum pH of cellulase is generally 4.5–6.5, while that of pectolyase Y-23 is 3.0 (Duquenne et al. 2007). The amount of mannitol and the pH of the enzyme solution may differ depending on the type of original plant material. In this work, the optimal mannitol concentration and pH for ginkgo were 0.7 M and 5.7, which were different from the conditions for holm oak mesophyll protoplasts of 0.6 M and 5.0, respectively (Kuzminsky et al. 2016). Interestingly, leaves of Arbutus unedo collected in spring needed to be digested with 0.6 M mannitol, while those collected in November required 0.8 M mannitol to obtain partial protoplasts (Choury et al. 2017).

**Effects of the nylon mesh size and centrifugation speed on protoplast purification**

To remove undigested tissue and broken protoplasts and maximize the integrity of the protoplasts, we filtered and centrifuged the enzyme solution digested under the optimal conditions. First, we compared the effects of different nylon mesh sizes on the protoplast purification. It was demonstrated that the protoplast yield and viability first increased and then decreased with increasing pore size of the nylon mesh (Fig. 3a). The highest total yield (5.23 × 10⁶ protoplasts g⁻¹ FW) and viability (81.08%) were obtained with 300 mesh. Above 300 mesh, larger volumes of protoplasts could not be filtered, resulting in a significant decrease in the protoplast yield. Next, we evaluated the protoplast yield and viability after centrifugation speed (Fig. 3b). The yield and viability first increased and then decreased with increasing centrifugation speed. The highest yield (4.94 × 10⁶ protoplasts g⁻¹ FW) and viability (79.83%) were obtained at 400 g⁻¹. Above 400 g⁻¹, the yield and viability decreased significantly.

**Table 2. Variations in the yield and viability of G. biloba mesophyll protoplasts obtained with the same experimental protocol carried out at different times.**

<table>
<thead>
<tr>
<th>Date</th>
<th>Total protoplast yield (10⁶ g⁻¹ FW)</th>
<th>Viable protoplast yield (10⁶ g⁻¹ FW)</th>
<th>Protoplast viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>2018.05</td>
<td>5.39</td>
<td>0.11</td>
<td>4.32</td>
</tr>
<tr>
<td>2019.05</td>
<td>4.94</td>
<td>0.09</td>
<td>3.95</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effects of the nylon mesh size and centrifugation speed on protoplast purification. (a) Effects of the nylon membrane mesh size on the yield and viability of G. biloba mesophyll protoplasts. (b) Effects of the centrifugation speed on the yield and viability of G. biloba mesophyll protoplasts. Different letters represent significant differences at p<0.05, and bars represent SEs.

**Fig. 4.** G. biloba leaf mesophyll protoplasts: (a and d) raw material for protoplast isolation; (b and e) FDA-stained protoplasts under bright light; (c and f) FDA-stained protoplasts under ultraviolet light. a, b and c correspond to enzymatic hydrolysis of protoplasts carried out in May 2018, and c, d, and e correspond to May 2019.
igation of the filtered solution at different speeds. The protoplast quantity and quality first increased gradually and then decreased slightly with increasing centrifugation speed (Fig. 3b). The largest number of protoplasts (5.31×10^6 protoplasts g⁻¹ FW) and the highest viability (81.25%) were obtained at a centrifugation strength of 50G. The yield and viability gradually decreased as the speed increased further, and some intact protoplasts were also broken at excessively high centrifugation speeds. Accordingly, the optimal nylon mesh size and centrifugation speeds were 300 and 50G, respectively.

Many other methods of purification have also been reported, such as flotation on dense sucrose or Ficoll solutions (Cocking et al. 1974). However, the protoplasts purified via such methods are more viscous than those purified by filtration and centrifugation and cannot be used in additional experiments except in protoplast regeneration. At higher centrifugation speeds, protoplasts are more likely to break. Yu et al. (2017) reported that low-speed centrifugation could effectively avoid protoplast breakage, and Sun et al. (2018) indicated that the optimal centrifugation speed for protoplast purification from Chinese kale was 179×G.

Ginkgo mesophyll protoplast isolation and purification performed in two different years

Ginkgo protoplast isolation and purification with the optimized method was carried out in two different years (Fig. 4). The first year, newly and healthily expanded young leaves (Fig. 4a) of plants growing in a greenhouse were collected for protoplast isolation. The prepared protoplasts were spherical and varied in size due to the variable presence of a complete cell membrane at different locations on the leaves; the protoplasts appeared green under UV light (300–400 nm) (Fig. 4b). After staining with FDA, the viable protoplasts turned green with excitation by blue light (450–520 nm) (Fig. 4c). To show the repeatability of the protocol, in the second year, we carried out experiments based on the results of the first year of this study. We used the material shown in Fig. 4d and obtained a large number of protoplasts after purification (Fig. 4e). The yellow–green fluorescence in Fig. 4f indicated that the mesophyll protoplasts had high viability. Table 2 also showed that high-quality protoplasts were obtained using this protocol. The protoplast yields are higher than those reported for female haploid protoplasts isolated from G. biloba (Laurain et al. 1993).

In fact, the optimization of the conditions affecting protoplast isolation is empirical and needs to be carried out for each type of raw material. It is difficult to efficiently and reproducibly obtain protoplasts from tree species (Redenbaugh et al. 1981) potentially because the exposure of leaves to the environment or the aging of leaves may cause some compounds, such as phenylpropanoids, to increase, in plant tissues, as these processes increase the concentrations of lignin and polyphenols, which harden the cell wall and confer resistance to enzymatic digestion (Ximenes et al. 2010).

In conclusion, we have shown a case study for the isolation of G. biloba mesophyll protoplasts with high viability and yield using digestion by 2.0% cellulase R-10, 0.1% pectolyase Y-23 and 1.5% macerozyme R-10 for 5 h in a solution containing 0.7 M mannitol. The solution pH was 5.7, the optimal nylon membrane pore size was 300 mesh, and the centrifugation speed was 50×G. The established protocol could enable further studies based on protoplasts that may include culture of isolated protoplasts and even the analysis of functional genes.

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References


