Preparation and Optimization of Leaf Protoplasts from *Salsola laricifolia* (Postprint)

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Abstract

Releasing high-quality protoplasts from plant tissues is a prerequisite for establishing other technical systems such as protein subcellular localization, transient expression analysis, and protein-protein interactions. To rapidly mine special functional genes in the C3-C4 intermediate plant *Salsola laricifolia*, establishing a simple and efficient method for preparing leaf protoplasts is essential. This study used true leaves from sterile tissue-cultured seedlings of *Salsola laricifolia* as material to analyze the effects of different concentration ratios of cellulase and macerozyme, as well as osmotic pressure, on protoplast isolation. The results showed that: using true leaves from 25-day-old sterile tissue-cultured seedlings, enzymatic digestion at 25 ℃ for 2 h in a digestion solution containing 2% cellulase + 0.5% macerozyme + 0.6 mol · L^{-1} mannitol, and purification with W5 solution at a rotation speed of 800 rpm · min^{-1}, the protoplast yield could reach 1.21$×10^{6}$ cells with a viability of 85%. Moreover, using the obtained *Salsola laricifolia* protoplasts as recipients, the pBI121-SaNADP-ME4-GFP plasmid vector was successfully transformed via PEG-mediated transformation, and SaNADP-ME4 was detected to be localized in chloroplasts. This study established an efficient preparation system for leaf protoplasts of *Salsola laricifolia*, laying a foundation for mining special gene functions in *Salsola laricifolia*.

Full Text

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Preparation and Optimization of Leaf Protoplasts from *Salsola laricifolia*

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Abstract

The release of high-quality protoplasts from plant tissues is a prerequisite for establishing technical systems such as protein subcellular localization, transient expression analysis, and protein-protein interaction studies. To rapidly explore the special functional genes of the C3-C4 intermediate plant *Salsola laricifolia*, it is essential to establish a simple and efficient method for preparing leaf protoplasts. In this study, the euphylla of sterile tissue culture seedlings of *S. laricifolia* were used as material to analyze the effects of different concentration ratios of cellulase and macerozyme, as well as osmotic pressure, on protoplast isolation. The results showed that using true leaves from 25-day-old sterile tissue culture seedlings, enzymatic digestion in an enzyme solution containing 2% cellulase + 0.5% macerozyme + 0.6 mol · L^{-1} mannitol at 25°C for 2 h, followed by purification at 800 rpm · min^{-1}, yielded protoplasts at **1.21$×10^{6}$** per 100 mg fresh weight with **85%** viability. Furthermore, using the obtained *S. laricifolia* protoplasts as receptors, the **pBI121-SaNADP-ME4-GFP** plasmid vector was successfully transformed via PEG-mediated transformation, and SaNADP-ME4 was detected to be localized in chloroplasts. This study establishes an efficient protoplast preparation system for *S. laricifolia* leaves, laying a foundation for mining the special gene functions of this species.

Keywords: *Salsola laricifolia*; leaf; protoplast; C3-C4 intermediate plant

Plant protoplasts refer to all structures of plant cells except the cell wall and are capable of certain life activities [1]. Since protoplasts lack the protection of cell walls, they can uptake exogenous substances such as plasmids and viruses [2],

enabling functional studies of exogenous genes, protein subcellular localization, and protein-protein interactions [3]. Consequently, protoplast preparation and transformation systems have been continuously developed. Although the preparation and transformation systems for model plants such as Arabidopsis and tobacco are well-established and readily accessible, gene expression in heterologous systems may differ from that in the native plant due to different genetic backgrounds [4]. While the principles of plant protoplast isolation are similar, there is no universal protoplast preparation and transformation system. Only a few researchers have established protoplast preparation and transformation systems suitable for angiosperm floral organs and fruits [5]. Therefore, establishing protoplast preparation and transformation systems for different species is crucial for validating and mining their own gene functions.

Protoplasts can be obtained through mechanical or enzymatic methods, with enzymatic digestion being the most widely used approach currently. Plant materials are placed in enzyme solutions that dissolve cell walls, releasing protoplasts after cell wall degradation [6]. Through continuous optimization of experimental conditions by researchers, the protoplast preparation technology, transformation, and regeneration systems for model plants such as *Arabidopsis thaliana* [7], tobacco (*Nicotiana tabacum*) [8], and rice (*Oryza sativa*) [9] have become relatively complete and widely applied. Non-model plants such as *Gerbera hybrida* [10], *Amaranthus tricolor* [11], *Camellia sinensis* [12], *Lolium perenne* [13], *Gossypium hirsutum* [14], *Vitis vinifera* [15], *Elaeis guineensis* [16], and *Prunus avium* [17] have also established protoplast preparation and transformation systems.

Salsola laricifolia belongs to the Salsoleae tribe of Chenopodiaceae family and is a small shrub mainly distributed in Mongolia, the former Soviet Union, Central Asia, and China [18], commonly found on gravelly slopes and in gravelly deserts [19]. Based on anatomical structure, ultrastructure, and enzyme activity indicators, *S. laricifolia* has been identified as a **C3-C4 intermediate plant** (an intermediate type between C3 and C4 photosynthesis) [20-22]. Studying the anatomical structure, physiological and biochemical characteristics, and special functional genes of C3-C4 intermediate plants helps explore the high photosynthetic efficiency mechanisms and evolutionary significance of plants. Therefore, *S. laricifolia* serves as a typical C3-C4 intermediate desert plant. Establishing a complete protoplast preparation system is a prerequisite for building other technical systems (such as protein subcellular localization, transient expression analysis, and protein-protein interaction studies), which is of great significance for mining the special functional genes and exploring the evolutionary implications of this species.

This study used the euphylla of sterile tissue culture seedlings of *S. laricifolia* as experimental material to conduct orthogonal experiments on four factors: cellulase concentration, macerozyme concentration, mannitol concentration, and enzymatic hydrolysis time, investigating the effects of enzyme solution composition and digestion time on protoplast preparation. Based on these results, the

PEG-mediated method was used to verify the transformation efficiency of the *S. laricifolia* NADP-malic enzyme gene (**SaNADP-ME4**), aiming to establish an efficient protoplast preparation and transformation system for *S. laricifolia*.

1.4 Protoplast Isolation

True leaves from sterile tissue culture seedlings of *Salsola laricifolia* were longitudinally sectioned from the middle with a sterile scalpel in a laminar flow hood, placed in **0.7 mol · L^{-1}** mannitol solution for plasmolysis for **10 min**, then transferred to enzyme solution (composition as shown in) for dark enzymatic digestion for several hours (digestion time as shown in). After digestion, the solution was gently inverted several times to fully release protoplasts.

1.5 Protoplast Purification

The digested solution was filtered through a **75 m** mesh to remove residual undigested plant tissue. An equal volume of **W5** solution was added to terminate the enzymatic reaction (by cutting off the pipette tip and slowly adding along the tube wall). The mixture was centrifuged at **800 rpm · min^{-1}** for **5 min**; the supernatant was discarded, and the protoplast pellet was resuspended in W5 solution for washing. After centrifugation again, the supernatant was discarded, and the protoplast pellet was resuspended in **200-500 L** W5 solution (with gentle operation throughout to avoid vigorous shaking). The resulting *S. laricifolia* protoplast suspension was stored at **4°C** for later use.

1.6 Protoplast Viability Determination and Counting

Viability determination was performed using Evans blue staining: **50 L** of protoplast suspension was placed on a microscope slide, **20 L** of **0.25%** Evans blue stain was added, and after **5 min**, viability was assessed under an optical microscope. Viability was calculated as: (number of unstained protoplasts / total number of protoplasts) × 100%.

Yield calculation was performed using a hemocytometer: The hemocytometer was cleaned with ethanol solution, rinsed with water, and dried with filter paper. The objective and eyepiece of the optical microscope were cleaned with lens paper. A clean cover slip was placed over the counting chamber, and protoplast suspension (with pipette tip cut off) was added to one side, allowing it to seep into the chamber. After the protoplasts settled, counting began. For the **25$×16∗ ∗hemocytometer, $fivemediumsquares(upperleft, lowerleft, upperright, lowerright, andcenter)werecounted,$ ∗16∗∗$smallsquares(total$∗∗80∗∗$smallsquares).Thenumberofprotoplastsinthese∗ ∗80∗∗$smallsquareswascountedthreetimespersample.Thecalculationformulafortheh$25×$16 hemocytometer was applied [36]: Protoplast number = (number of protoplasts in 5 medium squares / 80) × 400 × 10^4 × dilution factor / 100 mg fresh weight.

1.7 Orthogonal Experiment for Protoplast Isolation

Based on preliminary experiments, a **four-factor three-level** orthogonal experimental design was adopted, with **9** treatment groups and **3** replicates per group, to compare the effects of cellulase concentration, macerozyme concentration, mannitol concentration, and enzymatic hydrolysis time on protoplast yield and viability from true leaves of *S. laricifolia* tissue culture seedlings ().

1.8 Transformation of Exogenous Genes in Protoplasts

The prepared protoplast solution was centrifuged at **800 rpm · min^{-1}** for **5 min**; the supernatant was discarded, and the protoplasts were resuspended in **1 mL MMG** solution (composition: **73.000 g · L^{-1}** mannitol, **0.853 g · L^{-1}** $MgCl_2$, **1.430 g · L^{-1}** MES, pH 5.8). For transformation using the widely applied and highly efficient **PEG** method [20]: **100 L** of protoplast solution was added to a **1.5 mL** tube, **10 L** of **pBI121-SaNADP-ME4-GFP** plasmid vector was added to the bottom and gently mixed by flicking the tube; **110 L** of **PEG** solution (composition: **400.000 g · L^{-1}** PEG4000, **36.500 g · L^{-1}** mannitol, **11.100 g · L^{-1}** $CaCl_2$, pH 5.8) was added, slowly inverted several times to mix, and incubated for **20 min** to complete transformation; **440 L** of **W5** solution was added to terminate the reaction and mixed well; centrifuged at **800 rpm · min^{-1}** for **3 min**; the supernatant was discarded, and the protoplasts were resuspended in **WI** solution (composition: **73.000 g · L^{-1}** mannitol, **0.853 g · L^{-1}** MES, pH 5.8) and incubated overnight in darkness; centrifuged at **800 rpm · min^{-1}** for **3 min**, the supernatant was discarded to concentrate the protoplast solution, and an appropriate amount was used for slide preparation. Protoplasts transformed with **pBI121-GFP** empty vector were used as controls, and GFP expression was observed under a laser confocal scanning microscope.

2.2 Effects of Enzyme Solution Composition, Mannitol Concentration, and Enzymatic Hydrolysis Time on Protoplast Yield and Viability

According to the orthogonal experimental design with four factors and three levels, protoplasts were isolated from true leaves of *S. laricifolia*, and yield and viability were statistically analyzed with range analysis performed on yield data (). The range analysis for protoplast yield showed that cellulase concentration had the highest range value, indicating that among the four experimental factors, cellulase concentration had the most significant effect on protoplast yield from *S. laricifolia* true leaves. Additionally, mannitol concentration had the second highest range value, indicating it is another important factor affecting protoplast yield. The range values for enzymatic hydrolysis time and macerozyme concentration were smaller, suggesting their effects on protoplast yield were less than those of cellulase and mannitol concentration under the tested conditions. For protoplast viability, enzymatic hydrolysis time showed the highest range

value, with mannitol concentration second, indicating that hydrolysis time had the greatest impact on viability, while osmotic pressure maintained by mannitol also significantly affected viability.

Analysis of yield and viability data from all orthogonal experimental groups showed that **group 9** achieved the highest protoplast yield, reaching **1.21$\times10^{6}$** per 100 mg fresh weight, with 85.0$\times10^{5}$ * * and * *6.99$\times10^{5}$ per 100 mg, respectively. Protoplasts from these three groups appeared as full spheres with clearly visible chloroplast **1}$ mannitol**, with **2 h** as the optimal enzymatic hydrolysis time.

Under an optical microscope, protoplasts from sterile tissue culture seedlings of *S. laricifolia* appeared as spherical bodies of varying sizes, smooth and full, with chloroplasts clearly distributed at the cell periphery ([Figure 2: see original paper]).

2.3 Transformation of Protoplasts

To verify that the prepared protoplasts could be used for transient transformation, protoplasts isolated under the optimal conditions were used as receptors. The plant transient expression vectors **pBI121-SaNADP-ME4-GFP** and **pBI121-GFP** empty vector (control) were introduced into protoplast cells via PEG-mediated transformation, incubated overnight at room temperature, and observed under a laser confocal scanning microscope for fluorescent signals of the SaNADP-ME4 protein. The results showed that protoplasts transformed with both plasmids appeared transparent and regular in shape without aggregation. When excited with blue light (**460-480 nm**), protoplasts carrying the **pBI121-SaNADP-ME4-GFP** plasmid showed green fluorescence in chloroplasts ([Figure 3: see original paper]), while protoplasts with the empty vector showed green fluorescence only in the cytoplasm without significant fluorescence in chloroplasts. In summary, the empty vector expressed GFP only in the cytoplasm, whereas the **pBI121-SaNADP-ME4-GFP** vector expressed fluorescence in chloroplasts, demonstrating that **SaNADP-ME4** is localized in chloroplasts.

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3 Discussion

Various factors affect protoplast yield and viability during isolation, including plant material selection, material pretreatment, enzyme solution composition, osmotic pressure selection, enzymatic hydrolysis time, purification speed, and temperature [6,30-31]. Plant material selection is fundamental for successful protoplast isolation. Young leaves are typically chosen due to simple sampling and abundant tissue cells, and sterile tissue culture seedlings can eliminate surface sterilization and reduce contamination risk [32]. This study used **25-day-old** sterile tissue culture seedling leaves. Material pretreatment greatly improves isolation efficiency; for example, sorghum leaves pretreated with mannitol solution for plasmolysis helps maintain osmotic pressure and improve isolation efficiency

[33]. This study used longitudinal sectioning of small leaves and plasmolysis pretreatment in **0.7 mol · L^{-1}** mannitol to increase yield.

Enzyme solution composition is a key factor for successful isolation [34]. Cellulase removes the main components of cell walls, while macerozyme is milder than pectinase for digesting pectin components in the cytoskeleton [35]. The combination of these two enzymes is most widely used [36]. However, due to differences in cell wall composition among plant species, the optimal concentration ratio varies. For example, *Gerbera hybrida* leaf protoplasts achieved optimal yield with **1.5% cellulase and 0.5% macerozyme** [10]; *Lolium perenne* leaf protoplasts showed optimal results with **2% cellulase and 0.5% macerozyme** [13]. This study used cellulase and macerozyme for digestion, with mannitol as osmoticum, achieving good results with **2% cellulase + 0.5% macerozyme + 0.6 mol · L^{-1} mannitol**.

Enzymatic hydrolysis time is another important factor affecting isolation efficiency. Overly long digestion damages the protoplast plasma membrane and increases debris, while overly short digestion yields insufficient protoplasts for subsequent experiments [13,33]. Digestion time varies greatly among species, ranging from **4-24 h** [6,10,13]. This study found that extending digestion time increased enzyme consumption but also increased cell debris and reduced viability, likely due to plasma membrane damage from prolonged enzyme exposure [37]. Enzymatic temperature also affects yield and viability, with **25°C** being optimal for enzyme activity without damaging protoplasts [38]. In the purification process, centrifugation speed is critical; excessive force causes mechanical damage, while insufficient force results in incomplete sedimentation [39]. This study achieved good separation using **800 rpm · min^{-1}**. In summary, using **25-day-old** seedling true leaves, sectioning longitudinally, pretreating with plasmolysis in **0.7 mol · L^{-1}** mannitol, digesting in **2% cellulase + 0.5% macerozyme + 0.6 mol · L^{-1} mannitol** for **2 h**, and purifying at **800 rpm · min^{-1}** yielded high protoplast quantity and viability suitable for subsequent experiments.

To verify the applicability of the prepared protoplasts for transient transformation, this study used chloroplast-localized **SaNADP-ME4** for transformation. The results showed **SaNADP-ME4-GFP** fusion protein localized to chloroplasts, consistent with previous studies localizing this gene to Arabidopsis chloroplasts [27], indicating that the established protoplast isolation and transformation method can be used for basic research in *S. laricifolia*, laying a foundation for gene function studies in this species.

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4 Conclusion

This study used true leaves from sterile tissue culture seedlings of *Salsola laricifolia* as material to analyze the effects of different cellulase and macerozyme concentration ratios, osmotic pressure, and enzymatic hydrolysis time on protoplast yield and viability. The conclusions are as follows:

Among the three influencing factors, cellulase concentration had a greater effect on protoplast yield, while enzymatic hydrolysis time had the most significant effect on protoplast viability. The optimal conditions were: **2% cellulase, 0.5% macerozyme, 0.6 mol·L^{-1} mannitol, and 2 h enzymatic hydrolysis**, yielding **1.21$\times$10^{6}$** protoplasts per **100 mg** fresh weight with **85%** viability. Using the prepared *S. laricifolia* protoplasts as receptors, the **pBI121-SaNADP-ME4-GFP** plasmid vector was successfully transformed via PEG-mediated transformation, and **SaNADP-ME4** was detected to be localized in chloroplasts. This study establishes an efficient protoplast preparation system for *S. laricifolia* leaves, providing a foundation for mining the special gene functions of this C3-C4 intermediate desert plant.

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Note: Figure translations are in progress. See original paper for figures.