Journal of Animal & Plant Sciences, 35(5): 2025, Page: 1281-1290
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2025.5.0109

AN EFFICIENT PROTOPLAST ISOLATION PROTOCOL FOR TRANSIENT EXPRESSION IN RICE

H. B. Kaya [1*]

[1]Department of Bioengineering, Manisa Celal Bayar University, Manisa, Türkiye
*Corresponding author's email: hilalbetul.kaya@cbu.edu.tr

ABSTRACT

Genome editing technologies like CRISPR/Cas9 have revolutionized crop improvement; however, they remain challenging and genotype-dependent. Guide RNAs (gRNAs) are essential for genome editing but often struggle to efficiently cleave target loci due to complex chromatin structures and a lack of tools to predict target site accessibility. This leads to wasted time and resources on ineffective editing experiments, as *in vitro* assays do not account for the native genomic context. Protoplasts are valuable in plant genome editing research because they provide a rapid and efficient way to test various parameters and reagents, enabling scientists to optimize their editing strategies more quickly than through the time-consuming process of testing directly on whole plants, which often requires multiple growth cycles to observe genetic modifications. Rice, due to its economic significance and compact genome, serves as an ideal model for such studies. Despite ongoing efforts to optimize protocols for protoplast isolation and transfection in rice, achieving stable and high-yield protoplasts remains a challenge. This study presents a detailed protocol for rice protoplast isolation, emphasizing critical procedural steps. As a result, this study achieved a yield of approximately 1 x 10^8 protoplasts from 120 seedlings (5 x 10^7 protoplasts/mL) of the japonica rice cultivar Nipponbare, representing the highest protoplast yield reported to date. Additionally, PEG-mediated transfection efficiency reached 65% using the 35S: GFP construct.

Keywords: Protoplast isolation, rice, transfection, transient expression

INTRODUCTION

The rapid development of genome editing technologies has revolutionized research on the plant genome, enabling scientists to accelerate crop improvement and breeding (Gao, 2021). Precise site-specific modifications, such as substitutions, insertions, and deletions can enhance crops by introducing desirable agronomic traits (Puchta, 2005). Among the various genome editing tools, the CRISPR/Cas system is the most widely used for its precision, efficiency, versatility, and ease of use (Ma *et al.,* 2016). The CRISPR/Cas9 tool has been extensively applied for trait improvement, method development, proof of principle, and gene functional analysis in various plants (Ricroch *et al.,* 2024).

One of the key obstacles in plant genome editing is the reliance on tissue culture, which is both time-intensive and expensive (Maher *et al.,* 2020). Tissue culture and regeneration techniques are crucial for achieving heritable gene modifications, as they are necessary after delivering CRISPR reagents into reproductive cells. Multiple factors influence the efficiency of CRISPR/Cas9 genome editing, including gRNA design, target DNA, Cas9, and the promoters driving Cas9 and sgRNAs (Čermák *et al.,* 2017; Ma *et al.,* 2016). The advent of new genome editing techniques, such as base editors (Ricroch, 2024; Nishida *et al.,* 2016) and prime editors (Anzalone *et al.,* 2019; Wang *et al.,* 2021), has increased the number of reagents that need testing. Due to the time-consuming nature of stable transfection for testing genome-editing reagents, protoplast cells offer a rapid and practical platform for evaluating genome-editing reagents (Jiang *et al.,* 2013). Protoplasts are totipotent and can regenerate into whole plants (Zhang *et al.,* 2011). Their ability to facilitate the easy delivery of foreign DNA makes them highly valuable in genome editing studies for transient gene expression and functional analysis (Davey *et al.,* 2005; Akkale, 2025). Transient protoplast transfection is an efficient and cost-effective system for validating genome editing reagents, as well as for understanding protein interactions, localization, and gene function (Chen *et al.,* 2006; Zhang *et al.,* 2011). In some plant species, protoplasts can regenerate into whole plants carrying heritable targeted mutations (Woo *et al.,* 2015). Protoplasts have been used to evaluate genome editing reagents across various plant species (Reed and Bargmann, 2021). A variety of protoplast isolation and transfection protocols have been developed for both dicot and monocot species, including Arabidopsis (Yoo *et al.,* 2007), tobacco (Lei *et al.,* 2015), tomato (Liu *et al.,* 2022), maize (Hu *et al.,* 2020), and wheat (Shan *et al.,* 2014). While Arabidopsis and tobacco protoplasts are widely used due to their ease of isolation and transformation, monocot species such as rice and maize often require more specialized procedures due to differences in cell wall composition and regeneration capacity (Xu *et al.,* 2022). These cross-species comparisons underscore the need for crop-specific optimization of protoplast protocols, particularly for genome editing applications.

Rice (*Oryza sativa* L.) is a key staple crop that feeds over half of the world's population (Kaya *et al.,* 2014; McCouch *et al.,* 2016). Beyond its economic significance, rice is a well-studied model for monocotyledonous plants, particularly in genome editing, due to its known and relatively small genome size (Endo and Toki, 2020; Mishra *et al.,* 2018). Over the past few decades, protoplast isolation techniques in rice have evolved considerably, with varying efficiencies influenced by cultivar, tissue type, enzyme composition, and handling procedures. Early efforts utilized cell suspension cultures, which, although yielding high viability, are labor-intensive and less accessible for many laboratories (Li and Murai, 1990; Utomo *et al.,* 1995). Subsequent protocols focused on etiolated seedlings due to their rapid growth and accessibility, leading to simplified workflows and improved transfection efficiencies (Chen *et al.,* 2006; He *et al.,* 2016; Shan *et al.,* 2014). More recently, the use of callus-derived protoplasts has gained attention for genome editing applications, particularly CRISPR-based approaches, due to their relatively high transformation efficiency (Poddar *et al.,* 2020). Comparative studies have highlighted that parameters such as enzyme concentrations (cellulase and macerozyme), osmotic stabilizers (mannitol concentrations), digestion duration, and tissue pre-treatment methods substantially impact protoplast yield and viability (Zhang *et al.,* 2011; Yoo *et al.,* 2007; Lin *et al.,* 2018). Despite these advances, there is still no consensus on a universally optimal protocol, and slight procedural modifications can lead to significant variability in outcomes. These gaps underscore the importance of systematically optimizing and reporting protoplast isolation parameters, particularly for laboratories aiming to adopt protoplast-based assays with limited resources. Various established protocols for rice protoplast isolation and transfection utilize plant tissues such as cell suspension cultures, seedlings, and callus (Li and Murai, 1990; Chen *et al.,* 2006; Poddar *et al.,* 2020). A summary of these protocols is presented in Table 1, highlighting how differences in enzymatic digestion, osmotic stabilizers, and pre-treatment steps affect yield and viability. This study provides a streamlined and reproducible protocol for rice protoplast isolation and transfection to enhance accessibility and consistency.

MATERIALS AND METHODS

Plant materials: Seeds of Oryza sativa L. japonica cv. Nipponbare were kindly supplied by Prof. Dr. Adam Bogdanove from Cornell University.

Seed surface sterilization and germination: Rice (*Oryza sativa* L.) cultivar Nipponbare seeds (60 seeds per growth jar) were dehulled and surface sterilized using 70% ethyl alcohol for 5 minutes, followed by a 30-minute treatment with 15% freshly prepared commercial bleach. The seeds were then washed five times with sterile ultra-pure water. The sterile seeds were sown in 10 cm diameter glass jars containing ½ MS media (Murashige and Skoog, Phyto Technology Laboratories, USA) with 3% sucrose (Merck, S0389) and 0.7% agar (Merck, A7921). The jars were placed in a growth chamber with a photoperiod of 12 hours of light at 30°C and 12 hours of darkness at 26°C for 14 days. All experiments were conducted in 2019 at Cornell University, Plant Pathology and Plant Microbe Biology Department, Bogdanove Lab, Ithaca, NY, USA.

Protoplast isolation: Protoplast isolation and purification were optimized by adjusting the key conditions based on previously reported methods (Zhang *et al.,* 2011; Shan *et al.,* 2014). A stepwise optimization of the protocol is detailed below. All steps, except for incubation and centrifugation, were performed under sterile conditions in a laminar flow hood.

Rice seedling stem tissue was cut in the direction of growth into approximately 0.5-1 mm strips using sterile razor blades, then immediately placed into the enzyme solution (approximately 60 stems in 10 mL of enzyme solution). The pre-incubation step in 0.6 M mannitol for 10 minutes in the dark was omitted, as noted by Shan *et al.,* (2014) and Zhang *et al.,* (2011). The strips in enzyme solution were vacuum infiltrated for 30 minutes at 400-500 mmHg in the dark, turning the vacuum on and off every ten minutes. While Zhang *et al.,* (2011) omitted vacuum infiltration, Shan *et al.,* (2014) applied a constant vacuum (~380–508 mmHg) for 30 min without interruption. After releasing the vacuum, the strips were incubated with enzyme solution in the dark for 8 hours with gentle shaking (100 rpm) at room temperature, in contrast to the shorter 4-6 hour incubation used by Shan *et al.,* (2014) and Zhang *et al.,* (2011).

Following enzymatic digestion, an equal volume of W5 wash buffer (10 mL) was added to the strips, which were vigorously shaken by hand for 1 minute to halt digestion. The mixture was filtered through a 40 µm nylon mesh into 50-mL centrifuge tubes to remove cell debris. Retained strips were washed 3-5 times with additional W5 buffer in a petri dish, and debris was gently squeezed using a sterile pipette tip or forceps to release protoplasts. It is critical to homogenize the strips in the buffer using a sterile pipette tip or forceps.

Table 1. Comparison of protoplast isolation methods in rice.

Cultivar	Source material	Pre-plasmolysis before digestion	Enzyme concentration (w/v)	Enzyme treatment	Yield	Viability	Ref.
Nipponbare, Taipei 309	Callus-derived suspensions	No pre-plasmolysis	4% cellulase RS, 1% macerozyme R-10	3 h	8 x10^6 protoplasts /g fresh weight	Unspecified	Li and Murai, 1990
Mercury, Lacassine, Maybelle, Cypress, and Lemont	Callus-derived suspensions.	No pre-plasmolysis	1% pectinase, 4% cellulose	3 h	5.3x10^6/g fresh weight[a]	Unspecified	Utomo *et al.,* 1995
Taipei 309	Stems and sheaths	No pre-plasmolysis	1.5% cellulase R-10, 0.3% macerozyme R-10	4 h	10-16 × 10^6 protoplasts from 105 seedlings	90%	Chen *et al.,* 2006
Taipei 309	Leaves	No pre-plasmolysis	1.5% cellulase R-10, 0.3% macerozyme R-10	12 h	3.4 x 10^5 protoplasts from 105 seedlings	Unspecified	Chen *et al.,* 2006
Nipponbare	Stems and Sheaths	Plasmolysis with 0.6 M mannitol for 10 min in the dark	1.5% cellulase RS, 0.75% Macerozyme R-10	4-5 h	1x10^7 protoplasts from 40-60 seedlings	95%	Zhang *et al.,* 2011
Nipponbare	Stems and sheaths	Plasmolysis with 0.6 M mannitol for 10 min in the dark	1.5% cellulase R10, 0.75% macerozyme R10	5–6 h	1x10^7 protoplasts from 100-120 seedlings	Unspecified	Shan *et al.,* 2014
Unspecified	Stems and sheaths	No pre-plasmolysis	1.5% cellulase, 0.3% macerozyme	4 h	1.5–2.5x10^6 protoplasts from ~100 seedlings	Unspecified	He *et al.,* 2006
Unspecified	Stems and sheaths	No pre-plasmolysis	1.5% cellulase R10, 0.75 macerozyme R10	3 h	4.8 × 10^6 protoplasts/g fresh weight	Unspecified	Lin *et al.* 2018
Nipponbare	Stems and sheaths	Plasmolysis with 0.6 M mannitol for 15 min in the dark	1.5% w/v cellulase RS, 0.75% macerozyme R10	4 h	Unspecified	Unspecified	Page *et al.,* 2019
Nipponbare	Callus	No pre-plasmolysis	1.5% cellulase R-10, 0.1% pectolyase or 0.75% macerozyme R-10	3 h	9.8x10^6 protoplasts from 5 g of rice callus[c]	-	Poddar *et al.,* 2020
Unspecified	Stems and sheaths	No pre-plasmolysis	1.20% cellulase R-10, 0.60% macerozyme R-10	Unspecified	4.31x10^7 protoplasts/g fresh weight	~90%	Ren *et al.,* 2021
Unspecified	Leaves	No pre-plasmolysis	Unspecified	Unspecified	No viable protoplasts	-	Ren *et al.,* 2021
Indica and Japonica	Stem	Plasmolysis with 0.6 M mannitol 10 min in the dark	1.5% Cellulase R10, 0.75% Macerozyme R10	5 h	2-5x10^7 protoplasts from ~80 seedlings[b]	91%	Panda *et al.,* 2024

[a]Highest yield reported for cultivar Mercury; [b]Highest yield reported for all plants used in the study; [c]Yield reflects only live protoplasts.

Protoplasts were harvested by centrifugation at 100 x g for 5 min at room temperature using a swinging bucket rotor. The supernatant was carefully decanted into a sterile flask rather than pipetted. The decanted supernatant was centrifuged again to collect protoplasts at 100 x g for 5 minutes at room temperature. Protoplast pellets were resuspended in 1 mL of MMG buffer, and the resuspended protoplasts were combined into one tube. All solution compositions are given in Table 2. Each experiment was performed with three independent replicates, and protoplast yield data are presented as mean values.

Table 2. Composition of buffers used in protoplast isolation and transfection.

Buffer	Composition
Extraction Buffer	1.5% Cellulase R10 (wt/vol) (Yakult Pharma. Ind. Ltd., Japan) 0.75 Macerozyme R10 (wt/vol) (Yakult Pharma. Ind. Ltd., Japan) 0.6 M Mannitol 10 mM $CaCl_2$ 10 mM MES (pH=5.7) 10% BSA (wt/vol)
W5 Buffer	154 mM NaCl 123 mM $CaCl_2$ 5 mM KCl 5 mM Glucose 2 mM MES (pH=5.7)
MMG Buffer	0.4 M Mannitol 100 mM $CaCl_2$ 15 mM $MgCl_2$ 4 mM MES (pH=5.7)
PEG Buffer	40% PEG 4000 (wt/vol) 0.4 M Mannitol 100 mM $CaCl_2$ 15 mM $MgCl_2$ 4 mM MES (pH=5.7)

To enhance clarity and comprehensiveness, a detailed flow diagram is included in Fig. 1, providing a step-by-step overview of the experimental procedures, and facilitating a better understanding of the processes described. Protoplasts were counted using a hemocytometer under microscopy.

Protoplast viability: The viability of protoplasts was evaluated through fluorescein diacetate staining (FDA) (Gupta and Don Durzan, 1986). FDA prepared in DMSO at a concentration of 5 mg/mL, was added to the protoplast solution to reach a final concentration of 0.05%, followed by a 2-minute incubation in the dark. Viable cells emitted a green fluorescence after staining, while dead cells and cellular debris appeared dark, enabling the visualization and assessment of protoplast viability using a fluorescence microscope (DM2000 LED, Leica, Germany). The viability percentage is determined by dividing the number of fluorescent protoplasts by the total number of protoplasts.

Preparation of plasmid DNA: *E. coli* strain transformed with pMOD_C3001 plasmid (which contains a 35S promoter driving the GFP gene, followed by a pea rbcsE9 terminator) (Čermák *et al.,* 2017) was cultured in LB (Luria–Bertani) medium. The culture was grown overnight in a 1 L baffled flask at 37°C, shaken vigorously at approximately 300 rpm. Plasmid DNA was extracted from *E. coli* using the HiSpeed Plasmid Maxi Plasmid Purification Kit (Qiagen Sciences, USA) following the manufacturer's guidelines.

Protoplast transfection using PEG-mediated method: Protoplasts were transfected as described by Shan *et al.,* (2014) based on the modifications described below. Approximately 1 x 10^6 protoplasts (instead of 5 x 10^5 protoplasts) in MMG were mixed with 15 µg of plasmid DNA at room temperature. An equal volume of freshly prepared PEG buffer (Table 1, PEG4000 (Sigma-Aldrich, cat. no. 95904)) was added and mixed gently. After 20 minutes of incubation at room temperature in the dark, 900 µL of W5 Buffer was added to stop the reaction, and the mixture was centrifuged at 100 x g for 5 min at the swinging bucket rotor. The protoplasts were then gently resuspended in 1 mL of MMG buffer (instead of WI), transferred to the 6-well plate, and incubated in the dark at room temperature for 24-48 hours. An empty vector was used to transfect protoplasts as a negative control. Protoplast transfection were conducted with three independent replicates.

Confocal imaging and fluorescence microscopy: Protoplasts were pelleted by centrifugation at 100 x g for 2 min, and a small drop of the protoplast suspension was then placed on a hemacytometer. A coverslip was carefully positioned on the sample with gentle pressure. Protoplasts were observed with a digital camera under a fluorescence microscope (DM2000 LED, Leica, Germany). Transfection efficiency was calculated by dividing the number of green-fluorescent protoplasts by the total number of protoplasts

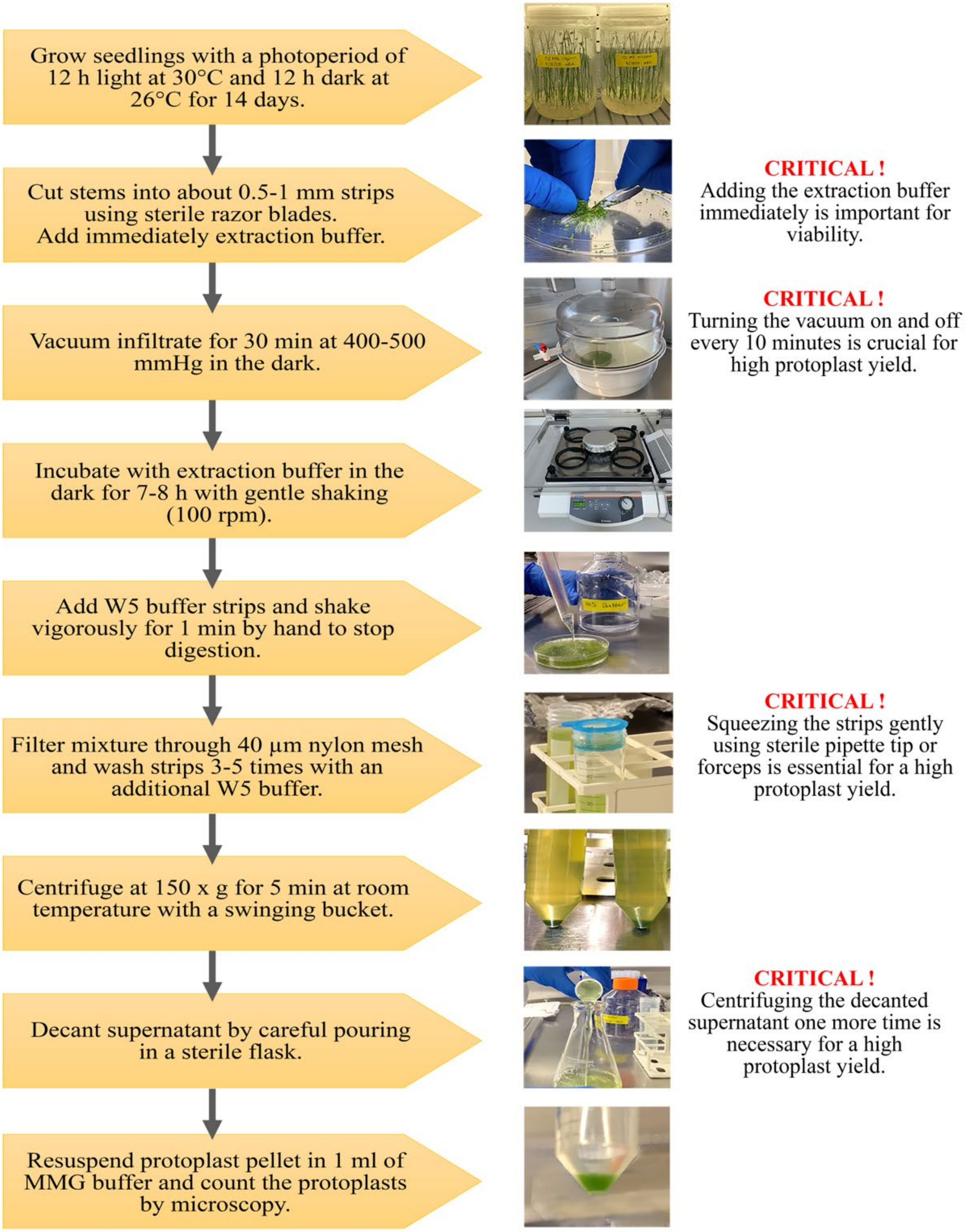

Fig. 1. Schematic overview of the protoplast isolation protocol.

RESULTS AND DISCUSSION

A high-efficiency protoplast isolation method from rice seedlings: This study provides evidence that small nuances and meticulous implementation of the protocol can enhance protoplast yield. While many existing protocols for rice are available (Table 1), this study achieved a yield of approximately 1 x 10^8 protoplasts from 120 seedlings (5 x 10^7 protoplasts/mL MMG), representing the highest protoplast yield reported to date.

Selection of the proper source of plant material is critical for isolating high-yield protoplast from plants. Germinated seedlings, suspension cultures, and calli have been used as source materials for rice protoplast isolation (Thompson *et al.,* 1986; Shan *et al.,* 2014; Poddar *et al.,* 2020). In particular, for monocotyledonous plants such as wheat (Jia *et al.,* 2016) and rice (He *et al.,* 2016), the mid-region of young seedlings is considered the optimal choice for protoplast isolation. In this study, young stem tissues from fourteen-day-old, germinated seedlings were selected as the source for rice protoplast isolation (Fig. 2). Stem tissue from older seedlings (over two weeks) was also used for protoplast isolation; however, lower protoplast yields were obtained using this older tissue source (data not shown). Similarly, Zhang *et al.,* (2011) used young stem rice tissues grown at 26°C under a 12-hour light cycle followed by 12 hours of darkness for protoplast isolation, but their efficiency (1.0 x 10^7) was relatively lower compared to this study.

Panda *et al.* (2024) used etiolated tissue from rice seedlings, achieving protoplast yields averaging between 2 x 10^7/mL and 5 x 10^7/mL across all plant species in their study, including rice, *Arabidopsis* and chickpea. They reported that etiolated tissue of rice increases the yield of viable protoplasts due to its tenderness and reduced lignification, which facilitate more efficient enzymatic digestion during the isolation process. However, their study did not include a direct comparison between etiolated and green rice seedlings.

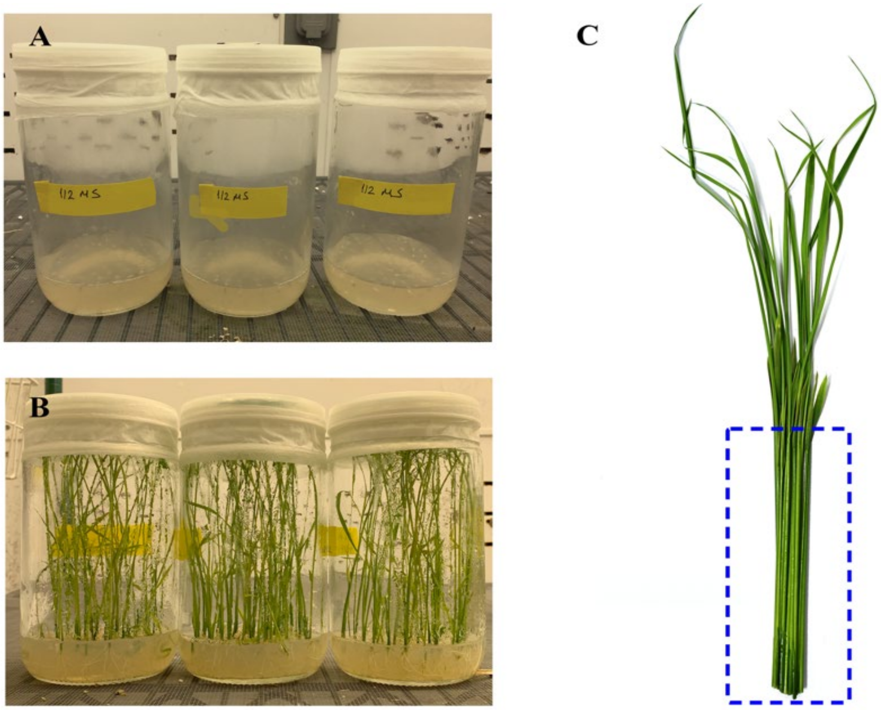

Fig. 2. Preparation of seedlings for protoplast isolation. A) Nipponbare seeds were sown in 10 cm diameter glass jars containing ½ MS media, B) Healthy rice seedlings, 14 days old, were used for protoplast isolation, C) Blue rectangular shows the stem of seedlings used for protoplast isolation.

The success of the isolation process also depends on the composition of the extraction buffer. In this study, the successful breakdown of rice stem tissue walls was achieved using an extraction buffer of 1.5% cellulase R10 and 0.75% macerozyme R10. Previous reports also used the same enzyme concentrations in rice protoplast isolation; however, they obtained lower yields compared to this study (Page *et al.,* 2019; Shan *et al.,* 2014). Shan *et al.,* (2014) obtained 1 x 10^7 protoplasts from 100–120 seedlings, while Chen *et al.,* (2006) reported an average yield of 10–16 x 10^6 protoplasts from approximately 105 rice seedlings.

To enhance the quantity of viable protoplasts obtained from tissue, pre-treatment can be employed. This involves improving the accessibility of enzymes used to break down the plant cell wall through physical tissue disruption (such as slicing leaf tissue), vacuum infiltration of extraction buffer, or pre-plasmolysis treatment (Reed and Bargmann, 2021).

The cut direction of strips affects the protoplast yield, and cross-section cutting resulted in high protoplast yield in rice (Shan *et al.,* 2014; Zhang *et al.,* 2011). Lin et al. (Lin *et al.,* 2018) used longitudinal cuts instead of latitudinal strips and reported a yield of 4.8 × 10^6 protoplasts per gram of fresh weight. However, since their results were reported based on tissue weight, while other studies including ours report yield based on the number of seedlings, direct comparison is challenging. In this study, we cut the rice seedlings into cross-section pieces, as this method has been associated with higher protoplast yields in previous reports. Vacuum is often used during protoplast isolation to facilitate the release of protoplasts from plant cells. The vacuum infiltration helps to increase the efficiency of enzyme penetration into plant tissue during enzymatic treatment, resulting in better digestion of the cell walls and higher protoplast yield. Previous protoplast isolation protocols from rice also highlighted the importance of vacuum infiltration during enzymatic treatment for higher protoplast yields (Chen *et al.,* 2006; Shan *et al.,* 2014). In this study, vacuum infiltration was implemented for 30 min with two abrupt breaks. There have also been studies in various plants of utilizing vacuum infiltration of plant tissue with the extraction buffer can enhance the protoplast yield (Nanjareddy *et al.,* 2016; Osakabe *et al.,* 2018). To initiate plasmolysis, Page *et al.,* (2019) placed cuttings directly into mannitol solution as a pre-treatment. Similarly, Shan *et al.* (2014) added 0.6 M mannitol to the rice strips and incubated them in the dark for 10 minutes to induce rapid plasmolysis. However, we obtained high yields of protoplasts without this treatment. Incubation with mannitol before enzymatic digestion was also implemented in our study, but it did not increase the yield of the protoplast (data not shown).

In this study, isolated protoplasts were subjected to FDA staining, revealing that the viability rates were consistently high, with an average of 90% (Fig. 3). This result indicates that the isolation protocol effectively maintained cell integrity and metabolic activity, which are crucial for subsequent applications such as transfection and regeneration. Similar viability results were reported in other studies (Table 1).

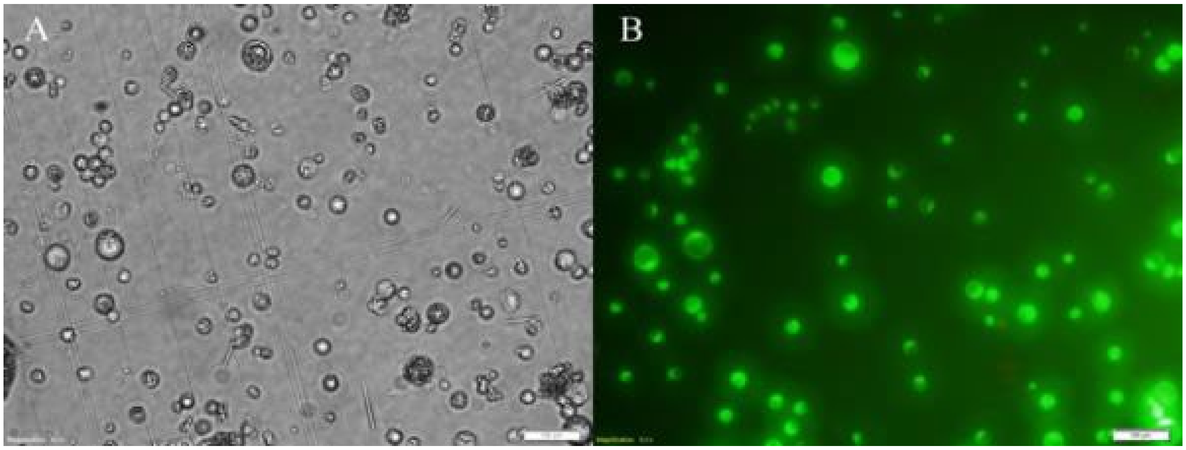

Fig. 3. Assessment of protoplast viability in rice isolated via FDA staining.

Efficient transfection of GFP inserted constructs into rice protoplasts: Rice protoplast transfection techniques have been extensively studied over the past decade to establish an efficient and reliable system (Zhang *et al.,* 2011; Shan *et al.,* 2014; Page *et al.,* 2019). The PEG-mediated transfection method is the most used approach for rice protoplast transfection (Chen *et al.,* 2006). Protoplasts are used in several biotechnological applications that are directly impacted by both the quantity and quality of protoplasts (Poddar *et al.,* 2020). One key application is establishment of a transient expression system, which is useful for high-throughput functional gene characterization. Achieving an efficient transient expression system requires high-quality and freshly isolated protoplasts (Yoo *et al.,* 2007). Freshly isolated protoplasts maintain their cellular individuality and exhibit high transfection efficiency, making them a valuable system for investigating gene functions and analyzing gene regulatory networks (Yoo *et al.,* 2007; Reed and Bargman, 2021). However, protoplast viability decreases over time. This study employed a PEG-mediated transfection protocol to assess the competency of freshly prepared protoplasts. Protoplasts were transiently transfected with plasmid DNA, including GFP as a visual marker. Transfection efficiency was measured two days post-transfection by counting GFP-fluorescing cells (Fig. 4). Transfection efficiencies ranged from 45-60%, with a mean efficiency 52%, using the 35S: GFP construct across three replicates, while Shan *et al.,* (2014) reported an efficiency of 80% using the same PEG-mediated transfection protocol. The primary differences between the studies were the number of protoplasts and the incubation buffer used after transfection: this study used 1×10^6 protoplasts (compared to 5×10^5 protoplasts used by Shan *et al.,* (2014) and employed MMG as the incubation buffer, while Shan *et al.,* (2014) used WI buffer. Different plasmids were also used in these studies. These differences could contribute to the varying transfection efficiencies. Increasing protoplast numbers does not necessarily enhance transfection efficiencies; in fact, depending on DNA and protoplast quality, higher cell numbers can sometimes yield lower transfection rates (Yoo *et al.,* 2007).

It is also known that plasmid size has a great impact on transfection efficiencies. Zhang *et al.* (2011) achieved a maximum transfection efficiency of 75% with a 4.5 kb plasmid, while larger plasmids (13 kb) yielded efficiencies of 45-66% when using 2 × 10^5 protoplasts. Similarly, maximum transfection efficiency (>80%) was recorded with smaller plasmid (10 kb), whereas larger plasmid (15 kb) obtained approximately 65% efficiency (Panda *et al.* 2024). Several factors influencing transfection efficiency, such as PEG concentration, transfection duration, protoplast density, and DNA concentration, have been examined in previous studies, and optimal conditions were reported for rice (Ren *et al.,* 2021; Panda *et al.,* 2024).

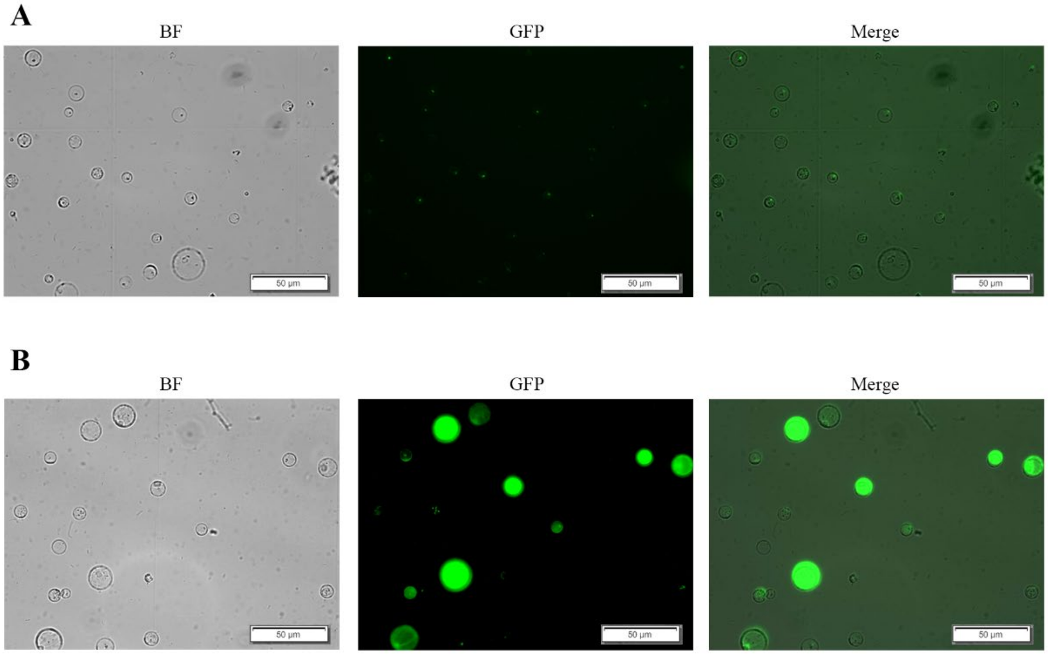

Fig. 4. A confocal image showing the rice protoplast after PEG-mediated transfection. A) Protoplasts transfected with an empty vector, B) Protoplasts transfected using the 35S: GFP construct. BF: Confocal brightfield channel; GFP: Green fluorescent protein channel; Merge: Merge of BF and GFP channels.

Although protoplast isolation efficiency can vary significantly among rice genotypes, the protocol described in this study provides a robust and reproducible framework optimized for Oryza sativa cv. Nipponbare. This method may serve as a starting point for adapting protoplast-based systems in other rice cultivars through genotype-specific adaptations. The high yield and viability achieved here make this protocol especially suitable for rapid, cost-effective evaluation of genome editing reagents, prior to investing in labor-intensive and time-consuming stable transformation approaches. Such applications are particularly valuable in functional genomics studies and early-stage breeding programs, where the ability to validate gene candidates or guide RNAs efficiently is critical. While further optimization may be required for use in other monocots, the core principles of this protocol, tissue selection, enzymatic digestion conditions, and handling steps, offer a practical foundation for developing similar approaches in other cereal crops. This expands the potential utility of the method across a broader spectrum of genome editing applications in plants.

Conclusions: As a valuable tool, plant protoplasts offer a promising alternative for rapidly assessing genome-editing reagents. With its economic importance and manageable genome size, rice remains a preferred model for studying genome editing. This study provided a comprehensive protocol for rice protoplast isolation, resulting in high yield and efficient transfection. Overall, these findings support ongoing efforts to enhance genetic manipulation in plants and accelerate agricultural advancements.

Acknowledgments: The author gratefully acknowledges Prof. Dr. Adam Bogdanove at Cornell University for his valuable discussion on the design of the study and for providing his laboratory resources. The author also thanks Dr. Li Wang from Cornell University and Dr. Simon Sretenovic from the University of Maryland for their helpful comments. HBK has received research support from the Scientific Research Projects funds of Manisa Celal Bayar University, under Project No: (2017-113).

REFERENCES

Akkale, H.B.K. (2025). Asma protoplastlarında CRISPR/Cas9 aracılı genom düzenleme. J. Agri. Faculty of Ege Univ. 62(1): 117-131.DOI: 10.20289/zfdergi.1432 614

Anzalone, A.V., P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy and A. Raguram (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576(7785): 149-157. DOI: 10.1038/s41586-019-1711-4

Burris, K.P., E.M. Dlugosz, A.G. Collins, C.N. Stewart and S.C. Lenaghan (2016). Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). Plant Cell Reports 35: 693-704. DOI: 10.1007/s00299-015-1928-7

Čermák, T., S.J. Curtin, J. Gil-Humanes, R. Čegan, T.J. Kono, E. Konečná and R.L. Greenstein (2017). A multipurpose toolkit to enable advanced genome engineering in plants. The Plant Cell 29(6): 1196-1217. DOI: 10.1105/tpc.17.00199

Chen, S., L. Tao, L. Zeng, M.E. Vega-Sanchez, K. Umemura and G.L. Wang (2006). A highly efficient transient protoplast system for analyzing defence gene expression and protein–protein interactions in rice. Molecular Plant Pathology 7(5): 417-427. DOI: 10.1111/j.1364-3703.2006.00350.x

Davey, M.R., P. Anthony, J.B. Power and K.C. Lowe (2005). Plant protoplasts: status and biotechnological perspectives. Biotechnology Advances 23(2): 131-171. DOI: 10.1016/j.biotechadv.2004.09.005

Endo, M. and S. Toki (2020). Genome editing in rice. Rice 13: 27. DOI: 10.1186/s12284-020-00389-6

Gao, C. (2021). Genome engineering for crop improvement and future agriculture. Cell 184(6): 1621-1635. DOI: 10.1016/j.cell.2021.02.013

Gupta, P.K. and J. Don Durzan (1986). Isolation and cell regeneration of protoplasts from sugar pine (Pinus lambertiana). Plant Cell Reports 5: 346-348. DOI: 10.1007/BF00272717

He, F., S. Chen, Y. Ning and G.L. Wang (2016). Rice (Oryza sativa) protoplast isolation and its application for transient expression analysis. Current Protocols in Plant Biology 1(2): 373-383. DOI: 10.1002/cppb.20022

Hu, Y., D. Song, L. Gao, B.S. Ajayo, Y. Wang, H. Huang, J. Zhang, H. Liu, Y. Liu, G. Yu, and Y. Liu (2020). Optimization of isolation and transfection conditions of maize endosperm protoplasts. Plant Methods 16(1), p.96. DOI: 10.1186/s13007-020-00636-y

Jia, X., X. Zhang, J. Qu and R. Han (2016). Optimization conditions of wheat mesophyll protoplast isolation. Agricultural Sciences 7(12): 850-858. DOI: 10.4236/as.2016.712083

Jiang, W., H. Zhou, H. Bi, M. Fromm, B. Yang and D.P. Weeks (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Research 41(20): e188. DOI: 10.1093/nar/gkt780

Ricroch, A. D. Eriksson, D. Miladinović, J. Sweet, K. Van Laere, and E. Woźniak-Gientka (2024). A Roadmap for Plant Genome Editing, p 561. Springer Nature. DOI: 10.1007/978-3-031-46150-7_2

Kaya, H.B., M. Demirci and B. Tanyolac (2014). Genetic structure and diversity analysis revealed by AFLP on different Echinochloa spp. from northwest Turkey. Plant Systematics and Evolution 300: 1337-1347. DOI: 10.1007/s00606-013-0970-4

Lei, R., W. Qiao, F. Hu, H. Jiang and S. Zhu (2015). A simple and effective method to encapsulate tobacco mesophyll protoplasts to maintain cell viability. MethodsX 2: 24-32. DOI: 10.1016/j.mex.2014.12.005

Li, Z. and N. Murai (1990). Efficient plant regeneration from rice protoplasts in general medium. Plant Cell Reports 9(4): 216-220. DOI: 10.1007/BF00232503

Lin, C.S., C.T. Hsu, L.H. Yang, L.Y. Lee, J.Y. Fu, Q.W. Cheng and R. Zhang (2018). Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnology J. 16(7): 1295-1310. DOI: 10.1111/pbi.12854

Liu, Y., M. Andersson, A. Granell, T. Cardi, P. Hofvander and A. Nicolia (2022). Establishment of a DNA-free genome editing and protoplast regeneration method in cultivated tomato (Solanum lycopersicum). Plant Cell Reports 41(9): 1843-1852. DOI: 10.1007/s00299-022-02881-z

Ma, X., Q. Zhu, Y. Chen and Y.-G. Liu (2016). CRISPR/Cas9 platforms for genome editing in plants: developments and applications. Molecular Plant 9(7): 961-974. DOI: 10.1016/j.molp.2016.04.003

Maher, M.F., R.A. Nasti, M. Vollbrecht, C.G. Starker, M.D. Clark and D.F. Voytas (2020). Plant gene editing through de novo induction of meristems. Nature Biotechnology 38(1): 84-89. DOI: 10.1038/s41587-019-0345-2

McCouch, S.R., M.H. Wright, C.-W. Tung, L.G. Maron, K.L. McNally, M. Fitzgerald and P. Korniliev (2016). Open access resources for genome-wide association mapping in rice. Nature Communications 7(1): 1-14. DOI: 10.1038/ncomms10532

Mishra, R., R.K. Joshi and K. Zhao (2018). Genome editing in rice: recent advances, challenges, and future implications. Frontiers in Plant Science 9: 1361. DOI: 10.3389/fpls.2018.01361

Nanjareddy, K., M.-K. Arthikala, L. Blanco, E.S. Arellano and M. Lara (2016). Protoplast isolation, transient transformation of leaf mesophyll protoplasts and improved Agrobacterium-mediated leaf disc infiltration of Phaseolus vulgaris: tools for rapid gene expression analysis. BMC Biotechnology 16: 1-14. DOI: 10.1186/s12896-016-0260-2

Nishida, K., T. Arazoe, N. Yachie, S. Banno, M. Kakimoto, M. Tabata and A. Kondo (2016). Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 353(6305). DOI: 10.1126/science.aaf8729

Osakabe, Y., Z. Liang, C. Ren, C. Nishitani, K. Osakabe, M. Wada and M. Poli (2018). CRISPR–Cas9-mediated genome editing in apple and grapevine. Nature Protocols 13(12): 2844-2863. DOI: 10.1038/s41596-018-0051-5

Page, M.T., M.A. Parry and E. Carmo-Silva (2019). A high-throughput transient expression system for rice. Plant Cell Environment 42(7): 2057-2064. DOI: 10.1111/pce.13549

Panda, D., S. Karmakar, M. Dash, S.K. Tripathy, P. Das, S. Banerjee and M.J. Baig (2024). Optimized protoplast isolation and transfection with a breakpoint: accelerating Cas9/sgRNA cleavage efficiency validation in monocot and dicot. aBIOTECH 1-18. DOI: 10.1007/s42994-024-00139-7

Poddar, S., J. Tanaka, J.H. Cate, B. Staskawicz and M.-J. Cho (2020). Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays. Plant Methods 16(1): 1-11. DOI: 10.1186/s13007-020-00617-9

Puchta, H. (2005). The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J. Experimental Botany 56(409): 1-14. DOI: 10.1093/jxb/eri025

Reed, K.M. and B.O. Bargmann (2021). Protoplast regeneration and its use in new plant breeding technologies. Frontiers in Genome Editing 20. DOI: 10.3389/fgeed.2021.666314

Ren, R., J. Gao, D. Yin, K. Li, C. Lu, S. Ahmad and F. Yang (2021). Highly efficient leaf base protoplast isolation and transient expression systems for orchids and other important monocot crops. Frontiers in Plant Science 12: 626015. DOI: 10.3389/fpls.2021.626015

Ricroch, A., D. Eriksson, D. Miladinović, J. Sweet, K. Van Laere and E. Woźniak-Gientka (2024). A Roadmap for Plant Genome Editing. Springer Nature, p. 561. DOI: 10.1007/978-3-031-46150-7

Shan, Q., Y. Wang, J. Li and C. Gao (2014). Genome editing in rice and wheat using the CRISPR/Cas system. Nature Protocols 9(10): 2395-2410. DOI: 10.1038/nprot.2014.157

Xu, Y., R. Li, H. Luo, Z. Wang, M.W. Li, H.M. Lam and C. Huang (2022). Protoplasts: small cells with big roles in plant biology. Trends in Plant Science 27(8): 828-829. DOI: 10.1016/j.tplants.2022.06.003

Thompson, J., R. Abdullah and E. Cocking (1986). Protoplast culture of rice (Oryza sativa L.) using media solidified with agarose. Plant Science 47(2): 123-133. DOI: 10.1016/0168-9452(86)90046-7

Utomo, H.S., S.S. Croughan and T.P. Croughan (1995). Suspension and protoplast culture of US rice cultivars. Plant Cell Reports 15(1): 34-37. DOI: 10.1007/BF00233348

Wang, L., H.B. Kaya, N. Zhang, R. Rai, M.R. Willmann, S.C. Carpenter and J.E. Leach (2021). Spelling changes and fluorescent tagging with prime editing vectors for plants. Frontiers in Genome Editing 3: 617553. DOI: 10.3389/fgeed.2021.617553

Woo, J.W., J. Kim, S.I. Kwon, C. Corvalán, S.W. Cho, H. Kim and J.-S. Kim (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nature Biotechnology 33(11): 1162-1164. DOI: 10.1038/nbt.3389

Yoo, S.-D., Y.-H. Cho and J. Sheen (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature Protocols 2(7): 1565-1572. DOI: 10.1038/nprot.2007.199

Zhang, Y., J. Su, S. Duan, Y. Ao, J. Dai, J. Liu and D. Feng (2011). A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. Plant Methods 7(1): 1-14. DOI: 10.1186/1746-4811-7-30.