MEDDOCS Open Access Publisher

ISSN: 2637-7721

Journal of Plant Biology and Crop Research

Open Access | Research Article

An Improved Method for Protoplast Isolation and Gene-Editing from Soybean Root, Callus and Transgenic Hairy-Roots

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Received: Sep 09, 2023

Accepted: Oct 16, 2023

Published Online: Oct 23, 2023

Journal: Journal of Plant Biology and Crop Research

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Protoplast; Soybean; Transfection; Gene-editing; CRISPR/Cas9; Roots; Hairy roots; Callus.

Abstract

Protoplast is an excellent model for evaluating genetic engineering reagents and serve as a resourceful system in plant biology that provides a platform for rapid analysis of diverse signaling pathways, studying gene function and a high-throughput tool for functional genomics. However, isolation of high-quality protoplast from variety of plant tissues is relatively challenging and this system is less exploited in many crops including soybean. In majority, protoplasts are isolated from leaf mesophyll tissue however, protoplast isolation from multiple tissue types provides greater flexibility and provide variety of cells for tissue specific experimentation e.g., single cell transcriptomics. It also helps ensure that the cells obtained are representation of the plant, rather than just a single tissue type. Finally, isolation of protoplast from transgenic tissues such as hairy-roots and transgenic callus provide a rapid method for studying gene function. Here, we developed an improved method for isolation, transfection, and gene-editing from non-transgenic (roots) and transgenic (hairy-roots and callus).

Introduction

Protoplast is a versatile system for conducting genomics, transcriptomics, proteomics, and genome engineering studies [1,2]. Given the advantages of protoplast system, it has been widely used for genetic engineering and genome editing in plants [3]. One of the main advantages of protoplasts in gene editing is that they are highly amenable to transformation, allowing for the efficient delivery of exogenous DNA, RNA and/or protein into the cell. Plant protoplast culture can also mimic the animal cell culture for transient gene analysis and serve as user-friendly tool to study gene function. Since, the advent of CRISPR/Cas9 system, direct modification of crop genomes has become the promising new breeding technology for trait discovery and crop improvement. However, inserting CRISPR/Cas9 transgene and guide RNA (gRNA) and development of gene-edited plants using *Agrobacterium* or gene-gun mediated transformation methods is time consuming and labor-intensive process [4]. Therefore, it is highly desirable to test these reagents before initiating the stable transformation experiment. In this case protoplasts serve as most rapid and reliable system to evaluate targeted mutagenesis efficiency at relatively faster and cost-effective setting. Secondly, gene-editing through transient expression of CRISPR/Cas9 and gRNA followed by regeneration of protoplast can achieve the desired genetic outcome within a single clonal regeneration and by avoiding the integration and segregation of transgene into the host genomes. However, protoplast re-

Cite this article: Lois Nwoko C, Arjun Ojha K, Devkar V, Patil GB. An Improved Method for Protoplast Isolation and Gene-Editing from Soybean Root, Callus and Transgenic Hairy-Roots. J Plant Biol Crop Res. 2023; 6(2): 1088.

generation is a major bottleneck that impedes its utility beyond in vitro assays. For example, protoplast is highly recalcitrant for plant regeneration and majority of protoplast cells can enter in cell division processes leading to mass of cells, but these cells do not reprogram towards gaining the pluripotency and fails to enter in dedifferentiation state. Therefore, unfortunately, not all plant species can be easily regenerated from protoplast, and this process is highly inefficient among majority of species and tissue types. On contrary, only few plants such as tobacco, carrot, potato and Arabidopsis can differentiate and regenerate relatively easily from isolated mesophyll protoplasts. Nevertheless, due to the genome editing technology, protoplast system is revitalized and becoming an essential tool for trait discovery and crop improvement, especially it serves as efficient method for transgene-free gene-edited plants [1].

A typical tissue used in protoplast isolation is derived from young leaves [5-8], and sometimes from shoots [9], immature seed tissues [2, 10] and suspension culture [11]. However, majority of the genes are specifically expressed in tissue/cell specific manner. Therefore, functional analyses of these genes or their regulatory network using advanced genomics (single cell transcriptomics) or gene editing requires tissue specific protoplast [9, 12]. For example, root tissues serve as ideal model to study water and nutrient uptake, and interaction with beneficial or pathogenic microbes. Similarly, transgenic hairy roots developed using *Agrobacterium rhizogenes* culture is important tool in plant science and has several important applications in numerous fields including fundamental plant biology, metabolite production, agriculture, and environmental sciences. For examples, hairy roots offer a good source of material for the development of transgenic protoplasts that express flag genes, which may be valuable for protoplast fusion experiments and monitoring the regeneration process. Therefore, protoplast isolated from transgenic hairy-roots offers several advantages to study the effect of transgene or gene-edited roots for gene functional characterization and several downstream investigations. However, isolation of protoplast from transgenic tissues is not well established in plant species.

During 1980-90s, protoplast isolation from soybean was first attempted [5, 10] and recently this method was improved for young trifoliate leaves [13] and immature seeds [2]. Although, some plant tissues are relatively amenable to protoplast isolation, leaves, and immature seeds pose challenges in term of developmental stages, reproducibility, cell types and tissue availability. Therefore, development of simple and efficient cell-based system is particularly important for rapid gene function analysis. Here, we sought to develop protoplast isolation from different radially available tissue types (transgenic and non-transgenic). We investigated multiple factors that affect protoplast yield and quality including different cell wall digesting enzymes, plasmolyse buffers, pre-treatment with vacuum and incubation times. With this improved method the highest yield of 4.06 x 10^6, 1.58 x 10^6 and 3.76 x 10^6 protoplasts was achieved from protoplasts of soybean roots, hairy roots, and callus tissues, respectively. Additionally, isolated protoplasts were evaluated for transient gene expression and gene knockout using CRIPR/Cas9 reagents. In summary, this method provides a new method to isolate high-quality protoplast from variety of tissue types and can be efficiently employed in several molecular biology experiments including but not limited to single-cell transcriptomics analysis, transient gene-expression, gene-knockout, and protein-protein interaction.

Materials

Chemicals and Stock solutions (Table 1, adapted from Patil et al. 2020):

1. Cellulase [Onozuka R-10 from RPI]: Concentration 2%. Mix 1 gm of Cellulase R10 in 40 mL Digestion solution and add additional dH_2O to the final volume of 50 mL.
2. Cellulase [RS from RPI]: Concentration 2%. Dissolve 1 gm of Cellulase RS in 40 mL Digestion solution and add additional dH_2O to the final volume of 50 mL.
3. Pectolyase [from RPI]: Concentration 0.5%. Dissolve 0.5 gm of Pectolyase in 80 mL Digestion solution and add additional dH_2O to the final volume of 100 mL.
4. Percoll [Sigma]: To make 20, 40, 60 and 80% percoll solution add 20, 40, 60 and 80 ml percoll solution to W5 solution to the final volume of 100 mL.
5. Macerozyme R-10: Concentration 0.5%. From RPI. Dissolve 1 g of Macerozyme R10 in 40 mL and add additional dH_2O to the final volume of 50 mL.
6. MES buffer (0.2 M, pH 5.7): dissolve 11.71 g 2-(N-morpholino) ethanesulfonic acid in 400 mL dH_2O. Adjust pH to 5.7 using 10 N NaOH and add additional dH_2O to the final volume to 500 ml.
7. Mannitol (0.8 M stock): For 500 mL, add 72.86 g mannitol first to 400 mL dH_2O in a flask with a stirring bar. Dissolve the chemical completely, then add additional dH_2O to the final volume.
8. Calcium chloride (1.0 M): For 500 mL, dissolve 73.05 g $CaCl_2 \cdot 2H_2O$ in 400 mL dH_2O, then add additional dH_2O to the final volume**.**
9. Magnesium chloride (2.0 M): For 500 mL, dissolve 95.21 g $MgCl_2$ in 400 mL dH_2O, then add additional dH_2O the final volume.
10. Potassium chloride (0.1 M): For 500 mL, dissolve 3.72 g KCl in 400 mL dH_2O, then add additional dH_2O to the final volume.
11. Sodium chloride (2.0 M): For 500 mL, dissolve 58.44 g NaCl in 400 mL dH_2O, then add additional dH_2O to the final volume.
12. PEG solution: For 50 mL. Dissolve 20 gm of PEG-4000 in 20 mL solution containing 0.2 M Mannitol and 100 mM $CaCl_2$ (see Buffer D; PEG-Calcium Transfection Buffer). Add additional solution D to the final volume.
13. Murashige and Skoog Media: Dissolve 4.93 g of MS powder (M530 Phytotech Laboratories), and 30 gm Sucrose in 900 ml water. Adjust pH to 5.8 then add 7 gm Agar and add dH_2O to make a final volume of 1L.

Solutions and media

Prepare working solutions and media as shown in Table 1.

Table 1: Reagents and Solutions for protoplast isolation and transformation from different tissue types.

#	Chemical	Stock Concentration	Working Concentration		
A.	**Digestion Solution**				
			Root	Hairy roots	Callus
1	Mannitol D (-)	0.8M	0.6M	0.6M	0.6M
2	Cellulase R-10	_	2%	_	_
3	Cellulase RS	_	_	2%	2%
4	Macerozyme	_	2%	_	_
5	Pectolyase	_	_	0.5%	0.4%
6	$CaCl_2$	1.0M	10mM	10mM	10mM
7	MES (pH 5.7)	0.2M	10mM	10mM	10mM
8	$MgCl_2$	2.0M	10mM	10mM	10mM
9	BSA	_	1%	1%	1%
B.	**W5 (Washing solution)**				
1	Mannitol D (-)	0.8M	0.6M		
2	$CaCl_2$	1.0M	10mM		
3	MES (pH 5.7)	0.2M	10mM		
C.	**WI Solution**				
1	Mannitol D (-)	0.8M	0.6M		
2	KCl	2.0M	20mM		
3	MES (pH 5.7)	0.2M	4mM		
D.	**MMG Buffer**				
1	Mannitol D (-)	0.8M	0.4M		
2	$MgCl_2$	2.0M	15mM		
2	MES (pH 5.7)	0.2M	4mM		
E.	**PEG Solution**				
1	Mannitol D (-)	0.8M	0.2M		
2	$CaCl_2$	1.0M	100mM		
3	PEG	-	40%		

Additional supplies and instruments

1. Glassware (beakers and flasks 100, 250, and 500 ml)
2. Laminar flow hood
3. Forceps and razor blade
4. Serological pipettes (5, 10, 25 ml)
5. Aluminum foil
6. 22 µM filter units [Product# Nalgene UX-06730-31]
7. Cell Strainer 40 µM [Product# Grainer 48TD63]
8. Vacuum Chamber (Bel-Art 42010)
9. Open Bench-top orbital shaker
10. Petri dishes (60- and 100-mm size)
11. 15- and 50-mL falcon tubes
12. Bucket centrifuge with 15- and 50-mL conical tube adapters.
13. Microcentrifuge for 1.5 mL and 2.0 mL tubes
14. Regular and wide bore pipette tips, 1000 µL and 200 µL
15. 6-well cell culture plate
16. Compound microscope with 10X, 20X and 40X lenses
17. Hemocytometer and cover glass
18. Fluorescent microscope, EVOS M5000 Imaging system, ThermoFischer Scientific with color fluorescence, transmitted light, and color images [Product # AMF5000].

Plasmid DNA Constructs

1. Green Fluorescent Protein gene construct: Gene construct carrying GFP under 35S promoter was obtained from Dr. Daniel Voytas, University of Minnesota, St. Paul, MN, USA. This construct available at Addgene #91042.
2. Gene editing construct: Guide-RNAs (gRNA) were cloned in pMOD_B2103 [Addgene #91061] using modular cloning method as described [2, 14]. The final construct pVD1 carrying GmUBI::Cas9 – CmYLCV::gRNA1-gRNA2 – 35S::GFP was developed.

Methods

Plant material, explant isolation, and preparation

Root tissues

1. Seeds of genotype "William 82" (W82) were surface sterilized in 70% ethanol for 1 min and followed by 30% bleach (7.2% sodium hypochlorite) solution for 15 min, washed 5 times in sterile H_2O and imbibed in water for 5-8 hrs.
2. Imbibed seeds were transferred to the Murashige-Skoog (MS) basal medium (PhytoTech Laboratories) [15] supplemented with 0.8% agar, 3% (w/v) sucrose and pH adjusted to 5.8 with 1M KOH. Sterile seeds were germinated in a 16/8 h light/dark photoperiod at 24°C

Hairy root tissue

1. Seed Sterilization and germination was performed.
2. Soybean hairy roots were induced using *Agrobacterium rhizogenes* (strain *K599*) transformation containing gene of interest using cotyledons (1 week old) as described [16]. After co-cultivation the cotyledons were transferred on solid MS media supplemented with 0.8% (w/v) agar, 3% sucrose, and 500 mg/L cefotaxime (Duchefa, The Netherlands) to control Agrobacterium growth. Cotyledon explants were kept for 15-20 days post infection and after 20 days, young hairy roots were collected for protoplast isolation as described below (Table 2).

Callus tissue

1. Seed Sterilization and germination was performed.

2. The imbibed seed cotyledons were removed with a scalpel to expose the embryos, seeds were cut longitudinally, and the two cotyledons split apart. Forceps were used to carefully remove whole embryos and place them in callus inducing medium containing 2mg/L 2,4- dichlorophenoxyacetic acid [(2,4-D). Petri dishes, each containing 10 embryos, were sealed with parafilm, and were kept in the dark at 25°C. Embryos were subcultured in a fresh supply of the same medium at 2 weeks intervals for 2 months.

Protoplast isolation and transfection

Tissue preparation

Prepare the tissue for protoplast isolation as described in Table 2.

Table 2: Steps involved in tissue preparation and protoplast isolation.

Steps	Roots	Hairy roots	Callus
Preparation	• Cut young root tissues from the tips (about 1 cm) from 7-day-old seedling into 1 mm slices. • Immerse them into 25 ml of enzymatic digestion solution (Fig.1D and 1E).	• Carefully harvest 2-3 weekold hairy roots induced by infection from *A. rhizogenes* into a Petri dish. • Keep hairy roots in sterile water to ensure that the roots do not dry out. • Cut roots into 1 mm slices (Fig.1B) and transfer to pre-plasmolyse buffer.	• Collect about 3-5 grams of callus tissues and gently crumble using the edge of a scalpel in a petri dish containing 25ml of digestion solution (Fig.1C).
Pretreatment	• Vacuum infiltrate for 15 mins (400-500 mmHg) (Fig. 1F).	• Pre-plasmolyse the root tissues in 25 ml 0.8M mannitol for 4-6 hrs. • Transfer plasmolyzed tissues into 25 ml of enzymatic digestion solution. • Vacuum infiltrate for 30 mins (400-500 mmHg) at 10 mins interval (Fig. 1F).	• Transfer callus containing digestion solution to a 50ml falcon tube and vortex briefly to break up callus aggregates. • Vacuum infiltrate for 10 mins (Fig. 1F).
Incubation time	• Incubate the protoplast containing digestion solution for 15hrs with gentle shaking (60-80 rpm) at room temperature in the dark at 24 °C .	• Incubate the protoplast containing digestion solution for 15 hrs with gentle shaking (60-80rpm) at room temperature in the dark at 24 °C.	• Incubate the protoplast containing digestion solution for 12 hrs with gentle shaking (60-80rpm) at room temperature in the dark at 24 °C.
Follow next steps for protoplast washing, transfection and gene-editing			

Protoplast wash

1. Following the tissue digestion, add 50 ml of W5 solution to the protoplast solution to stop the digestion.
2. Gently swirl the petri dish by hand for 1 min to release the protoplasts.
3. Collect the protoplasts by filtration through 40 µm cell strainer nylon filters (Fig 1G).
4. Rinse the roots 2 times with W5 solution of 15 ml for each time.
5. Centrifuge the protoplast solution at 300 rcf for 3 minutes in a swinging-bucket rotor at room temperature.
6. Discard the supernatant carefully.
7. Resuspend pellet in 2ml of W5 solution (Fig 1H).
8. For protoplast purification, prepare 2ml of 80%, 60%, 40% and 20% percoll solution, respectively and slowly layer each solution in a gradient form in the above order with 20% percoll solution at the top in a sterile 15 ml centrifuge tube.
9. Carefully layer 2 ml of unpurified protoplast on top and centrifuge at 1000g for 10 mins using a swing bucket centrifuge (Fig 1I).
10. Intact protoplasts will be harvested in the milky colored band formed in a layer between the 40% and 20% percoll solution (FIG 1J).
11. Carefully pipette the protoplasts into a sterile 15ml tube.
12. Wash protoplasts twice in W5 solution, centrifuging at 300 rcf for 3 mins.
13. Resuspend pellet in 5ml WI solution.
14. Keep protoplast on ice until further use.

Protoplast transfection using PEG

1. Centrifuge protoplasts for 5 min at 300 rcf at 22 °C room temperature (centrifuge settings Accel=9 Decal=9).
2. Remove supernatant using pipette and quickly replace with 1 mL of MMG solution.
3. Estimate protoplast number using hemocytometer. Adjust the final density to 1 x 10^6/mL in MMG buffer
4. Prepare a DNA sample for transformation by mixing 20 µg of DNA constructs and remaining MMG solution to a total volume of 100 µL in a 2 mL tube. Note: Multiple constructs can be transformed simultaneously if DNA concentration does not exceed 25µg.
5. Add 200 µL of protoplast in MMG solution using a wide bored tip to each transformation tube and flick gently. Approximately 2 x 10^5 cells per transformation was used.
6. Incubate this DNA/protoplast solution at 22 °C temperature in the dark for 5-10 minutes.
7. Add an equal volume of PEG4000 (approx. 300 µL) and mix well either by swirling slowly or by flicking gently.
8. Incubate the tubes at room temperature (22°C) for 30 minutes in the dark.
9. Stop the transfection by adding 1mL of WI solution. Invert tubes very gently several times until the PEG4000 is completely dissolved.
10. Centrifuge at 300 rcf for 3min. Remove supernatant using 200 µL pipette tip without disturbing the pellet.
11. Wash the pellet with 1 mL of solution and centrifuge at 300 rcf for 3 min.
12. Carefully remove the supernatant using a 1 mL pipette (do not disturb the pellet).

13. Add 1mL of plating media to resuspend the pellet, invert gently 4-5 times. Centrifuge at 300 rcf for 3 min.

14. Remove supernatant using 200 µL pipette tip. Add 1 µL of plating media, invert gently until protoplasts are resuspended.

15. Transfer the protoplast solution to a low retention 6-well sterile culture plates.

Analysis of transfection efficiency

1. Using a wide bore pipette tip, add 10 µL of GFP transfected protoplasts to a microscope slide after 48 h.

2. Examine the protoplast using a florescence microscope (EVOS M5000, Invitrogen).

3. Choose five fields of view for picture and visualization (Fig 4).

4. Determine the total number of protoplasts and fluorescent cells visible

5. Repeat for each sample.

6. Calculate the transfection efficiency by dividing the number of fluorescent protoplasts by the total number of protoplasts of protoplasts observed.

Analysis of mutation

1. Extract genomic DNA from the transfected protoplasts.

2. Amplify the target region by PCR using specific primers flanking the gRNA (Forward primer 5′-ACTACAAGGGAGATGGGGTCA-3′, reverse primer 5′-TTAGCTTTGCGTTACCAGCTT-3′ using Q5 High Fidelity 2X Master mix (NEB)

3. Make a 25µL PCR reaction of 12.5µL Q5 Master mix + 1.25µL forward primer + 1.25 µL reverse primer + DNA 3 µL (50 -100ng) + 7 µL deionized water. Thermocycling conditions are 98 °C for 30 sec, 30 cycles of (98 °C for 30 sec, 66 °C for 45 sec, 72 °C for 45 sec) and a final hold of 72 ° C for 2 min

4. Analyze the PCR product by gel electrophoresis to detect modifications (Fig 5)

5. Purify the PCR product with Nucleospin Gel and PCR cleanup kit

6. Clone purified PCR product into cloning vector according to manufacturer's manual (CloneJet PCR cloning kit was used).

7. Isolate plasmid DNA from colonies and send for Sanger sequencing as per manufacturer's instructions. To determine mutation frequencies and type of mutations, design deep amplicon sequencing primers for each gRNA and perform nested PCR to amplify fragments with approximate lengths of 500 bp.

8. The mutation frequency from each sequenced sample was analyzed using CRISPResso2 and TIDE software [16,17].

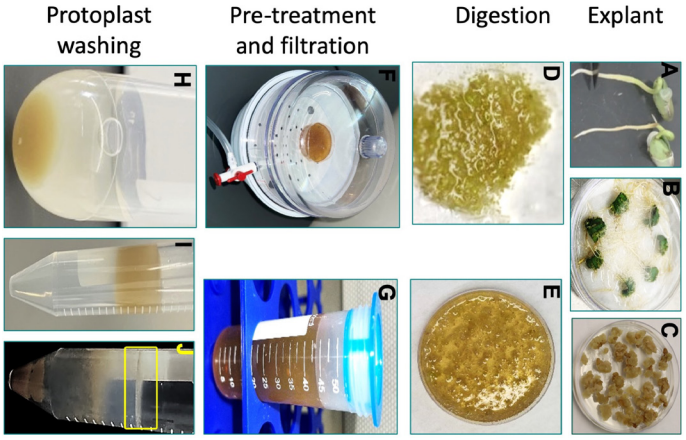

Figure 1: Steps involved in protoplast isolation. Soybean roots **(A)**, Hairy roots **(B)** and Callus **(C)** are selected. Explants were sliced into small pieces (**D** and **E**). Vacuum infiltration of tissues was carried out in presence of digestion solution **(F)** and infiltrated tissues were kept in the dark at appropriate time. Digested explants were passed through cell strainers **(G)**. Following the step 5 in section 3.3.2, protoplast settle at the bottom of the tube after centrifugation **(H)**. Overlaid protoplast solution on percoll gradient **(I)**. Protoplasts form visible layer, marked by yellow box **(J)**.

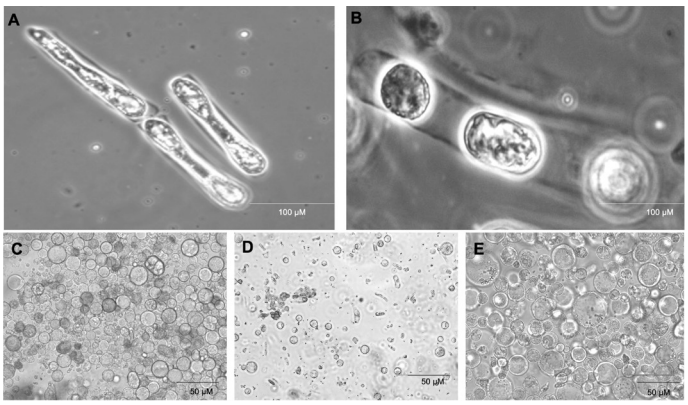

Figure 2: Effect of pre-plasmolysis **(A)** before plasmolysis treatment of hairy roots. **(B)** After a 4hr plasmolysis treatment of hairy roots. Freshly isolated protoplast from **(C)** Roots **(D)** Hairy roots and **(E)** callus. Magnification 20X.

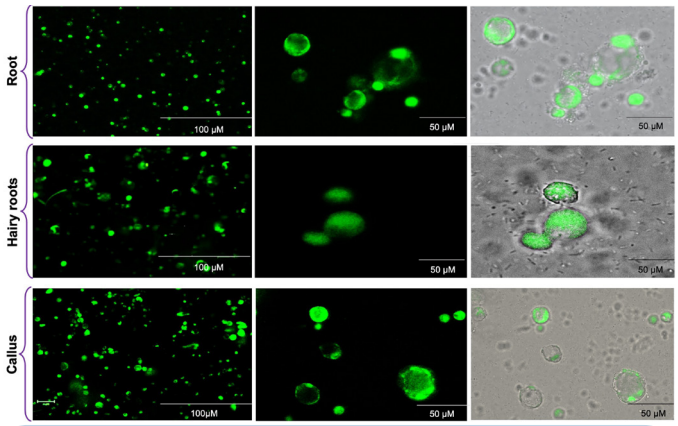

Figure 3: Soybean protoplast expressing GFP after 48 h of transformation. The plasmid pMOD_C3001 is used as a marker to determine the transfection efficiency. Cells fluorescing green are successfully transfected under 10x magnification (left), 40X magnification (middle), and merged (right).

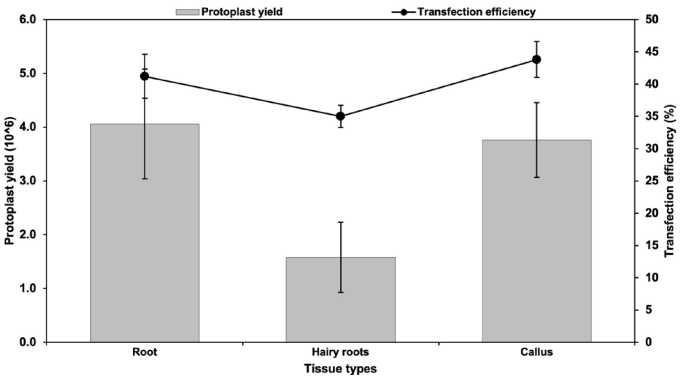

Figure 4: Protoplast yield and average transfection efficiency using 35S:GFP from different tissue types. Yield is calculated 10^6.

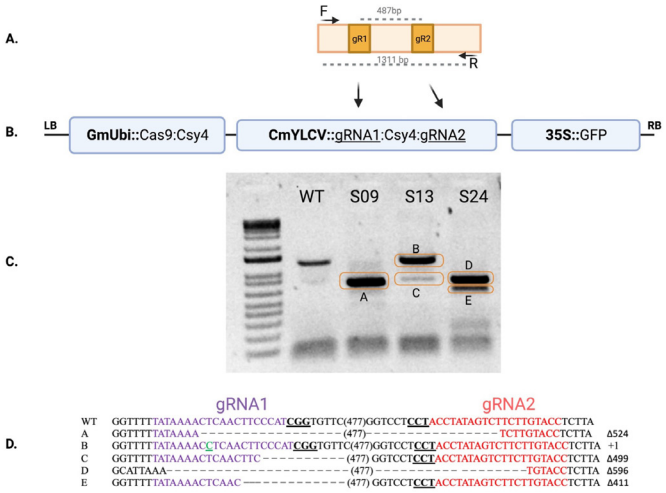

Figure 5: Schematic representation of the construct and gene editing. **(A)** Two CRISPR gRNAs (gR1 and gR2), 487bp apart at the promoter sequence of Glyma.08G107700 were designed. F and R are the primers designed to amplify the region covering both guide RNAs with the amplicon size of 1311bp. **(B)** Selected gRNAs were cloned under CmYLCV promoter as described. The final construct with Cas9, gRNAs and GFP was transformed into protoplast. **(C)** PCR amplification and gel electrophoresis of WT (non-transfected control) and three selected samples (transfected with pVD1) showing small and large mutations **(A - E)**. (D) Detection of mutations using Sanger sequencing.

Results & Discussion

Although protoplast isolation methods for several plant species is well established, the protocol used for one plant species, or one tissue type is not always amenable to other plant species or tissues. In a nutshell, there are several factors that can affect the yield and quality of the protoplast and some of the common challenges are cell wall composition, selection of cell wall digesting enzymes, concentration of enzymes and incubation time and tissue handling. Since the advent of gene editing and cell specific transcriptomics technologies, protoplast isolation from different tissues needs to be optimized to obtain high yield and quality protoplast. There have been few reports on soybean protoplast isolation from leaf [13], immature seeds [2] and one report on the isolation of protoplasts from soybean roots [19], and studies on the isolation of protoplasts from transgenic tissues (hairy roots and callus) are incredibly uncommon [20, 21]. In the current research, we tested several parameters that affect the overall yield and viability of protoplast from different tissue types.

In general, cellulase R10 and macerozymes R10 are commonly used enzymes in protoplast isolation including soybean leaf and immature seeds [13, 2]. However, use of similar enzyme types and their concentration conceded lower protoplast yield from hairy roots and callus tissue. Therefore, we tested 2% cellulase RS with different concentration of Pectolyse and found that 0.4 – 0.5% Pectolyse yielded relatively more protoplast (3.76×10^6 for callus tissue and 1.58×10^6 for hairy root tissue) as compared to cellulase R10 and macerozyme R10. It has been shown that cellulase RS, a mutant form of Cellulase R-10 can dissolve the cell walls of a larger variety of plants and has higher digestive activity [20]. The combination of cellulase R10 and macerozyme R10 yield highest protoplast yield (4.06×10^6). In agreement with other studies, we observed that. pectolyse Y-23 (that contains two highly active pectinase) release cells more effectively from the recalcitrant plant tissue [23].

We found that pre-plasmolysis (before enzymatic digestion) with 0.8M osmatic solution; mannitol and vacuum infiltration, allow efficient cell separation as compared to cells without plasmolysis treatment (Fig 2A and 2B). Plasmolysis provides loss of turgor and detachment of protoplast from the cell wall. Therefore, osmatic concentration of 0.6 to 0.8 M provide space between the cell wall and protoplast passage apparently providing pathway for diffusion of the cell wall digesting enzymes [24, 25]. Additionally, we observed that incubation time of 14 h – 16 h was more effective for root, hairy root, and callus tissues. Following the cell wall digestion and release of individual protoplast cell, it is pre-requisite to separate the intact and viable protoplast from remaining cell debris by centrifugation using sucrose or percoll gradient. In the current method we identified that percoll gradient was more effective than sucrose (Fig 1J). The larger cell debris was collected at the bottom of the centrifuge tube while viable and intact protoplast was captured on the top-most layer.

The present study aimed to develop an improved method for the isolation, transfection, and gene-editing of protoplasts from a variety of non-transgenic (root), and transgenic (hairy root and callus) tissues from soybean to assess the potential of protoplasts as versatile and efficient tool for transient gene expression experiments in soybean. High-quality, viable protoplast isolated with our improved method, the transfection efficiencies of 41.2%, 35%, and 43.8% (in the root, hairy root and callus respectively) were achieved (Fig 3 and Fig 4). This is comparable to or higher than those reported in previous studies on soybean protoplast transformation isolated from other tissue types [2, 11, 13]. To evaluate the efficacy of protoplasts for gene editing, all three types of protoplast cells were transformed with a pVD1 construct targeting two gRNAs in the promoter region of the Glyma.08G107700 gene. The gRNAs were designed approximately 487bp apart with the intention to induce larger deletions. Following the transfection and incubation for 72hrs, protoplast cells were harvested to isolation genomic DNA from individual samples and amplification of the target gene was carried out using flanking primers (Fig 5). Amplified products were run on agarose gel to identify putative mutants with large deletions. We identified variety of small insertion and deletions mutations. We specifically selected three samples showed larger deletion (Fig 5) and analyzed for type of mutations. To further characterize the mutation type at the target sites, the PCR fragments were recovered and cloned into pJET vectors for Sanger sequencing. The results indicated the presence larger deletion up to 596bp (Fig 5). Overall, we developed a method to isolate high-quality protoplast from different types of soybean tissues

and demonstrated the potential of protoplasts as a valuable tool for both transient gene expression experiments and gene editing in soybean.

Acknowledgements

We would like to thank Dr. Daniel Voytas and Dr. Colby Starker for providing gene-editing tool kit.

Funding

The work was carried out with the financial support of Governor's University Research Initiative (GURI) to GP.

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