Methods in Molecular Biology 2464

Springer Protocols

Kan Wang · Feng Zhang *Editors*

Protoplast Technology

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-0716-2163-9 ISBN 978-1-0716-2164-6 (eBook)
https://doi.org/10.1007/978-1-0716-2164-6

Preface

A tissue culture procedure that was considered obsolete is now drawing great attention from the plant community, thanks to the advent of genome editing technology. Plant protoplast culture system, an "ancient" procedure to establish a wall-less single-cell plant culture, is revitalized and becoming an important tool for many laboratories in their endeavor of plant genome engineering using molecular reagents such as CRISPR/Cas. Plant protoplast culture can mimic mammalian cell culture in the transient evaluation of gene function or CRISPR/Cas efficacy using transfection. The automation and high-throughput potential of the protoplast culture system make it even more powerful when combined with the genomic and single-cell technologies. Moreover, for many plant species, their protoplasts can be further cultivated to form multicellular callus culture. These callus cultures can differentiate and ultimately regenerate into plants that can be brought to maturity. Because of this totipotency, protoplast culture can be used as an effective system to produce gene-edited plants without incorporating any exogenous genes into the genome.

This book invites scientists who are actively engaged in developing or using protoplast technology to share their protocols with the community. The book contains two parts. Part I collects 14 chapters that focus on basic protoplast techniques and their utilities. This part presents protocols of protoplast isolation, transfection, and regeneration as well as examples of how to use protoplasts for genome editing and gene function analysis in a number of major crop or model plant species. Chapters 1 and 2 describe how to make protoplasts from single-cell plant species *Physcomitrium patens* and *Bienertia sinuspersici*, respectively. Chapter 3 demonstrates a highly efficient protocol for protoplast isolation and transfection in Arabidopsis. It also provides examples of utilizing the system for gene functional analysis in this important model plant. Two chapters deal with another model *Nicotiana* species, one for *N. tabacum* (Chap. 4) and the other one for *N. benthamiana* (Chap. 5). Both chapters include protocols of using the protoplast system for CRISPR/Cas-mediated genome editing as well as protoplast regeneration. Five chapters focus on important grain crops, i.e., rice (Chap. 6), maize (Chaps. 7–9), and wheat (Chap. 10). The three maize chapters include protocols for the isolation of protoplasts from maize mesophyll tissue (Chap. 7), endosperm (Chap. 8), and shoot apical meristem (Chap. 9). These protocols can be leveraged for future single-cell genomics research. Chapter 11 provides isolation, transfection, and regeneration protocols for recalcitrant forage and turf grasses. Chapters 12–14 teach how to handle industrial oil crops, including winter oilseed pennycress and camelina (Chap. 12), soybean (Chap. 13), and oil palm (Chap. 14).

Part II invites chapters from three groups who are at in the forefront of protoplast automation, large-scale functional genomics, and synthetic biology. Chapter 15 describes a method of fluorescence-activated cell sorting for the enrichment of a specific protoplast population in Arabidopsis. This technique enables the quantification and evaluation of CRISPR/Cas reagents in desired cell types. Chapter 16 illustrates protoplast transfection protocols in both low- and high-throughput (96 well) fashion in *Setaria viridis*. It also demonstrates the use of the system for characterizing programmable transcription activators. Finally, Chap. 17 offers a soybean protoplast system that is designed for automated high-throughput transfection and quantitative analysis.

The basic principle of protoplast preparation and transfection are straightforward. However, applying the principle to each plant species can result in different outcomes. Some slight modifications in enzyme concentrations, incubation time, temperature, and even the sources of the chemicals can make large differences in protoplast yields and transfection frequency. This book will give our readers not only the protoplast protocols for their specific plant of interest but also showcase how experts deal with other different species. It is hoped that this collection will inspire the new generation of researchers to further improve the protocols and apply the technology to accelerate plant genomic study.

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Part I

Basic Techniques and Their Applications

Chapter 1

Physcomitrium patens Protoplasting and Protoplast Transfection

Florence Charlot, Guillaume Goudounet, Fabien Nogué, and Pierre-François Perroud

Abstract

Protoplast production with the moss *Physcomitrium* (*Physcomitrella*) *patens* has a long and successful history. As a tool, it has not only been the base of reverse genetic studies covering research fields as diverse as development, metabolism, or gene network regulation but also allowed its development as a bioengineering platform for protein production. We present here a standardized protocol for protoplast production from *Physcomitrium* (*Physcomitrella*) *patens* protonemata. Additionally, we detail procedures for their transfection, their plating for optimal regeneration, and three alternative selection approaches. To improve the consistency of protoplast regeneration, we describe a new option for protoplast embedding. The use of an alginate matrix to regenerate moss protoplast alleviates the use of warm agarized medium. Thus, it optimizes transformed protoplast survival without any morphological detrimental effect or impact on transfection efficiency.

Key words Alginate, CRISPR-Cas9, Homologous recombination, PEG-mediated transfection, *Physcomitrella patens*, Protoplast, Reverse genetics

1 Introduction

Isolating a plant cell without its cell wall, the protoplast, is a goal achieved by whom we can call the first plant cell biologists at the end of the nineteenth century [1]. The mechanical method used during this early developmental period did not allow the protoplast production in large number, and its use was mostly restricted to descriptive approach and small-scale culture trials. The use of a biochemical approach, e.g., the digestion of the cell wall using pectolytic enzyme(s), changed the paradigm. Inspired by similar experiments performed on bacteria and fungi, E.C. Cocking isolated for the first time protoplasts from tomato root tip cells using cellulase extract [2, 3]. This led to subsequent trials with other plants and together with the development of reliable

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_1,

commercial cell wall-digesting enzymes, purified or in mixtures, finally allowed to protoplast tobacco leaf cells and subsequently regenerate a whole plant from a single protoplast [4, 5]. The 1970s and 1980s were very active in this field, so much so that a survey counted in 1989 212 seed plant species for which protoplasts had been obtained [6]. The establishment of a consistent protocol for viable protoplast isolation was, and still is for new species, not a small endeavor as the type of tissue (developmental stage, age) as well as the cell wall-degrading enzymes mixture and its utilization is varying with each new species and need specific optimization. Additionally, the protoplast regeneration into a whole plant also demands a species-specific procedure that is not available today for the majority of the tested species.

In this context, bryophytes, particularly *Physcomitrium* (previously *Physcomitrella*) *patens* and the family of Funariaceae, ended up being a very good source of material for protoplast. Even with suboptimal pectolytic enzyme mix, the protoplasts isolated from protonemal tissue displayed an incredible regeneration property: as long as it is generated and initially maintained in the appropriate osmotic environment, a regenerating protoplast will recapitulate normal developmental program without any phytohormonal input [7]. The use of the enzyme mix Driselase, a crude extract of basidiomycetes, provided the optimal protoplast production conditions in terms of yield without changing the regeneration properties of these isolated cells [8]. The use of *P. patens* protoplasts has followed the development of the plant cell and plant molecular genetic approaches in this model plant. They were initially used as a source of single cells to study moss cell polarity, notably with respect to photo-polarotropism [9]. Protoplasting has been used to individualize single cell regenerant from tissue mutagenized by chemical [10] or directly mutagenized chemically or by UV irradiation [11]. The procedure to fuse protoplasts issued from different strains allowed successful complementation studies between mutants [8, 12]. Recently, this approach associated with deep genome sequencing allowed the identification of the mutant *nog1*, a mutant unable to form gametophore [13]. The recent development of microfluidic approaches demonstrated that *P. patens* protoplasts could be used successfully in small percolation chamber. Protoplast regeneration, protonemal growth, and budding processes can be followed in a dynamic way in bright-field and fluorescence microscopy [14–16]. Additionally, precise controlled hormonal and pharmaceutical treatments can be performed and analyzed over several days [14, 16].

The development of PEG-mediated transfection of *P. patens* protoplasts using naked DNA [17] opened the way to the discovery that made this moss the model plant it is today: the efficient homologous recombination repair pathway of its vegetative cell [18]. It allows for the efficient gene knock-out and knock-in, and

today, most of published reverse genetic studies in *P. patens* use this method to generate their specific plant material. With the development of these techniques surfaced another important property of *P. patens* protoplasts: upon DNA uptake, a plasmid containing a plant resistance cassette is maintained episomally as long as a selection pressure is applied [19]. This property allows for the efficient short-term transient expression of proteins. For example, using fluorescent reporters coupled with a flow cytometry approach, transiently transfected protoplasts can be used to study gene expression in a qualitative and quantitative manner [20]. The maintenance of a construct transiently allowed also the successful development of a robust RNAi-mediated silencing reverse genetic procedure [21], very useful in the study of the filamentous growth processes in *P. patens* (*see* notably [22–24]). Finally, *P. patens* protoplast transfection lends itself particularly to the use of the CRISPR-Cas9 editing tools either to edit a specific genomic sequence [25, 26] or to integrate a new transgene sequence [27]. After transfection, regenerating protoplasts expressing transiently CRISPR-Cas9 and guide RNA constructs can be selected for a short time period with antibiotic selection, and the majority of such selected plants will display an edited genomic target. The constructs, on the other hand, are eliminated from the mutated plant when the selection pressure is removed.

The procedures presented here will focus specifically on four major experimental phases encountered when dealing with protoplast in *P. patens* (Fig. 1): their production (Subheading 3.1) and plating (Subheading 3.3) common to standard procedures leading to protoplast regeneration to whole plant (transfected or not), protoplast transfection (Subheading 3.2), and three distinct procedures of selection (Subheadings 3.4, 3.5 and 3.6) corresponding to three strategies of gene modulation or modification, RNAi, homologous recombination, and CRISPR-Cas9. Most of the protocols we present here are based on well-established and published methods [18, 22, 28] except the protocol of plating of the protoplasts that is, to our knowledge, completely new. This protocol is based on the polysaccharide alginate that has been since a long time proposed alternative to top agar methods for plant protoplast embedding [29] including for microencapsulation of *P. patens* protoplasts in the context of protein production [30]. The alginate-based method presented here to regenerate *P. patens* protoplasts removes the need to use a warm agar solution to plate protoplasts and displays to our eyes a clear improvement in regeneration rate consistency. Such protoplasts are regenerating (Fig. 2) morphologically in a similar way to protoplasts embedded in agar and show, in our experience, the same properties in terms of DNA transfection.

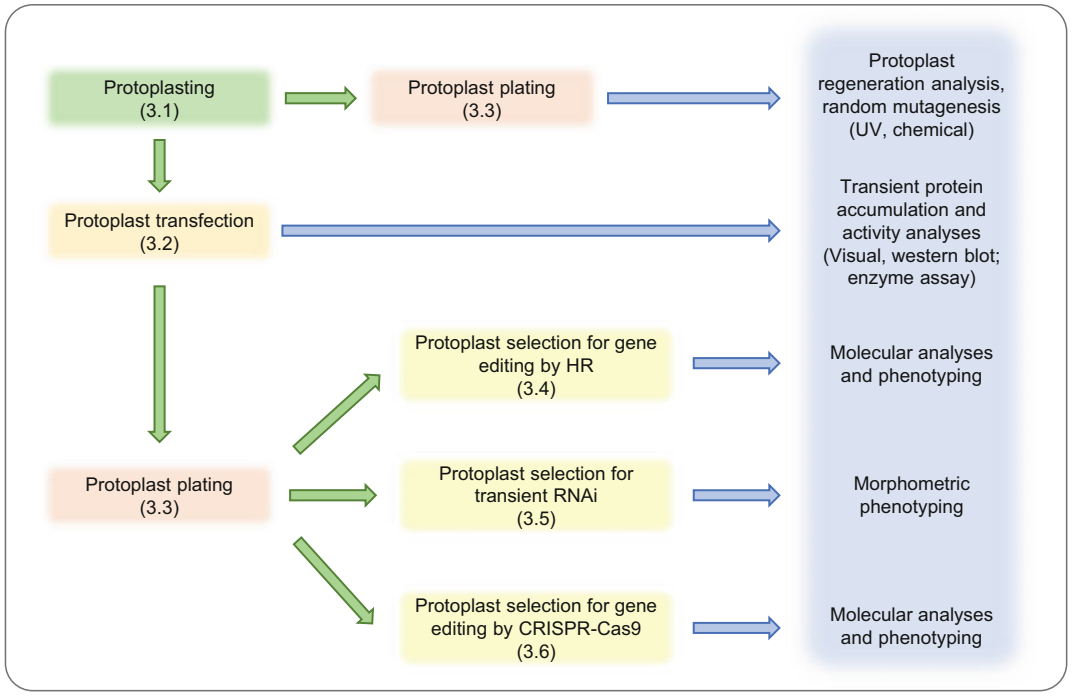

Fig. 1 Layout of the protocols. The green, orange, pink, and yellow shading indicate protocols described, with numbers representing corresponding subheadings in the chapter. The blue shading illustrates downstream use for protoplasts processed with the described procedures

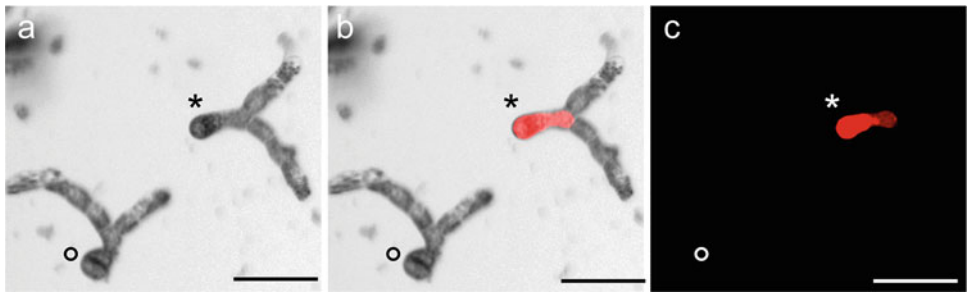

Fig. 2 Transfected *Physcomitrium patens* protoplasts. Protoplasts were produced as described in Subheading 3.1 and transfected according to Subheading 3.2 with 15 μg of the pTHUbi:mCherry vector. They were subsequently incubated for a week in a liquid dilution medium in a Petri dish. Images were acquired with a Zeiss Axio Zoom.V16 Stereo Microscope fitted with a Cy3 Sp Red_AHF_ 36750_Exc586/20_Dichroique605_Emi 628/32 set. (**a**) Bright-field image. (**b**) Merged bright-field and fluorescent image. (**c**) Fluorescent image. (°) points to a non-transformed protoplast. (*) denotes a transformed protoplast. Bar: 100 μm

2 Materials

All media described here are prepared in distilled water (dH_2O), autoclaved, and stored at room temperature (20–24°C) for up to 2 months, unless specified otherwise.

2.1 Plant and DNA Materials

1. Moss material: Axenic culture of the moss *Physcomitrium patens* (previously *Physcomitrella patens*). The protocols described here have been used successfully with different ecotypes of this species, initially mostly with Gransden but also with Villersexel and Reute. Wild-type *P. patens* strains are available at the International Moss Stock Center (IMSC) (https://www.moss-stock-center.org/en/), and specific mutant can be easily shipped as start-up culture from research laboratories across the scientific community.
2. pTHUbi:mCherry: Construct used for visual confirmation of successful transfection. This vector contains a strong fluorescent protein expression cassette. This cassette is formed of the mCherry fluorescent protein gene driven by the maize ubiquitin promoter that permits an easy fluorescent detection of a transfected protoplast. For the detailed vector map and vector assembly, *see* ref. 31.
3. pBNRF: Standard backbone vector used for gene targeting. This vector contains the gene *nptII* under the control of the Cauliflower Mosaic Virus 35S promoter which confers G418 antibiotic resistance in plants. This cassette is flanked by two multicloning sites which permit the cloning of targeting genomic sequences specific to the locus of interest. Detailed map and sequence can be found in [32].
4. pTUGGi: This vector is used to target and degrade through RNAi-mediated pathway both a transcript of interest and the fluorescent marker constitutively nuclear fluorescent marker of *P. patens* NLS-4 strain. Upon plant regeneration and selection, this permits to visually confirm RNAi construct efficiency, e.g., the loss of nuclear GFP, and the phenotype generated by targeting the transcript of interest. Detailed description and use of these vectors can be found in [21, 23].
5. pACT-Cas9, psgRNA, and pBNRF: Routinely, co-transformation of three vectors is used to target a given locus by CRISPR-Cas9. The first vector, pACT-Cas9, contains the Cas9 cDNA, codon optimized for eukaryotes, driven by the rice Actin promoter. The second, psgRNA, contains the guide RNA (sgRNA) specific to the locus of interest under the *P. patens* U6 promoter. The third and last vector, pBNRF, contains a plant resistance cassette to G418 necessary for transient selection of the transformed protoplasts. Detailed description and use of these vectors can be found in [26].

2.2 Stock Solutions

1. Phosphate (184 mM, 1000×): For preparing 100 mL solution, add 25 g KH_2PO_4 to 90 mL of dH_2O, adjust to pH 7.0 using 10 M KOH, and bring final volume to 100 mL with dH_2O.

2. Microelements (1000×): Dissolve 5.5 mg $CuSO_4 \cdot 5H_2O$, 5.5 mg $ZnSO_4 \cdot 7H_2O$, 61.4 mg H_3BO_3, 38.9 mg $MnCl_2 \cdot 4H_2O$, 5.5 mg $CoCl_2 \cdot 6H_2O$, 2.8 mg KI, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$ in 100 mL dH_2O.
3. $CaCl_2$ (1 M): Dissolve 11.1 g $CaCl_2$ in 100 mL dH_2O. Autoclave and store at room temperature up to a year.
4. Tris–HCl (1 M, pH 7.2): Dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to 7.2 by adding concentrated HCl and bring the volume to 1 liter with H_2O. Autoclave and store at room temperature up to a year.
5. Hygromycin B 1000× (20 mg/mL): Dissolve 25 mg of hygromycin B in sterile water. Store at −20°C until use.
6. G418 1000× (30 mg/mL): Dissolve 30 mg of G418 in sterile water. Store at −20°C until use.

2.3 Media

1. Solid $PpNH_4$ medium: In 1 L dH_2O, add 0.8 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.0125 g $FeSO_4 \cdot 7H_2O$, 0.5 g ammonium tartrate dibasic, 1 mL of phosphate (1000×), 1 mL of microelements (1000×), and 7 g plant tested agar. Sterilize by autoclaving and pour 25 mL of medium per 9 cm Petri dish.
2. Liquid regeneration medium: In 1 L dH_2O, add 0.8 g $Ca(NO_3)_2$ $4H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.0125 g $FeSO_4 \cdot 7H_2O$, 85 g mannitol, 1 mL of phosphate (1000×), and 1 mL of microelements (1000×). Sterilize by autoclaving. Before use, add 10 mL of sterile 1 M $CaCl_2$.
3. Solid regeneration medium: In 1 L dH_2O, add 0.8 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.0125 g $FeSO_4 \cdot 7H_2O$, 0.5 g ammonium tartrate dibasic, 66 g mannitol, 1 mL of phosphate (1000×), 1 mL of microelements (1000×), and 7 g plant tested agar. Sterilize by autoclaving, and before use, add 10 mL of sterile 1 M $CaCl_2$. Then pour 25 mL of medium per 9 cm Petri dish.

2.4 Protoplast Handling Solutions

1. Mannitol solution (8.5%): Dissolve 85 g of mannitol to 1 L of dH_2O. Sterilize by autoclaving and store at room temperature.
2. Driselase stock solution: In 100 mL dH_2O, add 8.5 g of mannitol and 2 g of Driselase (*see* **Note 1**). Mix the solution well for 20 min at room temperature. Centrifugate the solution at 4500 × *g* for 20 min. Collect the supernatant and filter it through a 0.2 μm filter unit. Aliquot the enzyme solution into sterile plastic tubes, 10 mL per tube. Store at −20 °C for up to 1 year.
3. Alginate stock solution: In 100 mL dH_2O, add 8.5 g mannitol and 1.5 g alginate. Sterilize by autoclaving and store at room

temperature. Filter the solution through a 0.2 μm filter to remove any precipitate before use.

2.5 Transfection Solutions

1. MMM solution: In 100 mL dH_2O, dissolve 8.5 g mannitol, 0.305 g $MgCl_2$, and 0.1 g MES [2-(N-morpholino)ethanesulfonic acid]. Adjust pH to 5.6 using about 25 μL of 10 N KOH. Filter sterilize using a 0.2 μm filter, and store the solution at 4 °C for up to 6 months.
2. PEG solution: In 50 mL dH_2O, add 3.5 g mannitol, 1.18 g $CaNO_3$, 0.5 mL of 1 M Tris–HCl (pH 7.2), and 20 g of polyethylene glycol (PEG) 4000 (*see* **Note 2**). Filter sterilize using a 0.2 μm filter, and store at −20 °C for up to 1 year.
3. TE solution: This contains 100 mM Tris–HCl and 10 mM EDTA (pH 8). Store at room temperature and filter sterilize using a 0.2 μm filter before use.

2.6 Other Supplies

1. Metallic forceps (sterilizable).
2. Sterile hood bench, e.g., laminar flow hood.
3. Water bath.
4. Sterile 9 cm Petri dish with vent.
5. Vent tape (3M Micropore™ paper surgical tape).
6. Sieve, 80–100 μm, sterile.
7. Sieve, 40–50 μm, sterile.
8. Sterile conical culture tube, 50 mL.
9. Sterile round bottom culture tube, 15 mL.
10. Cellophane, sterile.
11. Centrifuge equipped with a swinging-bucket rotor and allowing precise low centrifugation (e.g., Eppendorf Centrifuge 5910).
12. Growth chamber or biological incubator for the incubation with light and temperature control.
13. Counting chamber, Malassez type or equivalent.
14. Stereomicroscope with magnification lens that permits protoplast counting (30 μm diameter).

3 Methods

The protocols described hereafter need to be performed at room temperature (20–24 °C) if not mentioned otherwise and in a sterile environment to protect the samples, e.g., vertical or horizontal laminar flow hood. Note that an ambient temperature above 26–27 °C is detrimental to protoplast regeneration. Standard

in vitro plant culture precautions concerning sterility (for surface, tools, and reagents) are required. Albeit not an absolutely necessary measure, we recommend turning off the light of the laminar flow hood to shade protoplasts from direct light during their production and transformation. Finally, for an optimum protoplast regeneration and protonemal growth, Petri dish with vent must be used and they must be sealed with 3 M Micropore tape (or equivalent) to allow normal gas exchange in the plate (no Parafilm seal). The growth culture conditions are uniform for tissue culture and protoplast regeneration: the temperature set between 22 and 25 °C and long-day photoperiod (16 h light/8 h darkness) with light intensity between 50 and 70 μmol/m^2/s.

3.1 Protoplast Production

1. Harvest and rapidly transfer tissue (do not let the tissue dry) from four Petri dishes of entrained 6–7-day-old moss protonemata grown on a $PpNH_4$ medium overlayed with cellophane (*see* **Note 3**) into 15 mL of 8.5% mannitol solution.
2. Add 5 mL of 2% Driselase solution from frozen stock for a final Driselase concentration of 0.5%. Incubate for 45 min at room temperature with occasional gentle plate swirling (*see* **Note 4**).
3. Filter the suspension through sterile 80–100 μm sieve to a sterile 100 mL beaker. Let the protoplast suspension incubate at room temperature for 15 min.
4. Filter the suspension through sterile 40–50 μm sieve to a new sterile 100 mL beaker. Pour the flow-through (containing the isolated protoplasts) into a tube that can withstand low-speed centrifugation (e.g., 50 mL conical culture tube).
5. Centrifuge for 5 min at 150 × *g* at the room temperature to sediment the protoplasts.
6. At this stage, a dark green pellet should be visible and the supernatant should be brownish (filtered Driselase solution color), not green. Discard the supernatant by tilting the tube.
7. Gently resuspended the pellet into 20 mL of 8.5% mannitol solution supplemented with 200 μL of 1 M $CaCl_2$ (10 mM final concentration).
8. Repeat **steps 5** and **6** above.
9. Resuspended the pellet into 20 mL of 8.5% mannitol supplemented with 150 μL of 1 M $CaCl_2$ (7.5 mM final concentration). Estimate the protoplast number in suspension using a counting chamber (*see* **Note 5**).
10. Centrifuge the tube at 150 × *g* for 6 min to sediment the protoplasts. Resuspend the pellet in the appropriate medium for further use.

You have now a known number of protoplasts that can be used for transfection (Subheading 3.2) or can be plated directly on

regeneration medium (Subheading 3.3) for any test of interest. Protoplast yield should be between 0.5 and 1 × 10^6 protoplasts per one 9 cm Petri dish containing 6–7 days entrained protonemal culture (*see* **Note 6**). A single protoplast transfection experiment will require between 3.6 and 4.8 × 10^5 protoplasts.

3.2 Protoplast Transfection

1. In a 15 mL sterile tube, add 15 μg of vector DNA resuspended in sterile TE with a maximum of 30 μL volume (*see* **Note 7**).
2. Add 300 μL of protoplast resuspended in MMM buffer. Protoplast concentration in the MMM buffer can be anywhere between 1.2 and 1.6 × 10^6/mL without affecting transfection efficiency with the volume ratio for each component presented in this protocol (*see* **Note 8**).
3. Add 300 μL of PEG transfection solution (from frozen stock) and mix gently (do not vortex or pipet up and down the suspension) but thoroughly, producing visually uniform solution.
4. Incubate the tube in a 44 °C water bath for 5 min.
5. Let the tube stand at room temperature for 10 min.
6. The PEG transfection solution is toxic to the protoplasts. Dilute the transfection mix by adding, at 1 min interval, five times 300 μL, and then five times 1 mL liquid regeneration medium. Mix gently by swirling the tube after each dilution step.
7. Let the tubes stand at room temperature for 30 min. At this stage, protoplasts have been transfected. The tube can be used as incubation chamber and transferred in growth chamber for further use. Figure 2 shows an example of 1-week-old protoplast transfected with the vector pTHUbi:mCherry [31] expressing transiently a red fluorescent protein. Alternatively, they can be plated for regeneration (*see* Subheading 3.3).

3.3 Protoplast Plating

Plating protoplasts unto solid medium (containing agar) allow them to regenerate into protonemal cell optimally (Fig. 3). A non-transfected protoplast population displays regularly 80% regeneration. But the regeneration of transfected protoplasts is significantly lower, between 10% and 30% for wild-type tissue. This number can be significantly reduced for mutant strains.

1. To concentrate the protoplast suspension, transfected or not, centrifuge for 5 min at 150 × *g* to sediment the protoplasts. Discard the supernatant.
2. For a typical protoplast transfection as described in Subheading 3.2, add 4 mL of fresh mannitol 8.5% supplemented with 7.5 μL of 1 M $CaCl_2$ (7.5 mM final concentration).
3. Gently resuspend the protoplasts by swirling the tube.

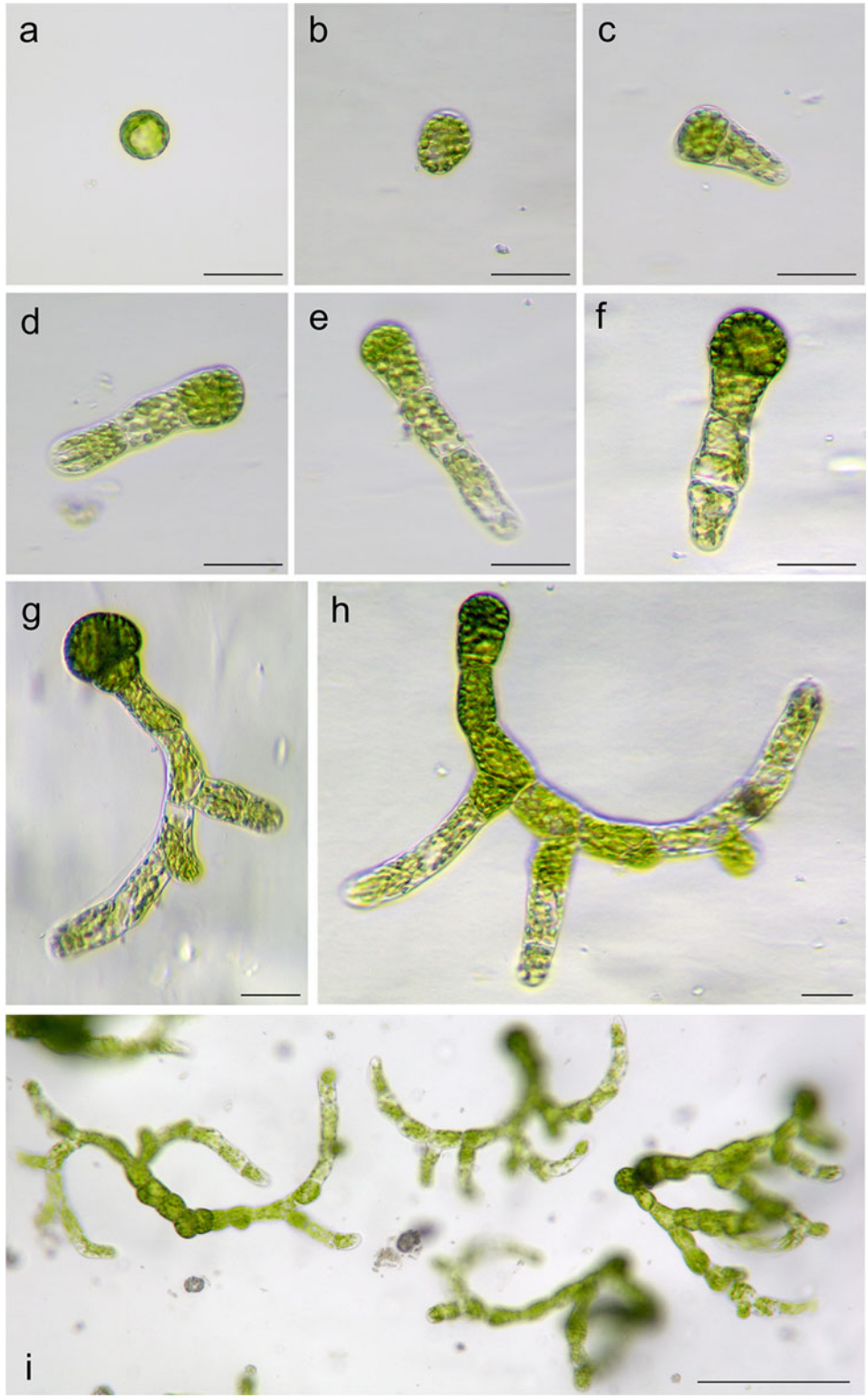

Fig. 3 *Physcomitrium patens* protoplast regeneration in alginate mixture. Protoplasts were produced as described in Subheading 3.1 and plated according to Subheading 3.3. Subsequently, they regenerated in

4. Add 4 mL of alginate storage mix to the protoplast suspension and mix gently by pipetting up and down (*see* **Note 9**).
5. Pour readily 2 mL of mixture per 9 cm Petri dish filled of solid regeneration medium overlayed with sterile cellophane (*see* **Note 10**). Each standard transfection is spread on four Petri dishes.
6. Spread equally the mixture by rotating the Petri dish and seal it with Micropore tape.
7. Let the mix set in the incubator for an hour and transfer into the growth chamber.
8. Let regenerate the protoplasts in standard growth conditions for a week (*see* **Note 11**). At this point, they should display the development shown in Fig. 3i.

3.4 Selection Procedure for Integration Event by Homologous Recombination

This protocol aims to select regenerated protoplasts that experienced integration of the transformation vector into their nuclear genome ("stable transformants") from (1) non-transfected regenerated protoplasts, killed with the first selection round, and (2) transfected regenerated protoplasts that did not experience integration of the transformation vector into their nuclear genome ("episomal transfectants" or "unstable transformants"), eliminated by the dilution of the episomal elements during the release step and killed during the second round of selection. Before using the protocol described in this section, the protoplasts must be already regenerated into plants. Plant in this context is defined as a protoplast which has achieved at least a cell division and one of the two cells started to elongate (*see* Fig. 3c). Such tissue displays characteristics of a protonemal cell. This protocol requires using antibiotics such as hygromycin B at 20 mg/L and G418 at 25 mg/L.

1. In order to evaluate transfection efficiency (regardless of the gene targeting efficiency), you must count the growing plant at the beginning of this step. This number is your effective number of plants submitted to selection (not the number of transfected protoplasts!).
2. Using sterile forceps, transfer the cellophane containing the regenerated protoplasts onto a $PpNH_4$ medium plate supplemented with an appropriate antibiotic (for which resistance is present in the transformation vector).
3. Incubate plates for 7 days in standard growth conditions (*see* **Note 12**).

Fig. 3 (continued) standard growth conditions for 1 week. Images were acquired with a Zeiss Axio Zoom.V16 Stereo Microscope at the following time points: (**a**) 1 h after plating; (**b**–**d**) 48 h after plating; (**g**–**h**) 5 days after plating; (**i**) 7 days after plating. Bar in (**a**–**h**): 50 μm. Bar in (**i**): 200 μm

4. Transfer cellophane with regenerating protoplasts to a fresh $PpNH_4$ plate devoid of antibiotic and incubate for 14 days in standard growth conditions (*see* **Note 13**).
5. Transfer cellophane onto $PpNH_4$ plate supplemented with the appropriate antibiotic (for which resistance cassette is present in the transformation vector) and incubate for 1 week in standard growth conditions (*see* **Note 14**).
6. At the end of **step 5**, pick, using a stereoscope placed in a laminar flow bench, any plant still growing and transfer them onto a fresh $PpNH_4$ plate for tissue amplification (*see* **Note 15**).
7. Optional: In case of doubt about the stability of a stable transformant, a strain can be tested at any time for antibiotic resistance by culturing a small tissue piece onto a $PpNH_4$ medium with selective agents (*see* **Note 16**).
8. As soon as enough material plant at the end of **step 6** (or 7) is available, it is ready to be submitted to genotyping procedure to evaluate the quality of the transformant (targeting, nonspecific insertion, copy number).

3.5 Selection Procedure for Transient Expression of RNAi Construct

This protocol aims to select transfected protoplasts that transiently express the RNAi construct targeting to the mRNA of choice and the resistance gene on the plasmid. The full phenotyping procedure is performed under selection to obtain a uniform construct expression in the whole plant. The identification of the RNAi impact is performed in comparison with the vector without a specific transcript sequence. To maximize the number of transfected plants for analysis, we recommend using the antibiotics hygromycin B at 10 mg/L and G418 at 12.5 mg/L for selection.

1. Transfer the cellophane containing the regenerating protoplasts onto a $PpNH_4$ plate supplemented with an appropriate antibiotic (for which resistance is present in the RNAi transformation vector).
2. Incubate for at least 4 days in standard growth conditions before starting the phenotype analyses. The observations must be performed on the same selective plate for 2 weeks. If the phenotype observation requires a longer growth period, transfer the culture to a fresh plate of selective medium.

3.6 Selection Procedure for Transient Expression of CRISPR-Cas9 Construct

This protocol aims to select transfected protoplasts that transiently express the Cas9 gene, the RNA guide(s) targeting the gene(s) of interest, and an antibiotic resistance gene. Hence, the plants selected here should not contain any insertion of the vectors but rather have their genome modified at the locus targeted by the RNA guide. The identification of the mutation comes in a second experimental phase during which the targeted locus/loci is

analyzed in the antibiotic-resistant plants for the presence of the mutation. It is recommended that the antibiotics hygromycin B at 20 mg/L and G418 at 25 mg/L should be used for this protocol.

1. Transfer the cellophane containing the regenerating protoplasts onto a $PpNH_4$ plate supplemented with an appropriate antibiotic (for which resistance is present in the transformation vector).
2. Incubate for 1 week under standard growth conditions.
3. Pick individual plant under a binocular lens and transfer onto $PpNH_4$ medium plates without antibiotic. Allow the plant to grow for 2 weeks or until you are comfortable to fragment the tissue and isolate enough plant material for genotyping.

4 Notes

1. Albeit several sources for the Driselase are commercially available, only the Driselase D8037 and D9515 from Merck KGaA, Darmstadt, Germany, are giving us a consistent satisfying result. Nevertheless, the Driselase activity can vary from batch to batch. Time of incubation can be reduced to 30 min if the protoplasts display a low regeneration rate, and if the protoplasting does not occur efficiently, final Driselase concentration can be raised up to 1%. Finally, note that Ca^{2+} has a strong Driselase inhibitory activity, so be careful to keep all components $CaCl_2$ free for this step.
2. PEG 4000 is commercially available from many sources. The brand that gives us the most regular positive result in terms of protoplast regeneration after transfection is the PEG 4000 #33136 SERVA Electrophoresis GmbH, Heidelberg, Germany.
3. The tissue should contain only protonemal cells. The presence of gametophores indicates that the tissue may be too old. Young tissue generates protoplasts more uniformly.
4. The first cells to be released are the tip cells of the tissue. It is possible to observe them after 10 min of treatment. Treatment longer than 1 h may release more protoplasts, but protoplast regeneration will be affected, resulting in no real gain in overall regeneration. After 45 min, the suspension should be green with a rapidly sedimenting colored component, the protoplasts. The tissue may not be totally protoplasted.
5. Different hemacytometer or counting-chamber device cells are available such as Malassez or Neubauer counting chamber. They all permit accurate cell counting, follow the manufacturer's instruction so to count properly the protoplasts.

6. It is possible to produce protoplast from protonemal culture as young as 3 days of growth after blending but protoplast number will be low until 5 days of growth, so days 6 and 7 represent the two best options to generate protoplasts used for transformation. Older culture (8–10 days' cultures) can generate more protoplasts, but in our hands, their transformation efficiency is dropping.
7. The DNA used for transfection can be produced by standard PCR amplification or plasmid prep amplification. For transient expression, plasmid amplified DNA needs to be used in its circular form. Except when the strategy for stable integration of the vector is based on the CRISPR-Cas9 technique [33], the transformation vector must be cut on each side of the transformation vector producing a linear, open-ended DNA fragment for stable transformation. This fragment type improves DNA integration in the genome and reduces the number of episomal transient transformants. TE and water work equally well as solute for the DNA, what is most important is the sterility of the solution. The use of DNA ethanol-precipitated vector DNA is recommended for all transfections. To avoid airborne contamination, perform this operation in a laminar flow hood.
8. Threefold proportional reduction of the volumes (1.2×10^5 protoplasts/5 μg DNA/100 μL PEG mix) to scale down the transfection reaction works successfully in our hands but gives of course three times less transfectants.
9. Alginate gelation is sensitive to divalent ion concentration, here $CaCl_2$. The present protocol $CaCl_2$ concentration in the plating mix is 3.75 mM, and it must be followed. Too much $CaCl_2$ leads the protoplast-alginate mix to gel too fast (and thus render proper plating not possible). On the other hand, too little $CaCl_2$ does not allow the gelation process to occur.
10. Cellophanes can be purchased from different sources; however, its quality can vary from manufacturer to manufacturer. The main problem seems to be the presence of chemical residue due to manufacture that led to poor protoplast regeneration. In order to clean them, we recommend autoclaving the cellophane in fresh Milli-Q® water (500 mL for 200 cellophanes) three times before using them for experiments. This simple method ensures their sterility and removes the compound (s) deleterious to protoplast regeneration. The cellophanes can be stored in the final water wash at room temperature until their utilization.
11. The regeneration time can be reduced to 4 days if wanted. However, it will reduce the final number of regenerants; therefore, the percentage of final transfection efficiency will be reduced.

12. Do not extend this step since antibiotics in general are light sensitive and selection pressure is decreasing with time, and hence after a week, episomal transfectants will be maintained and interfere with selection of integrative transformants. During this step, most of the plants should die, leaving 100–500 plants growing per Petri dish.
13. These 2 weeks of selection-free incubation will allow strong growth of the plants leading to dilution of the episomal elements (transformation vector not integrated into the genome). The plant that did not integrate the vector in their genome should end up being antibiotic sensitive and dying in the next selection step. Note that at this stage a majority of the growing plants are episomal transformants, not integrative one.
14. This step will kill up to 90% of the remaining growing plants. You should be left with stable, i.e., chromosome integrated, transformants.
15. Look carefully at the filament growing at the edge of your plants, since they should grow well in stably transformed plants, but will be the most sensitive cells of any episomal transfectant. Strong episomal transfectant may display a green tissue at the center of the plant, but the tissue at the edge of the plant will be dead.
16. In case of doubt about the stability of any given mutant, it is important to ensure it does not harbor any episomal element. Hence, before retesting the plant for antibiotic resistance, the plant needs to be sub-cultivated at least twice onto a nonselective medium for 2 weeks.

Acknowledgments

The Institut Jean-Pierre Bourgin benefits from the support of the LabEx Saclay Plant Sciences (SPS) (ANR-10-LABX-0040-SPS).

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Chapter 2

Protoplast Isolation and Transfection in the Single-Cell C_4 Species *Bienertia sinuspersici*

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Abstract

We have developed an optimized protocol for isolating protoplasts from chlorenchyma cells of the single-cell C_4 species *Bienertia sinuspersici*. The isolated protoplasts maintained the integrity of the unique single-cell C_4 intracellular compartmentation of organelles as observed in chlorenchyma cells after cell wall digestion. Approximately over 80% of isolated protoplasts expressed the fusion reporter gene following the polyethylene glycol-mediated transfection procedures. Overall, fluorescent protein fusion tagged with various intraorganellular sorting signals validated the potential use of the transient gene expression system in subcellular localization and organelle dynamics studies.

Key words C_4 photosynthesis, Chlorenchyma cells, Intracellular protein targeting, PEG-mediated transformation, Transient gene expression

1 Introduction

In mature *Bienertia* leaves, the chlorenchyma cells display a unique intracellular anatomy in that the cytoplasm of each cell is divided into the central cytoplasmic compartment (CCC) and the peripheral cytoplasmic compartment (PCC) (Fig. 1c). The CCC forms a distinctive spherical structure in the center of the cell containing tightly packed chloroplasts and mitochondria, whereas the PCC comprises a thin layer of cytoplasm with scattered chloroplasts and peroxisomes [1, 2]. Given the extraordinary subcellular organization of *Bienertia* chlorenchyma cells, earlier studies aimed at elucidating the mechanisms that regulate the development and maintenance of the subcellular organelle compartmentalization primarily involved using limited cellular and molecular techniques [2, 3]. Further understanding of this novel single-cell C_4 photo-

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_2,

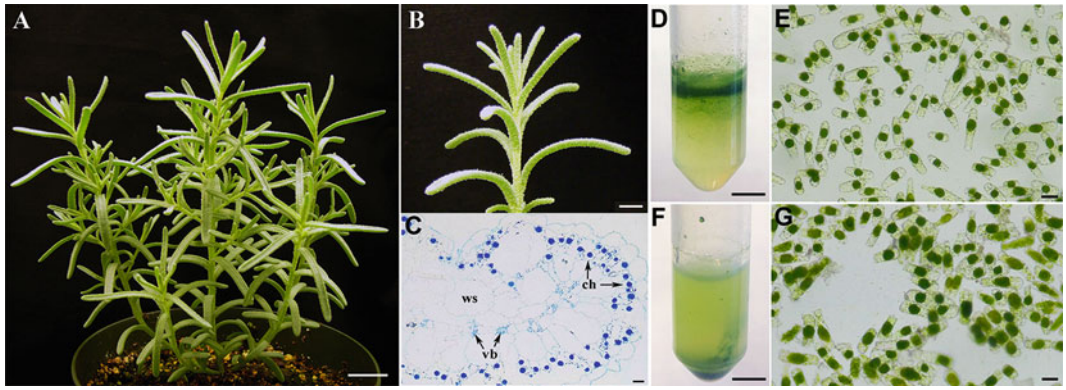

Fig. 1 (**a**) A 4-month-old seed-derived *Bienertia sinuspersici* plant used for protoplast isolation. (**b**) A branch with healthy leaves. (**c**) Transverse section of a mature leaf stained with Stevenel's blue showing chlorenchyma cells (ch), vascular bundles (vb), and water storage cells (ws). (**d**) Healthy chlorenchyma cells form a floating layer in CS buffer. (**e**) A bright-field image of a homogeneous population of healthy chlorenchyma cells taken from the floating layer of panel **d**. (**f**) Unhealthy and stressed chlorenchyma cells sink to the bottom of the solution. (**g**) A bright-field image of a homogeneous population of unhealthy chlorenchyma cells taken from the bottom of the solution of panel **f**. Scale bars: **a** = 2 cm; **b** = 0.5 cm; **c**, **e**, **g** = 50 μm; **d**, **f** = 5 mm

synthetic system is, therefore, hindered by the lack of established tools for manipulating gene expression.

Although significant advancement in the stable transformation methodologies has been developed for many plant species, some plants are still recalcitrant to this process, making protoplast transfection one of the most reliable methods to characterize gene functions [4]. Since the first successful report of protoplast isolation from root tips of tomato [5], optimized protocols have been established for many plant species. Protoplast-based transient expression systems are becoming more attractive in plant research because it enables rapid data collection compared to the difficult, lengthy, and inefficient stable transformation method [6]. Furthermore, protoplasts also have important applications for gene function analysis by transient gene expression and intracellular protein trafficking can be monitored qualitatively and quantitatively in a nondestructive manner within hours post-transfection.

In this chapter, we present a simple and rapid procedure for isolating a large number of viable protoplasts from healthy leaves of *Bienertia sinuspersici*, a model single-cell C_4 terrestrial plant, and an efficient method for the transient transformation of protoplasts that can be used for gene characterization studies [7, 8]. Our transfection rate of over 80% is comparable to that reported for *Arabidopsis* [4, 9]. In addition, the transformation protocol enables us to examine the selective targeting of various fusion constructs to organelles to each subcompartment of the single-cell C_4 system and more important its potential application in gene editing experiments.

2 Materials

Prepare all solutions using autoclaved ultrapure Milli-Q water and molecular grade reagents. Unless specified, all stock solutions are sterilized by autoclaving and stored at room temperature (22–25 °C). Refer to the appropriate Material Safety Data Sheets and standard operation procedures of your institution for proper handling of equipment and hazardous materials used in this protocol.

2.1 Plant Material and Plasmid DNA

1. Three- to four-month-old *Bienertia sinuspersici* plants derived from seeds or from vegetative propagation (Fig. 1a; *see* **Note 1**).
2. Plasmid DNA: All fluorescent protein fusion constructs were made using the pSAT6-EGFP vector series [8, 10].

2.2 Buffer and Media

1. Cell-stabilizing (CS) buffer: 0.7 M sucrose, 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 6.5), 0.005 M KCl, and 0.001 M $CaCl_2$ (*see* **Note 2**).
2. Enzyme buffer: 1.5% (w/v) cellulase, 0.1% (w/v) bovine serum albumin (BSA) in CS buffer (*see* **Note 3**).
3. Cell/protoplast viability staining solution: 0.2% (w/v) fluorescein diacetate (FDA) in acetone (*see* **Note 4**).
4. W5 buffer: 0.002 M 2-(N-morpholino)ethanesulfonic acid (MES)-KOH (pH 5.7), 0.154 M NaCl, 0.125 M $CaCl_2$, 0.005 M KCl.
5. MMS buffer: 0.004 M MES-KOH (pH 5.7), 0.015 M $MgCl_2$, 0.4 M sucrose.
6. PEG solution: 40% (w/v) polyethylene glycol (PEG) 4000, 0.4 M sucrose, 0.1 M $CaCl_2$.
7. WI buffer: 0.7 M sucrose, 0.004 M MES-KOH (pH 6.5), 0.005 M KCl.

2.3 Other Supplies

1. Mortar and pestle.
2. 40 μm nylon mesh filter.
3. Petri dishes (100 × 15 mm).
4. Centrifuge with a swing bucket rotor.
5. 15 mL Falcon tube.
6. Flat-bottomed depression slides.
7. Cover slips.
8. Petri dish (35 × 15 mm).
9. 2.0 mL microcentrifuge tube.

10. Commercial plasmid DNA extraction kit (midi or maxi kit size).
11. Growth chamber.
12. Fluorescence or confocal laser scanning microscope.

3 Methods

3.1 Protoplast Isolation

1. Obtain 20 leaves (ca. 2 g fresh weight) from healthy plants and gently press using a mortar and pestle in 10 mL of cell-stabilizing (CS) buffer to isolate the chlorenchyma cells (Fig. 1a, b).
2. Transfer the cell suspension onto a piece of 40 μm nylon mesh filter to remove excess solution by blotting with a stack of absorbent paper underneath (*see* **Note 5**).
3. Transfer the chlorenchyma cells from the nylon mesh filter by soaking in a 100 mm Petri plate containing 5 mL of enzyme solution (Fig. 1e; *see* **Note 6**).
4. Incubate the isolated chlorenchyma cells in the enzyme solution at room temperature in the dark without shaking for 4 h (*see* **Note 7**).
5. Transfer the cells/enzyme solution to a 15 mL Falcon tube and centrifuge the mixture at 100 × *g* for 2 min.
6. Carefully collect the floating layer of protoplasts by aspirating with a micropipette and transfer to a new tube (Fig. 2a; *see* **Note 8**).
7. Wash the protoplasts twice with 5 mL of CS buffer and centrifuge at 100 × *g* for 2 min (*see* **Note 9**).
8. Remove the solution and the pellet containing cell debris as much as possible without disturbing the floating layer of protoplasts.
9. Adjust the floating layer of protoplasts to 1 mL with W5 buffer.
10. Determine the number of protoplasts using a hemocytometer and assess their viability using fluorescein diacetate staining solution (Fig. 2b, c; *see* **Note 10**).

3.2 Protoplast Transfection

1. Incubate the isolated protoplasts in W5 solution on ice for 30 min to pellet the protoplasts.
2. Remove the supernatant and add MMS buffer to obtain a cell concentration of approximately 2×10^5 protoplasts per mL.
3. Transfer 100 μL of protoplasts (2×10^4) to a 2.0 mL tube containing 5 μg of plasmid DNA and mix gently by tapping (*see* **Note 11**).

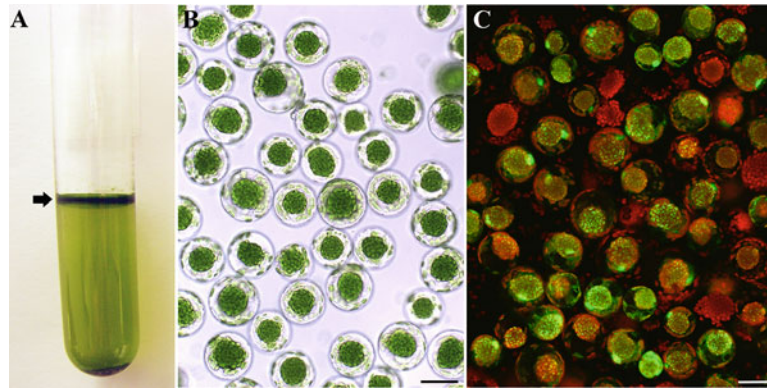

Fig. 2 (**a**) Protoplasts isolated in 0.7 M sucrose after a 2 min centrifugation step at 100 × *g* in the floating layer (arrow). (**b**) A bright-field image of healthy protoplasts from the floating layer. (**c**) Isolated protoplasts stained with FDA to assess their viability and observed under epifluorescence microscopy. Healthy and viable protoplasts displayed green fluorescence, while unhealthy or nonviable protoplasts exhibited only chlorophyll autofluorescence (red). Scale bars in **b**, **c** = 50 μm

4. Add 110 μL of PEG solution to the protoplast/DNA mixture, mix gently by inverting the tube four to six times, and incubate at room temperature for 5 min (*see* **Note 12**).
5. Add 800 μL of W5 buffer, mix by inverting the tube four to six times to stop the transfection, and incubate at room temperature for 20 min to settle the protoplasts.
6. Remove the supernatant and add 1 mL of WI buffer.
7. Transfer the transfected protoplasts in WI buffer to a 35 mm Petri plate and incubate in a growth chamber at 23 °C and a light intensity of 30 μmol/m^2/s for up to 16 h (*see* **Note 13**).
8. Mount 100 μL of the transfected protoplasts in a flat-bottom depression slide and examine under an epifluorescence microscope or a confocal laser scanning microscope (Fig. 3).

4 Notes

1. Seeds of *Bienertia sinuspersici* were provided by Mr. Abdulrahman Alsirhan from Kuwait. Seed-derived or vegetative propagated plants were grown in controlled environment chambers under high light intensity (ca. 500 μmol/m^2/s) with a day/night temperature of 25/18 °C and a photoperiod of 14/10 h. Intermediate leaves from 3- to 4-month-old healthy plants should be used for protoplast preparation. We find that the overall yield of isolated protoplasts is dependent on the health of starting plant materials. Healthy leaves that are

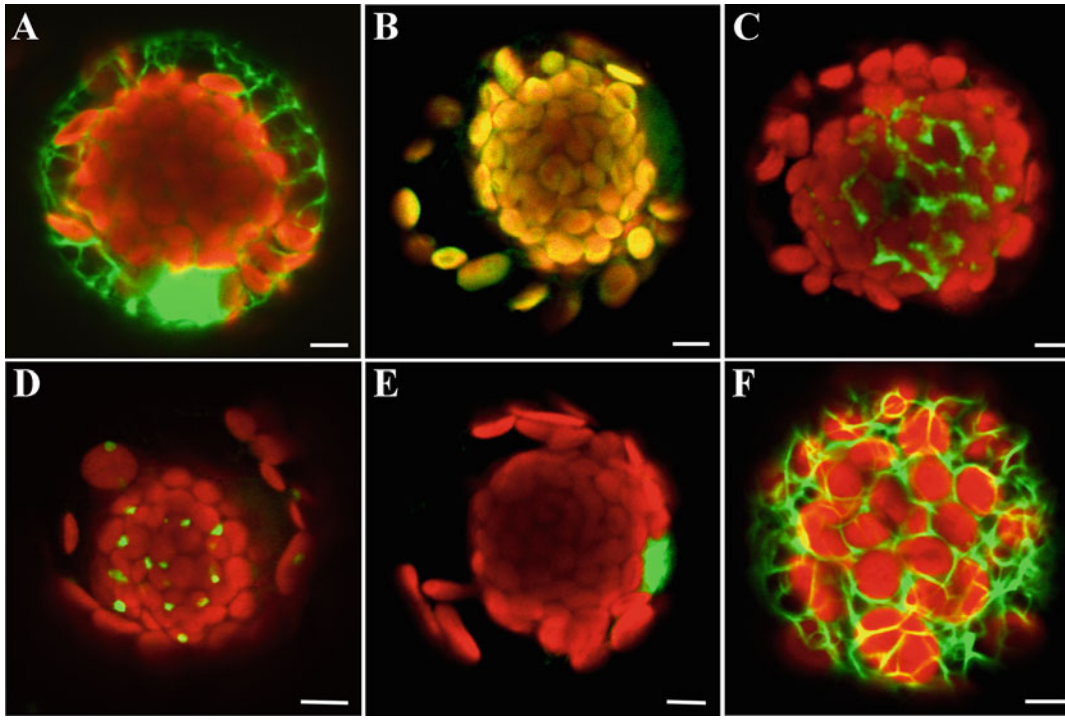

Fig. 3 Transient expression of fusion proteins (EGFP) targeted to different subcellular organelles in transformed protoplasts. (**a**) Protoplast transformed with an empty EGFP vector showing cytosolic and nuclear localization. (**b**) Protoplast transformed with the transit peptide of Rubisco small subunit-EGFP fusion construct targeted EGFP to the chloroplast stroma. (**c**) The mitochondrial targeting signal targeted EGFP to the mitochondria in the CCC. (**d**) Addition of the Ser-Lys-Leu (SKL) tripeptide, a peroxisome targeting signal 1 (PTS1), at the C-terminus of EGFP resulted in peroxisomal targeting. (**e**) Adding a nuclear targeting signal (NLS) at the N-terminus of EGFP targeted it exclusively to the nuclei. (**f**) Fusing EGFP with talin, an actin-binding protein, resulted in an extensive network of actin filaments. Scale bars = 5 μm

ideal for protoplast isolation should appear turgid, dark green in color, and covered with salt glands (Fig. 1b).

2. Weigh 23.96 g sucrose and transfer to a 200 mL beaker. Add about 60 mL of autoclaved ultrapure water and mix until the sucrose is completely dissolved. The sucrose can be dissolved faster if the water is warmed to about 50 °C. To simplify the preparation of solutions, prepare 10- or 100× stocks of all reagents. Add the appropriate volumes of the required reagents to get the final concentration and top it to 100 mL with water. The solution can be stored at 4 °C for up to a month.
3. Preheat CS buffer at 70 °C for 10 min. Cool the solution to 55 °C and add cellulase. Incubate further at 55 °C for 10 min to completely dissolve cellulose and cool it down to room temperature before adding 0.1% (w/v) BSA.

4. Weigh 2 mg fluorescein diacetate and dissolve in 1 mL of acetone. Wrap the solution in aluminum foil and store in −20 °C.
5. The breakage of water storage cells increased the cell suspension volume significantly and that we have to remove the excess sap to concentrate the isolated chlorenchyma cells on a piece of 40 μm nylon mesh filter prior to the cellulase treatment. Occasionally, if the procedure produces a lower yield or significantly fewer healthy protoplasts, then transfer the chlorenchyma cells into 5 mL of fresh CS buffer and centrifuge the suspension at 100 × *g* for 2 min. The floating layer should contain healthy cells (Fig. 1d, e), whereas unhealthy or stressed cells will sink to the bottom (Fig. 1f, g).
6. The enzyme solution must be freshly prepared each time. High-quality cellulase (Yakult Pharmaceutical Onozuka R-10) should always be used as the impurities in the lesser grade enzymes often lead to lower yield and fewer viable protoplasts. Since we are working directly with an isolated homogeneous population of healthy chlorenchyma cells (Fig. 1e), we omit the macerozyme R-10 from the enzyme solution.
7. We find that incubating the cells in the dark without shaking produced higher and healthier protoplasts.
8. Although cellulase digestion of cell wall was most active at pH 5.5, the highest viable protoplast yield was obtained in weak acidic pH 6.5. This is mainly due to a higher number of nonviable chlorenchyma cells or protoplasts in extreme acidic or alkaline buffers.
9. We examined the effects of various concentrations of different osmotica (D-sorbitol, D-mannitol, D-glucose, and sucrose) on the yield of protoplasts and found that in addition to the highest yield of protoplasts, the integrity of cytoplasmic compartments and the maximum cell viability were preserved in 0.7 M sucrose.
10. Add 4 μL of the FDA staining stock solution to 200 μL of protoplasts in CS buffer and incubate at room temperature for 15 min. After centrifugation at 100 × *g* for 2 min, wash the floating layer of cells twice with 200 μL of CS buffer and view the stained cells under a fluorescence microscope or a confocal laser scanning microscope.
11. Transformation of protoplasts with 5 μg of plasmid DNA extracted using commercial kit is highly recommended as it is often resulted in the highest transformation efficiency. Plasmid concentration of 0.5 μg/μL or higher should also be used as lower DNA concentrations will dilute the final PEG concentration producing lower transformation efficiency.

12. We find that it is best to prepare the PEG solution fresh each time.
13. The maximum number of protoplasts expressing the reporter gene was observed after a 12 h incubation period under low light intensity, whereas less than half of the protoplasts showed expression of the reporter gene after a 12 h incubation period in the dark.

Acknowledgments

This research was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) RGPIN-2017-04416 to SDXC.

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Chapter 3

Protoplast for Gene Functional Analysis in *Arabidopsis*

Yanlin Liu and Yan Xiong

Abstract

Protoplast, a plant cell without cell wall, can be readily transfected by exogenous macromolecules (DNA, RNA, protein) and therefore offer a versatile single cell-based functional analysis system to rapidly assess these exogenous macromolecules' functions. Properly prepared *Arabidopsis* leaf mesophyll protoplasts exhibit similar responses as intact plants to diverse abiotic and biotic stress signals as well as different hormones and nutrients, based on well-established reporter and marker gene assays. The protoplast transient expression system has been proven to be a vital and reliable tool for elucidation of the activities of transcription factors and protein kinases, protein subcellular localization and trafficking, protein-protein interaction, and protein stabilities in various signal transduction pathways. Moreover, protoplast also offers a platform for single cell-based plant regeneration, gene silencing, and genome editing. Healthy protoplasts isolated from plant tissues and the high transfection efficiency are key steps for successful use of the protoplast transient expression system. In this chapter, we describe the detailed methods of the protoplast transient expression system in *Arabidopsis*, including plant material preparation, high-quality maxi-plasmid DNA extraction, non-stressed protoplast isolation, highly efficient PEG-calcium transfection of plasmid DNA, and protoplast culture and harvest. We also provide several examples of gene functional analysis using this protoplast transient expression system.

Key words Protein kinase activity, Protein-protein interaction, Protein subcellular localization, Transient expression, Transcription factor activity

1 Introduction

The first isolation of plant protoplasts was succeed by Cocking from tomato roots in 1960 [1]. Subsequently, protoplasts were isolated from diverse plants and tissue types and were used to study cellular processes and activities, for example, cell wall synthesis, cell division and differentiation, photosynthesis activity, and ion channel responses to environmental stimulus [2]. Later, with the development and efficiency improvement of exogenous macromolecules (DNA, RNA, protein) transfection methods (PEG-calcium, electroporation, and microinjection), protoplasts were employed for functional analysis involving in multiple biological and signaling

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_3,

processes, e.g., calcium, light, stress, and hormone signaling in many plant species [2].

In the past decades, genome sequencing and global gene expression profiling, transgenic and genome editing technology, and the functional analysis of the loss- and gain-of-function mutants and transgenic plants have offered invaluable opportunities in understanding the gene function at the cellular and molecular levels in plants [2–5]. However, generation of the transgenic plants and isolation mutants from T-DNA insertion libraries or by genome editing approaches are highly time- and cost-consuming processes [2–4, 6]. Moreover, these approaches are also limited for analyzing closed members of gene families which often show high functional redundancy and genes that would cause embryonic or developmental lethality when deleted or overexpressed in intact plants.

As an alternative approach for plant gene functional analysis, the protoplast system has many advantages, including, but not limited to, rapid and efficient analysis of gene functions (2–6 h) [2], high-throughput screen and identify potential candidate genes of a particular signaling pathway (even from a whole set of subfamily clades [7]), functional analysis of genes that are normally absent, or low expression from the tissues where the protoplasts are derived (e.g., cell cycle S-phase gene regulation in well-expanded mesophyll protoplasts [8]). These advantages make the protoplast transient expression system a powerful tool to study diverse gene functions in multiple biological processes.

Arabidopsis is one of the most popular plant model systems. *Arabidopsis* mesophyll protoplasts have been utilized to study gene expression, protein trafficking or localization, protein-protein interaction, protein and enzyme activities, and protein stabilities in diverse plant signal transduction pathways, to analyze related metabolic production in cellular metabolism pathways, and also served as an ideal platform for single cell-based plant regeneration, gene silencing, and genome editing [2, 4, 9–11]. More information about plant protoplasts overview can be found in an excellent review [2] or in a transient expression in *Arabidopsis* mesophyll protoplast (TEAMP) procedure [12].

Here, we present the detailed methods how to prepare and set up an *Arabidopsis* leaf mesophyll protoplast system for gene functional analysis, including plant material preparation, maxi-plasmid preparation, protoplast isolation, PEG-calcium-mediated plasmid DNA transfection, and protoplast culture and harvest, and also provide several examples of gene functional analysis using this protoplast transient expression system. In our presented protocol, the yield of protoplast is about 2×10^6 cells per 0.6 g fresh leaf, and the transfection rate of protoplast is higher than 90%.

2 Materials

2.1 Plant Material, Plasmid, and Bacterial Strain

1. *Arabidopsis thaliana* (Col-0): For the details of plant growth conditions, please refer to Subheading 3.1.
2. *Escherichia coli* strain MC1061: A strain for gene cloning and maxi-plasmid preparation (*see* **Note 1**).
3. Plant transient expression vector HBT95 (GenBank: EF090408.1): A small plasmid with the backbone of pUC18, which carries the selectable marker gene *Amp* (resistant to ampicillin) and multiple cloning sites (MCS) which is regulated by the CaMV35S promoter and NOS terminator (*see* **Note 2**).

2.2 Stock Solutions

Specific description of the terms: autoclave, 121 °C for 20 min; filter sterilize, 0.22 μm Stericup or syringe; room temperature, 22–25 °C; ddH_2O, double distilled water.

1. Sodium hydroxide (5 M): Dissolve 200 g of NaOH in 1 L ddH_2O. No sterilization needed; the solution can be stored at room temperature for up to 1 year.
2. EDTA (0.5 M, pH 8.0): In 800 mL ddH_2O, add 186.1 g ethylenediaminetetraacetic acid disodium salt ($Na_2EDTA{\cdot}2H_2O$). Adjust pH to 8.0 using 5 M NaOH. Add additional ddH_2O to bring to 1 L. Autoclave, and store at room temperature for up to 1 year.
3. SDS (20% (W/V)): Dissolve 200 g of sodium dodecyl sulfate in 1 L ddH_2O. No sterilization needed; the solution can be stored at room temperature for up to 1 year.
4. Mannitol (0.8 M): Dissolve 145.7 g of mannitol in 1 L ddH_2O. Filter sterilize and store at room temperature for up to 1 year.
5. Potassium chloride (2 M): Dissolve 149.1 g of KCl in 1 L ddH_2O. Autoclave and store at room temperature for up to 1 year.
6. MES (0.2 M, pH 5.7): Dissolve 39.05 g of 2-(N-morpholino) ethanesulfonic acid (MES) in 1 L ddH_2O. Adjust pH to 5.7 using 5 M NaOH. Filter sterilize and store at room temperature in the dark for up to 6 months.
7. Calcium chloride (1 M): Dissolve 110.99 g of $CaCl_2$ in 1 L ddH_2O. Filter sterilize and store at room temperature for up to 1 year.
8. BSA (10%): Take 1 g of bovine serum albumin (BSA), dissolve in 10 mL ddH_2O, filter sterilize, and store at 4 °C for up to 6 months.

9. Sodium chloride (5 M): Dissolve 292.2 g of NaCl in 1 L ddH_2O. Autoclave and store at room temperature for up to 1 year.
10. Magnesium dichloride (1 M): Dissolve 203.3 g of $MgCl_2{\cdot}6H_2O$ in 1 L ddH_2O. Filter sterilize and store at room temperature for up to 1 year.
11. Potassium hydroxide (5 M): Dissolve 280.5 g of KOH in 1 L ddH_2O. No sterilization needed. Store at room temperature (22–25 °C) for up to 1 year.
12. Carbenicillin disodium (50 mg/mL): Dissolve 0.5 g carbenicillin disodium in 10 mL sterilized ddH_2O. No sterilization needed. Aliquot and store at −20 °C for up to 2 years.
13. Tris–HCl (1 M, pH 6.8): Dissolve 121.14 g of Tris-base in 850 mL ddH_2O. Adjust pH to 6.8 using 12 M HCl. Add additional ddH_2O to bring to 1 L. Autoclave and store at room temperature for up to 1 year.
14. EB (10 mg/mL): Dissolve 0.5 g ethidium bromide in 50 mL ddH_2O. No sterilization needed. Store at 4 °C for up to 6 months.
15. Rapamycin (10 mM): Dissolve 18.3 mg rapamycin in 2 mL dimethyl sulfoxide (DMSO). No sterilization needed. Store at −80 °C for up to 6 months.
16. Torin2 (1 mM): Dissolve 2.16 mg Torin2 in 5 mL DMSO. No sterilization needed. Store at −80 °C for up to 6 months.

2.3 Media and Buffers

1. Half Strength Murashige and Skoog (½MS) solid growth medium: In 900 mL ddH_2O, dissolve 2.215 g MS salt (with vitamins), 5 g glucose (final 0.5% (w/v)), 1 g MES (final 0.1% (w/v)). Adjust pH to 5.7 using 5 M KOH. Add additional ddH_2O to bring to 1 L. Add 8 g Phytoagar (final 0.8% (w/v)). Autoclave. Then pour into 10 cm × 1.5 cm Petri dishes after the medium has cooled down to ~55 °C (25 mL per dish).
2. LB bacterial growth medium: In 900 mL ddH_2O, dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl. Add additional ddH_2O to bring to 1 L. Add 15–20 g bacto-agar (final 1.5–2% (w/v)). Autoclave. Add 1 mL of 50 mg/mL of carbenicillin disodium (final 50 μg/mL) when the medium has cooled down to ~60 °C, and then pour into 9 cm × 1.5 cm Petri dishes (20 mL per dish).
3. Terrific broth (TB) medium: In 900 mL ddH_2O, dissolve 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4, 16.4 g K_2HPO_4. Add additional ddH_2O to bring to 1 L. Autoclave. Add 1 mL of 50 mg/L of carbenicillin disodium (final 50 μg/L) when the medium has cooled down to ~60 °C.

Use 2 L Erlenmeyer flask, 200 mL of TB medium per bottle (*see* **Note 3**).

4. Maxi-plasmid DNA preparation solution I: In 490 mL ddH_2O, add 10 mL of 0.5 M EDTA (final 10 mM; pH 8.0). No need to readjust pH. Cool to ~4 °C before use.
5. Maxi-plasmid DNA preparation solution II: In 930 mL ddH_2O, add 20 mL of 5 M NaOH (final 0.1 M) and 50 mL of 20% SDS (final 1%). Prepared freshly before use.
6. Maxi-plasmid DNA preparation solution III: Dissolve 250 g potassium acetate in 700 mL ddH_2O, then add 150 mL acetic acid. Add additional ddH_2O to bring to 1 L. Cool to ~4 °C before use.
7. Enzyme solution: To prepare 10 mL, mix 0.125 g cellulase R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.03 g macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 5 mL of 0.8 M mannitol (final 0.4 M), 100 μL of 2 M KCl (final 20 mM), 1.0 mL of 0.2 M MES (final 20 mM; pH 5.7). Heat the solution at 55 °C for 10 min. Cool it to room temperature before adding 100 μL of 1 M $CaCl_2$ (final 10 mM), 3.0 μL β-mercaptoethanol (optional), 100 μL of 10% BSA (final 0.1%). Add additional ddH_2O to bring to 10 mL. The enzyme solution should be prepared freshly and passed through a 0.45 μm filter (*see* **Note 4**).
8. W5 solution: For 1 L, mix 30.8 mL of 5 M NaCl (final 154 mM), 125 mL of 1 M $CaCl_2$ (final 125 mM), 2.5 mL of 2 M KCl (final 5 mM), 10 mL of 0.2 M MES (final 2 mM; pH 5.7). Add additional ddH_2O to bring to 1 L. Filter sterilize and store at 4 °C for up to 6 months.
9. PEG-Ca^{2+} solution: To prepare 10 mL, mix 4 g PEG4000 (final 40%), 3 mL ddH_2O, 2.5 mL of 0.8 M mannitol (final 0.2 M), 1 mL of 1 M $CaCl_2$ (final 0.1 M). In order to dissolve PEG completely, PEG-Ca^{2+} solution should be prepared at least 1 h before transfection. Preparing fresh PEG solution is highly recommended (*see* **Note 5**).
10. WI solution: In 800 mL ddH_2O, dissolve 91.09 g mannitol, 20 mL of 0.2 M MES (final 4 mM; pH 5.7), 10 mL of 2 M KCl (final 20 mM). Add additional ddH_2O to bring to 1 L. Filter sterilize and store at room temperature for up to 6 months.
11. MMg solution: Add 15 mL of 0.8 M mannitol (final 0.4 M), 450 μL of 1 M $MgCl_2$ (final 15 mM), 600 μL of 0.2 M MES (final 4 mM; pH 5.7). Add additional ddH_2O to bring to 30 mL. Preparing fresh MMg solution is highly recommended, and no sterilization is needed.
12. 2× SDS protein extraction buffer: 800 μL of 1 M Tris–HCl (final 80 mM; pH 6.8), 0.4 g SDS (final 4%), 200 μL

β-mercaptoethanol, 800 μL glycerol (final 4 mM), 2.0 mg bromophenol blue (final 0.02%). Add additional ddH_2O to bring to 10 mL. No sterilization needed. Store at 4 °C for up to 6 months.

2.4 Equipment and Related Supplies

1. Swing-out rotor centrifuge.
2. High-speed centrifuge.
3. Ultracentrifuge.
4. 37 °C shaker.
5. Room temperature platform shaker.
6. Fluorescence microscope.
7. Confocal microscope.
8. Real-time PCR detection system.
9. Microcloth.
10. 5.2 mL Quick-Seal centrifuge tube (Beckman, cat. no. 342412).
11. 0.22 μm Stericup quick release Durapore.
12. 0.22 μm syringe sterilization filter.
13. Modulus microplate reader (Turner Biosystems).
14. Luminometer (Monolite3010; Pharmigen).
15. Generic razor blade, single-edged blade.
16. Petri dish, 9 cm × 1.5 cm.
17. Nylon mesh, 75 μm.
18. Hemacytometer.
19. 250 mL tube (Beckman, cat. no. 325620).
20. 50 mL round-bottom tube.
21. 2 mL round-bottom natural microcentrifuge tube.
22. 6-well tissue culture plate.
23. Jiffy-7 peat soil pellets (Jiffy group).

3 Methods

All of the steps should be performed at room temperature (22–25 °C), unless otherwise specified.

3.1 Plants Preparation

This section should be performed at least 30 days before the experiments.

1. The *Arabidopsis* seeds are surface sterilized by 75% ethanol for 5 min at room temperature, and wash with sterilized ddH_2O

for three times. The surface sterile seeds are cold treated for 24–48 h at 4 °C in the dark.

2. Sow the cold-treated seeds directly on the wet Jiffy-7 peat soil pellets, 2–3 seeds per pellet, or grow the sterile seeds on the ½MS solid medium plates (10 cm × 1.5 cm), 9 seeds per plate.
3. Grow the plants in a greenhouse or an environment-controlled growth chamber with a relatively short photoperiod (10 h light at 23 °C/14 h dark at 20 °C) under low light condition (50–75 μmol m^{-2} s^{-1}) with 65% relative humidity for 4 weeks (on soil) or 3 weeks (on the ½MS solid medium) (*see* **Note 6**).
4. For the soil-grown seedlings, at the seventh day, leave one strongest seedling on each Jiffy-7 peat soil pellet and remove other seedlings (be careful not to hurt the seedling you want). Keep the routine care of the plants, and do not add extra nutrient when watering.
5. The plants grown on the ½MS medium plates do not need any extra operations before harvest.

3.2 Plasmid DNA Preparation (See Note 7)

This section should be done 2 days before the day of the experiments.

1. Plasmid transformation: Pick a single transformed MC1061 cell from a plate to 5 mL TB with antibiotic, grow for 3–4 h at 37 °C with shaking (220 rpm), $OD_{600} = 0.4–0.5$.
2. Transfer 1 mL cells into a 200 mL TB medium with antibiotic, grow for 10–12 h at 37 °C with shaking (220 rpm). Collect the cell pellet in a 250 mL centrifugal bottle and freeze at −80 °C for at least 30 min.
3. Add 40 mL solution I, vortex, resuspend cell pellet completely. Then add 80 mL fresh solution II, mix well (stay no more than 5 min for this step). Add 40 mL solution III, mix by gently inverting the bottle five to six times. Wait for 3–5 min at room temperature.
4. Spin at 3124 × *g* for 15 min at 4 °C. Pass the supernatant through a double-layer microcloth covered 250 mL centrifugal bottle.
5. Add isopropanol to fill the 250 mL bottle, invert the bottle to mix.
6. Spin at 3124 × *g* at 4 °C for 15 min, and discard the supernatant. Rinse the pellet gently with 5 mL absolute ethanol and dry the pellet in the air for 5–10 min.
7. Resuspend the pellet with 4 mL solution I. Transfer all the solution to a new 15 mL tube, and add 1 mL phenol:chloroform:isoamyl alcohol = 25:24:1, then vortex for 10 s.

8. Spin at 3184 × *g* for 10 min. Transfer supernatant to a new 15 mL tube.
9. Add 5.8 g CsCl in the supernatant, then add solution I to 5.5 mL. Add 200 μL EB (10 mg/mL), mix well, and spin at 3184 × *g* for 15 min.
10. Transfer the supernatant to a 5.5 mL Quick-Seal tube, seal the tube. Spin at 386,380 × *g* for 3 h or 217,339 × *g* for 12 h.
11. Pull the DNA band to a new 15 mL tube using a 3 mL syringe with a 20-gauge needle.
12. Extract with 7 mL of n-butanol (saturated with 1 M NaCl) for two to three times until the solution in the lower layer become colorless.
13. Add 3 mL of ddH_2O, do not mix. Remove the n-butanol layer and leave 3 mL of the aqueous layer at the bottom of the tube.
14. Add two volume of absolute ethanol (6 mL) to precipitate the DNA, spin at 3184 × *g* for 3–5 min, and discard the supernatant.
15. Resuspend the pellet with 5 mL 75% ethanol, spin at 3184 × *g* for 3–10 min, and discard the supernatant.
16. Dry by inverting the tube, then add 500 μL ddH_2O to dissolve the plasmid ($OD_{260/280} = 1.8$ and $OD_{260/230} = 2.3$), store at −20 °C. Dilute the plasmids into 1 μg/μL with ddH_2O before use.

3.3 Protoplast Isolation

1. Prepare enzyme solution freshly, then filter the enzyme solution through a 0.45 μm filter into a Petri dish (9 cm × 1.5 cm Petri dish for 10 mL enzyme solution).
2. Take well-expanded and healthy leaves from 4-week-old plants (fifth to seventh true leaves) grown on the Jiffy-7 peat soil pellets or from 3-week-old plants (second to fourth true leaves) grown on the ½MS solid medium plates (*see* **Note 8**).
3. On a piece of clean white A4 paper, remove the upper and bottom ends of the leaf, and cut the middle part of a leaf into 0.5–1 mm strips using a fresh sharp razor blade (*see* **Note 9**).
4. Dip the leaf strips quickly and gently into the prepared enzyme solution, make sure both sides of the strips are well immerged with the enzyme solution. For routine experiments, 20 leaves (fifth to seventh true leaves of 4-week-old plants growth on Jiffy-7 peat soil pellets, 0.6 g) in 10 mL enzyme solution will get about 2×10^6 protoplasts, which are sufficient for about 50 samples (4×10^4 protoplasts per sample).
5. After cutting all the selected leaves, put the Petri dish containing the enzyme solution with leaf strips in a vacuum desiccator and apply vacuum infiltration for 30 min in the dark.

6. Continue the digestion for another 3–5 h without shaking or 1–2 h with gentle shaking (40 rpm on a platform shaker) in the dark (*see* **Note 10**).
7. The enzyme solution should turn green, indicating the release of protoplasts into the solution. Release the protoplasts by shaking on a platform shaker at 80 rpm for 1 min. It is not recommended for 100% release of the protoplast.
8. Add the equal volume of ice-cold W5 solution to the enzyme/protoplast suspension, mix well by gently shaking.
9. Filter the suspension through a 75 μm nylon mesh to remove undigested leaf tissues.
10. Spin at 390 × *g* to pellet the protoplasts in a 50 mL round-bottom tube for 2 min (*see* **Note 11**).
11. Remove the supernatant as much as possible and resuspend the protoplast pellet with ice-cold W5 solution (equal volume with enzyme solution) by gentle swirling.
12. Repeat **steps 10** and **11** in this section. Put the tube with resuspended W5/protoplast suspension on ice in the dark for 30 min.
13. Heathy protoplasts should be settled down at the bottom of the tube by gravity after 30 min incubation on ice. Remove the supernatant as much as possible without touching the protoplast pellet.
14. Resuspend the protoplasts with MMg solution, count cells under the microscope using a hemacytometer, and make the final density of protoplasts at 2×10^5/mL (*see* **Note 12**).
15. Keep the protoplasts on ice. These protoplasts are ready for PEG-calcium-mediated transfection.

3.4 PEG-Ca^{2+}–Mediated Transfection

1. Aliquot 20 μL (1 μg/μL, total 20 μg per transfection) plasmid DNA in a 2 mL round-bottom tube (*see* **Note 13**).
2. Add 200 μL protoplasts (4×10^4 protoplasts) and mix gently.
3. Add 220 μL of PEG-Ca^{2+} solution, and mix completely by gently finger-tapping the tube.
4. Incubate the mixture for 5 min.
5. Add 1 mL of W5 solution, and mix completely by gently inverting the tubes five to six times to stop the transfection process.
6. Spin down at 390 × *g* for 2 min (accelerated speed 3) in a swing-out rotor Eppendorf centrifuge.
7. Remove the supernatant and add 100 μL W5 solution to resuspend the protoplasts.

8. Transfer the protoplasts into 1 mL WI solution in each well of a 6-well tissue culture plate (*see* **Note 14**).

3.5 Protoplast Culture and Harvest

1. Incubate the protoplasts under low light condition (20–30 μmol/m^2/s) at room temperature for 2–16 h based on the experimental needs (*see* **Note 15**).
2. If additional treatments are needed for your gene functional analysis, in this step, chemicals, stimuli, or hormones could be applied to the protoplasts.
3. At the end of the incubation, resuspend and harvest protoplasts in a round-bottom tube and spin down at 390 × *g* for 2 min.
4. Remove the supernatant, add the buffers or extraction solutions for the gene functional analysis, e.g., luminescence measurement, or freeze the samples in liquid nitrogen, then store them at −80 °C for further analysis, e.g., RNA or protein isolation.

3.6 Example 1: Functional Analysis of the Transcription Factor Activity in Mesophyll Protoplast

E2F transcription factors are conserved key regulators of S-phase genes governing cell cycle progression and DNA replication in plants and mammals. It has been reported that E2Fs are the well-established targets of the universal CYC-CDK-RBR (Cyclin-Cyclin-Dependent Kinase-Retinoblastoma-Related Protein) cascade initiating cell cycle. E2Fa protein contains the amino-terminal putative regulatory domain, DNA-binding-dimerization domain, and the carboxy-terminal transcription activation/RBR-interacting domain [8, 13]. A sensitive protoplast-based assay was applied to investigate the functions of these different domains of E2Fa on S-phase genes' activation by ectopic expression of full-length and various truncated E2Fa proteins (Fig. 1a) in non-division and fully differentiated leaf mesophyll protoplasts [8].

1. Transfection experiment was performed using 5 μg of E2Fa's full-length or truncated variant plasmid DNA in 1 mL protoplast suspension (2×10^5 in MMg solution) (*see* Subheading 3.4; *see* **Note 16**).
2. The transfected cells are incubated for 4 h in 5 mL WI solution in a Petri dish (9 cm × 1.5 cm) under low light condition (20–30 μmol/m^2/s) at room temperature by the method described in Subheading 3.5 (*see* **Note 17**).
3. 1/10 volume of samples are harvested for protein blot analysis to evaluate whether full-length or various truncated E2Fa constructs express relative equal amount of E2Fa protein.
4. 9/10 volume of samples are harvested for total RNA isolation and gene expression analysis.
5. Isolate the total RNA by using TRIzol Reagent according to the manufacturer's instructions (Ambion, 15596018).

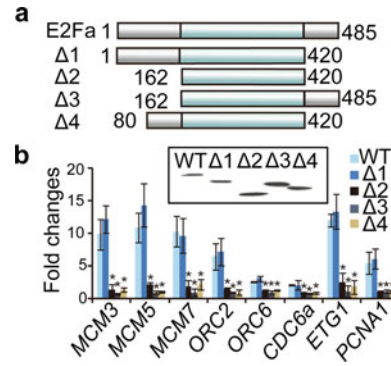

Fig. 1 The activity of E2Fa and its variants on regulation of S-phase gene expression in non-division and fully differentiated protoplast cells. (**a**) Schematic diagram of WT or various truncated E2Fa proteins. (**b**) Activation of S-phase gene expression by WT or various truncated E2Fa proteins in protoplast cells. RT-qPCR analysis. Means ± s.d., $n = 3$. $^*P < 0.05$. Protein blot analysis (inset) indicates the expression level of various E2Fa proteins. (Reproduced from ref. 8)

6. Reverse transcription of 1 μg of total RNA by using M-MLV reverse transcriptase kit according to the manufacturer's instructions (Promega, M1708).
7. Analysis of S-phase gene expression (*MCM3/5/7*, *ORC2/6*, *CDC6a*, *ETG1*, *PCNA1*) by qRT-PCR by using a CFX96 real-time PCR detection system with iQ SYBR Green Supermix (Bio-Rad, 172-5124). *TUB4* (At5g44340) was used as a loading control.

As shown in Fig. 1b, full-length E2Fa can strongly activate S-phase gene expression, and removal of C-terminal RBR-interacting domain (Δ1) does not affect E2Fa activation of S-phase genes. By contrast, deletion of N-terminal putative regulatory domain (Δ2) of E2Fa completely abolishes its transcriptional activation of S-phase gene expression without affecting protein translation/stability (Fig. 1b, inset). When the deletion of N-terminal region of E2Fa is narrowed from 1–162 AAs (Δ2) to 1–80 AAs (Δ4), the activity of E2Fa could still not be recovered. These data indicate that the N-terminal region (1–80 AAs) is essential for E2Fa transcriptional activation of S-phase gene expression.

3.7 Example 2: Protein Subcellular Localization Analysis—EIN2 Localization in Protoplast Cells

Protein subcellular localization provides an essential information for its function interpretation. Protoplast is an ideal system to investigate protein subcellular localization. Ethylene Insensitive 2 (EIN2) is a central integrator that shuttles between the cytoplasm and the nucleus in response to different stimuli [14]. Target of rapamycin (TOR) kinase is an evolutionarily conserved master regulator that integrates energy, nutrients, growth factors, and

stress signals to promote survival and growth in all eukaryotes. The dynamic subcellular localization of EIN2 in response to TOR kinase inhibitors' treatments is studied in leaf mesophyll protoplasts [15].

1. Co-transfect with 10 μg mCherry-EIN2-GFP and 10 μg CFP-HDEL (HDEL is ER localization signal) plasmids in 200 μL protoplast suspension (4×10^4 in MMg solution; *see* Subheading 3.3) and incubate for 10 h in 1 mL WI solution in 6-well plates by the methods described in Subheadings 3.4 and 3.5.
2. Add TOR inhibitor (rapamycin (Rap, 10 μM) or Torin2 (1 μM)) and incubate for another 3 h.
3. Transfer 50 μL protoplast to a single concave microscope slide and covered by a coverslip (*see* **Note 18**).
4. Observe the mCherry (587 nm), GFP (486 nm), and CFP (449 nm) signals by using a confocal microscope (Leica SP8).
5. Scan the photos of different channels and merge them together to analyze EIN2 localization patterns under different treatment conditions.

As shown in Fig. 2, under nontreatment condition, the GFP and mCherry fluorescent signals co-localize well with the CFP fluorescent signal in the cytosol, suggesting that full-length EIN2 protein localizes to the ER. By contrast, Torin2 or rapamycin treatment causes significant co-localization of GFP and mCherry but not the CFP fluorescent signal in the nucleus, whereas the CFP, GFP, and mCherry fluorescent signals still co-localize well in the cytosol, indicating that full-length EIN2 is able to move from the ER to the nucleus under TOR inhibition conditions.

3.8 Example 3: Protein-Protein Interaction—Split Luciferase Assay of TOR-FRB and FKP12 Interaction

Many methods could be used to examine protein-protein interaction in vivo [16]. Most of them could also be performed in protoplast systems. Here, we provide one example by using a split luciferase assay for detecting the dynamic protein-protein interaction. Rapamycin, a natural antibiotic produced by the soil bacterium *Streptomyces hygroscopicus*, can specifically inactivate TOR kinase in yeast, mammals, and plants. The inhibitory effect of rapamycin is mediated by the formation of a specific protein complex, in which rapamycin forms noncovalent bonds between the FK506-binding protein 12 (FKP12) and the FRB (FKP-rapamycin binding) domain of TOR proteins. A split luciferase complementation assay is employed to examine whether *Arabidopsis* FKP12 (AtFKP12) or human FKP12 (HsFKP12) associates with the *Arabidopsis* TOR-FRB domain in a rapamycin-dependent manner in *Arabidopsis* leaf protoplasts [17].

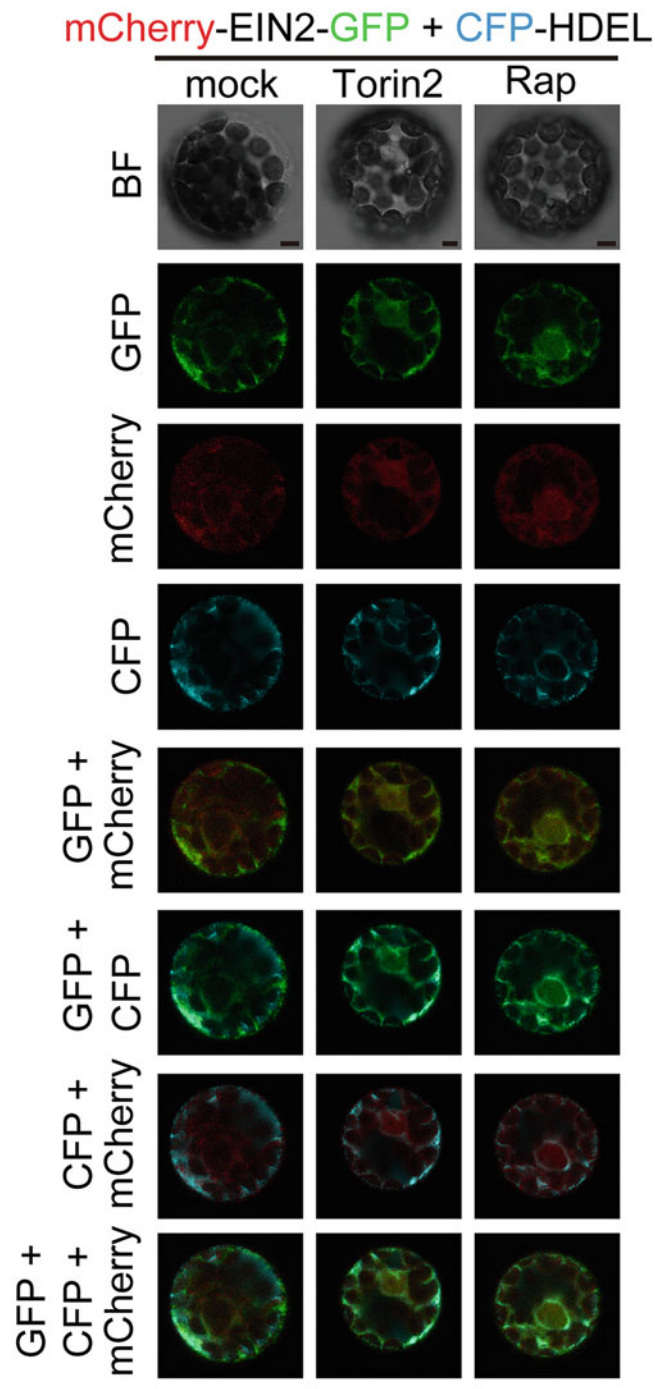

Fig. 2 mCherry-EIN2-GFP localization in protoplast cells under the treatments of TOR kinase inhibitors. *Arabidopsis* mesophyll protoplasts transiently transfected with mCherry-EIN2-GFP and CFP-HDEL (ER marker) were treated with or without rapamycin (Rap, 10 μM) or Torin2 (1 μM) for 3 h. Scale bar: 5 μm. (Reproduced from ref. 15)

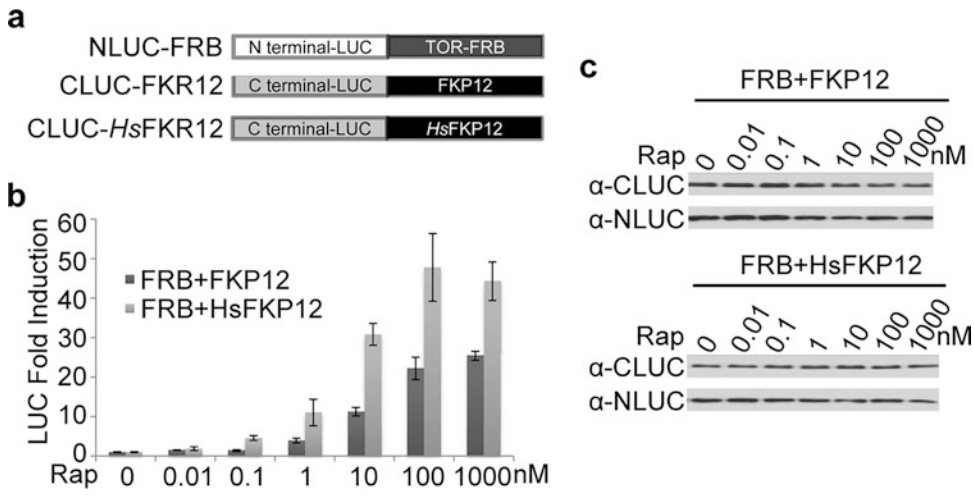

Fig. 3 Detection of protein-protein interaction by split luciferase assay in *Arabidopsis* leaf mesophyll protoplasts. (**a**) Schematic diagram of NLUC-FRB and CLUC-FKP12 constructs. (**b**) Relative interaction intensity between the TOR-FRB domain and FKP12s. Rapamycin induces the direct interactions between the *Arabidopsis* TOR-FRB domain and both *Arabidopsis* and human FKP12 in a concentration-dependent manner. Results represent means ± s.d., $n = 3$. (**c**) Protein expression level of NLUC-FRB and CLUC-FKP12s. (Reproduced from ref. 17)

1. The N- and C-terminal fragments of firefly luciferase (NLUC and CLUC) are translationally fused to TOR-FRB and FKP12, respectively (Fig. 3a).
2. Co-transfect with 10 μg NLUC-FRB and 10 μg CLUC-AtFKP12 or CLUC-HsFKP12 in 200 μL protoplast suspension (4×10^4 in MMg solution; *see* Subheading 3.3), and then incubate for 6 h in 1 mL WI solution in 6-well plates by the methods described in Subheadings 3.4 and 3.5.
3. Add luciferin (25 μg/μL) to the protoplast/WI mix solution, then incubate for another 30 min.
4. Add rapamycin with the indicated concentrations, and incubate for 5 min.
5. Transfer the samples to 96-well plates (100 μL per well), and read the output using a modulus microplate reader (Turner Biosystems). As shown in Fig. 3b, rapamycin even at 1 nM can quickly and significantly trigger the increase of luminescence intensity, which reaches the maximum at 1 μM. These results indicate that rapamycin induces the direct interactions between the *Arabidopsis* TOR-FRB domain and both *Arabidopsis* and human FKP12 in a concentration-dependent manner in plant cells.
6. Harvest 1/10 volume of samples for protein blot analysis. As shown in Fig. 3c, rapamycin treatment does not affect the expression of NLUC-FRB and CLUC-FKP12 proteins.

3.9 Example 4: Protein Kinase Activity—TOR Activated by Upstream Component ROP2 in the Protoplast System

Protein kinases play a vital role in plant signaling transduction networks responding to diverse internal and external stimuli. Detection of its kinase activity is a key step to analyze the upstream signals and regulators for a protein kinase. Many useful assays were developed to detect the kinase activity in vitro or in vivo [18]. Here, we list an example of analyzing the in vivo TOR kinase activity in *Arabidopsis* leaf protoplasts. ROP GTPases in plants have been considered as central molecular switches to regulate many developmental processes. To test whether ROP2 activates TOR kinase activity, S6K1-Flag is co-expressed with either ROP2-HA or constitutively active (CA) form of ROP (CA-ROP2-HA) in *Arabidopsis* leaf protoplasts, and the TOR activity is monitored by measuring its specific substrate S6K1 T449 phosphorylation level by protein blot analysis [19].

1. Co-transfect with 10 μg S6K1-Flag and 10 μg ROP2-HA or CA-ROP2 or sGFP in 200 μL protoplast suspension (4×10^4 in MMg solution; *see* Subheading 3.3), and incubate for 6 h in 1 mL WI solution in 6-well plates by the methods described in Subheadings 3.4 and 3.5.
2. One of S6K1-Flag plus sGFP sample is treated with TOR inhibitor Torin2 (25 μM) for 1 h before sample harvest which serves as a negative control.
3. Harvest protoplast samples, and then the cell pellet is lysed in 80 μL of 2× SDS protein extraction buffer.
4. Boil at 95 °C for 5 min. Cool down on ice, then spin at 13,523 × *g* for 2 min at 4 °C.
5. Take 20 μL supernatant of each sample to run in 8% SDS-PAGE gel.
6. Protein blot analyses are performed by using anti-Flag, anti-HA, and anti-S6K1(T449P) antibodies, respectively (Fig. 4a).
7. Results are quantified by ImageJ (Fig. 4b). As shown in Fig. 4, in non-stressed leaf cells, TOR kinase is already well activated, but CA-ROP2 can further increase TOR kinase activity compared with ROP2.

4 Notes

1. Compared with DH5α, MC1061 cells grow and proliferate more rapidly in TB (terrific broth) medium, therefore, MC1061 is suitable for large amount of plasmids DNA preparation.
2. To get high transient transfection rate (>90%) in the protoplast system, relative small backbone (3–5 kb) transient expression

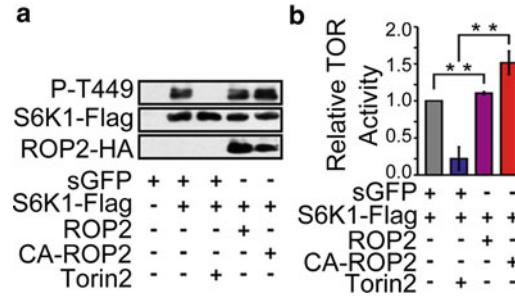

Fig. 4 Protein kinase activity detection in the protoplast system by protein blot analysis. (**a** and **b**) In leaf cells, S6K1-Flag was co-expressed with either ROP2-HA or CA-ROP2-HA, and the TOR activity was monitored based on the phosphorylation status of T449 in S6K1. In non-stressed leaf cells, TOR kinase is already well activated, but CA-ROP2-HA could further increase TOR kinase activity compared with ROP2-HA or control, while Torin2 completely blocks TOR kinase activity. (Reproduced from ref. 19)

vectors are highly recommended, e.g., HBT95 plasmid. Binary vectors usually have low transfection rate (<10%).

3. To get high-quality and quantity plasmid DNA for transient transfection assay, we recommend using the terrific broth (TB) medium, not LB medium, in a 2 L Erlenmeyer flask with a 200 mL medium. The three points presented above (using TB, in a 2 L bottle, and with a 200 mL medium) are important for preparing enough and healthy cells for maxi-plasmid preparation.
4. The volume of the enzyme solution is based on the leaves that you want to lyse. We usually use 10 mL to digest 20 leaves. Heating the enzyme solution at 55 °C for 10 min can help to inactivate DNases and proteases and to enhance enzyme solubility. After heating, the ready enzyme solution is clean and shows a light brown color.
5. The volume of PEG-Ca^{2+} solution is based on the transfection samples you want. We usually prepare 1–2 mL more than the needed volume. PEG4000 (Sigma, Cat# 81240) is highly recommended.
6. Clean air, water, soil, and suitable light period, intensity, and quality are needed for plant material preparation. Please try to maintain a constant environment as much as possible since all genotypes of *Arabidopsis* are sensitive to all kinds of environmental changes, and to avoid transplanting the seedlings from soils to soils when preparing your plants. Two methods for preparing plant materials are presented here with their own advantages and limitations. Soil-grown plants are bigger and more protoplasts could be harvested, but it takes more time and patience to maintain the constant growth environment.

Plants grown on the 1/2 MS medium are smaller, but it takes relative shorter time and is easier to control the growth conditions.

7. High purity and quality of plasmid DNA is one of the key points for high transfection rates and reproducible experimental results. We highly recommended the CsCl-based DNA Maxi-Prep method, although other methods, e.g., Qiagen Plasmid Maxi-Prep columns (Qiagen), could also be used.
8. Selection of healthy and well-expanded leaves is another key point for high transfection rates and reproducible experimental results. Protoplasts must be isolated from healthy and well-expanded leaves to ensure that the cell populations are active and homogeneous, which will offer low stress background and for high transfection efficiency (>90%). This is especially important for investigating the function of genes, which are involving in the stress, defense, and hormonal related pathways.
9. It takes some practice and patience to do a good job for cutting leaves gently but quickly. Be careful to avoid extra wounding or crushing on the leaf strips.
10. Digestion time needs to be optimized empirically; it depends on the material (ecotype, genotype) and your experiment scopes. Prolonged digestion time in the dark might produce more stress background, decrease the physiological response, and reduce the transfection efficiency.
11. A round-bottom tube and a swing-out rotor centrifuge are strongly recommended, which will decrease the damage of protoplasts by shearing force created in the centrifuge processes.
12. Assess the quality (cell size and cell status) and quantity (cell concentration) of the resuspended protoplasts by microscopy observation. Then, adjust the final concentration of protoplasts to 2×10^5/mL. About 10^3 to 10^4 protoplasts are sufficient for one LUC- or GFP-based reporter assay, 10^4 to 10^5 protoplasts are required for co-immunoprecipitation, and 10^5 to 10^6 protoplasts are recommended for RNA extraction and transcriptome analysis.
13. The amount of total plasmid used in one transfection with 4×10^4 protoplasts is highly recommended to no more than 20 μg. But it could be adjusted based on optimization. Multiple plasmids could be transfected in one transformation, but the total amount of plasmid for transfection needs to be maintained.
14. The culture plates are recommended to be pre-coated by 5% calf serum. For larger-scale experiments (50–100 samples),

12-well (0.5 mL WI per well) or 24-well (0.25 mL WI per well) plates could be used. Protoplast transfection can be scaled up or down depending on the experimental needs.

15. In general, for subcellular localization, protein-protein interaction, protein kinase and enzyme activity, and gene expression analysis, 4–16 h incubation after transfection is enough. It might need longer time for gene silencing or genome editing assay (24–36 h). The most suitable incubation time requires optimization on a case-by-case basis.
16. The amount of plasmid used for transfection requires optimization. Keep in mind that transfection of high amount of plasmid DNA may cause overaccumulation of the target proteins, which will lead to a hyper-saturated state of gene expression regulation.
17. The most suitable protoplast incubation time requires optimization. Longer incubation time may cause overaccumulation of the target proteins and more broken protoplasts.
18. A concave microscope slide is highly recommended. Protoplasts are plant cells without the cell wall. It is easily crushed by a coverslip when using a normal slide.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant 31870269 to Y.X., 31800199 and 32170273 to Y.L.), and the funding from Fujian Agriculture and Forestry University (Y.X.).

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Chapter 4

Application of Protoplast Regeneration to CRISPR/Cas9 Mutagenesis in *Nicotiana tabacum*

Fu-Hui Wu, Yu-Hsuan Yuan, Chen-Tran Hsu, Qiao-Wei Cheng, and Choun-Sea Lin

Abstract

Protoplast transfection is widely used in plant research to rapidly evaluate RNA degradation, reporter assay, gene expression, subcellular localization, and protein-protein interactions. In order to successfully use protoplast transfection with the newly emerging clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein editing platform, high yield of protoplasts, stable transfection efficiency, and reliable regeneration protocols are necessary. The *Nicotiana tabacum* transient protoplast transfection and regeneration system can effectively obtain target gene mutations in regenerated plants without transgenes and is thus a very attractive technique for evaluating gene editing reagents using CRISPR/Cas-based systems. Here, we describe in detail sterilized seed germination, culture conditions, isolation of *Nicotiana tabacum* protoplasts from tissue culture explants, construction of a vector containing the Cas protein and sgRNA cassette, highly efficient polyethylene glycol-calcium transient transfection of plasmids delivered into protoplasts, evaluation of mutagenesis efficiency and genotype analysis from protoplasts and regenerated plants, and the regeneration conditions to obtain CRISPR-edited plants from single protoplasts.

Key words CRISPR, Protoplast regeneration, Protoplast transfection, Ribonucleoprotein, Single protoplast genotyping

1 Introduction

Accurate and efficient gene editing is important for the advancement of life science research in general and for crop breeding. With the rapid development of technology, various gene editing techniques have been developed such as zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) proteins. These emerging gene editing methods are able to identify specific sequences of the target gene, enabling their deletion or modification [1–7].

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_4,

Prior to CRISPR, genome engineering approaches like ZFNs or TALENs required scientists to design and generate a new nuclease pair for every genomic target. CRISPR systems only contain two components, a CRISPR-associated endonuclease (Cas protein) and a guide RNA (gRNA or sgRNA). The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas protein-binding and user-defined ~20 nucleotide spacer that defines the genomic target to be modified. Thus, one can change the genomic target of the Cas protein by simply changing the target sequence present in the gRNA. Because of its convenience and efficiency, plant scientists have focused particularly on the establishment of CRISPR/Cas gene editing technology and applied it to plant research and crop breeding. Many research groups have focused on actively developing and publishing various gene editing vectors for monocot and dicot crops using different Cas proteins and sgRNAs [2, 8–17]. In plants, *Agrobacterium*-mediated stable transformation is commonly used for CRISPR/Cas gene editing. Plant cells can be subjected to transgenic selection by adding selectable markers of the plasmids. Using this method, CRISPR/Cas genes from plasmids can be inserted into plant chromosomes, and Cas proteins and gRNAs can be expressed for gene editing of the target sequence in plant cells. Because the edited regenerants contain transgenic genes introduced by *Agrobacterium*, the segregation screen can be achieved through offspring self-crossing and chromosome segregation to remove the introduced transgene. As a result, offspring with superior traits similar to those of traditional breeding and without transgenes can be obtained, and the time required for breeding is reduced [18–22].

Protoplasts are usually obtained by plant cell wall digestion with cellulose and macerozyme solution, transient transfection via polyethylene glycol (PEG)-calcium (Ca^{2+}), or electroporation of plant protoplasts [23, 31–33]. The plasmid containing CRISPR/Cas can be used to transiently express Cas proteins and gRNAs [24, 25]; or Cas proteins and sgRNAs can be directly assembled into ribonucleoprotein (RNP) complexes [26] in vitro and then introduced into the protoplasts [27, 28]. Regardless of whether plasmid DNA or RNP complexes are introduced into protoplasts for gene editing, protoplasts can be regenerated from a single cell into a gene-edited plant by inducing cell wall regeneration, callus initiation, and tissue differentiation [24, 25, 27, 29, 30].

Regenerating protoplast is derived from a single cell. When the target gene in the genome is edited before cell division, the target gene is edited in all the cells of the regenerants. Therefore, the edited genes seen in the genotype analysis of vegetative cells can be transmitted to the offspring [24, 25, 27]. CRISPR/Cas9 DNA only has to be transiently transfected into the protoplast, and no chromosome insertion is required for gene editing. Here, we present a detailed method for protoplast isolation, transfection, and

CRISPR/Cas9 mutagenesis regeneration, including sterile explant preparation and protoplast isolation, plasmid DNA via the PEG-Ca^{2+} transfection, for which 70% protoplast transient transfection can be achieved [24, 33]. By selecting suitable target sites [34, 35] and achieving highly editing efficiency, the target gene-edited protoplasts can be regenerated into plants using tissue culture with plant growth regulators, even without using antibiotics or a special phenotype as a screening marker. From our established protoplast regeneration platform, we can successfully obtain up to 60% mutagenized regenerated tetraploid tobacco plants containing at least one mutated allele [24].

2 Materials

2.1 Supplies and Equipment

1. 0.1 mm deep hemocytometer.
2. 0.22 μm syringe sterilization filter, PES membrane or MCE membrane.
3. 15 mL centrifuge tube.
4. 40 μm nylon mesh.
5. Autoclave.
6. Cell culture dish (Φ35 mm × H10 mm, Φ90 mm × H15 mm).
7. Glass Petri dish (Φ60 mm × H15 mm).
8. Glass screw cap culture tubes (round-bottomed tube, Φ13 mm × H100 mm; *see* **Note 1**).
9. Lamina flow hood.
10. Microcentrifuge and swing-out rotor centrifuge.
11. Optical microscope.
12. Orbital shaker.
13. Plant DNA purification kit.
14. Plasmid purification kit.
15. 5× polymerase chain reaction (PCR) mixture reagent.
16. Surgical blade.
17. Test tubes (Φ20 mm × H150 mm).
18. Thermal cycler for performing polymerase chain reaction (PCR).
19. Bath type ultrasonicator.
20. NanoDrop™ Microvolume UV-Vis Spectrophotometer or spectrophotometer.
21. Wide mouth glass bottle with cap (Φ50 mm × H80 mm).

22. Spreader.
23. Parafilm.

2.2 Plant Material and Vector Construct

1. Plant material: Tobacco (*Nicotiana tabacum*, Wisconsin 38) seeds. We used seeds which were a gift from the Transgenic Plant Laboratory, Academia Sinica. Growth condition please refer to Subheading 3.1.
2. CRISPR target site selection and DNA construct: We selected and screened target sites using CRISPR-P v.2.0 website [35], choosing higher on-score candidate target sites and cloning the sgRNA sequence into the pYLCRISPR/Cas9P35S-N system [10]. The plant codon optimized Cas9 gene (Cas9p) sequence including the nuclear localization signal peptide was designed with codon optimization for plants, including higher GC content at the 5′ terminal region for Gramineae genes [36]. The Cas9p was linked to the cauliflower mosaic virus 35S promoter (P_{35S}) in intermediate plasmids, and the cassettes were cloned into binary vectors derived from pCAMBIA1300, which had the Neomycin Phosphotransferase II gene. A fragment containing a modified ccdB flanked by two *Bsa*I sites was cloned into the vectors to produce the CRISPR/Cas9 binary vectors. *E. coli* strain Top10 F′ was used for maintaining the binary vectors. The target sgRNA sequences were synthesized using overlapping PCR performed by a thermal cycler. Target site sequence linkage with AtU3 or AtU6 promoters was introduced into the binary vector pYLCRISPR/Cas9P35S-N using Golden Gate cloning and transformed into *E. coli* strain DH5α. The AtU3b, AtU3d, AtU6-1, and AtU6-29 promoter sequences were amplified from *Arabidopsis* Columbia-0.

2.3 Chemicals and Stock Solutions

Prepare all solutions by using ultrapure water (prepared with pure deionized water to achieve a sensitivity of 18 MΩ-cm at 25 °C). Autoclave or filter all sterile prepared solutions and operate in a sterile environment. Special instructions: autoclave, 121 °C for 20 min; sterile filtration, 0.22 μm Stericup or syringe; room temperature, 22–26 °C.

1. MES (0.1 M, pH 5.7): Dissolve 1.95 g of 4-morpholineethanesulfonic acid (MES) in ~90 mL water. Adjust pH level to 5.7 with 1 M KOH and final volume to 100 mL. Filter sterilize and store the solution at room temperature for up to 1 month.
2. KCl (0.2 M): Dissolve 1.49 g of potassium chloride in water and adjust to a final volume of 100 mL. Filter sterilize and store the solution at room temperature for up to 2 months.

3. Glucose (0.5 M): Dissolve 4.5 g of glucose in water and adjust to a final volume of 50 mL. Filter sterilize and store the solution at 4 °C for up to 6 months.
4. D-mannitol (0.8 M): Dissolve 7.29 g of D-mannitol in water and adjust to a final volume of 50 mL. Filter sterilize and store the solution at room temperature for up to 2 months.
5. KOH (1 M): Dissolve 0.56 g of potassium hydroxide in water and adjust to a final volume of 10 mL. Filter sterilize and store the solution at room temperature for up to 6 months.
6. Kinetin (1 mg/mL): Dissolve 20 mg of kinetin in 2 mL of 1 M KOH solution. Adjust to a final volume of 20 mL. Store the solution at 4 °C for up to 6 months.
7. NAA (1 mg/mL): Dissolve 20 mg of 1-naphthaleneacetic acid (NAA) in 2 mL of 1 M KOH solution. Adjust to a final volume of 20 mL. Store the solution at 4 °C for up to 1 year.
8. TDZ (1 mg/mL): Dissolve 10 mg of thidiazuron (TDZ) in 2 mL dimethyl sulfoxide (DMSO). Adjust to a final volume of 10 mL with sterile water. No sterilization needed. The solution can be stored at 4 °C for 1 year.
9. $CaCl_2$ (2 M): Dissolve 29.40 g of $CaCl_2 \cdot 2H_2O$ in water. Adjust to a final volume of 100 mL. Filter sterilize and store the solution at room temperature for up to 6 months.
10. Sucrose (20%): Dissolve 10 g of sucrose in water and adjust to a final volume of 50 mL. Filter sterilize the solution and store at 4 °C for up to 1 month.
11. NaCl (3 M): Dissolve 17.53 g of sodium chloride in water and adjust to a final volume of 100 mL. Filter sterilize the solution and store at room temperature for up to 6 months.
12. Bleach solution: Mix 1 mL of commercial bleach (contains 6% sodium hypochlorite) with 5 mL water. Freshly prepare before being used for seed sowing in vitro.
13. X-gal (20 mg/mL): Dissolve 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 5 mL DMSO. No sterilization needed. The solution can be stored in the dark at −20 °C for years.
14. IPTG (50 mg/mL): Dissolve 250 mg of isopropyl β-D-1-thio-galactopyranoside (IPTG) in water and adjust to a final volume of 5 mL. Filter sterilize. The solution can be stored at −20 °C for years.
15. Cellulose R-10 (*see* **Note 2**).
16. Macerozyme R-10 (*see* **Note 2**).

2.4 Working Solution and Media

1. ½MS liquid medium: Dissolve 0.22 g of commercial MS stock powder (Murashige and Skoog Basal Medium with Vitamins),

7.29 g of mannitol (final 0.4 M), and 3 g of sucrose (final 3%) in ~90 mL water. Adjust pH to 5.7 with 1 M KOH and final volume to 100 mL. Filter sterilize the solution and store at room temperature for up to 1 month.

2. ¼MS liquid medium: Dissolve 0.11 g of commercial MS stock powder, 7.29 g of mannitol (final 0.4 M), and 3 g of sucrose (final 3%) in ~90 mL water. Adjust pH to 5.7 with 1 M KOH and final volume to 100 mL. Filter sterilize the solution and store at room temperature for up to 1 month.
3. Digestion solution: Dissolve 0.1 g of Cellulose R-10 and 0.05 g of Macerozyme R-10 in 10 mL ¼MS liquid medium. Filter sterilize the solution. Digestion solution needs to be freshly prepared before protoplast isolation (*see* **Note 2**).
4. W5 solution: To make 200 mL, in an autoclaved beaker, add 10.3 mL 3 M NaCl (final 154 mM), 12.5 mL 2 M $CaCl_2$ (final 125 mM), 5 mL 0.2 M KCl (final 5 mM), 4 mL 0.1 M MES (pH 5.7; final 2 mM), 2 mL 0.5 M glucose (final 5 mM) to 160 mL autoclaved sterile water. Adjust final volume to 200 mL. The solution can be stored at room temperature for up to 2 months (*see* **Note 3**).
5. Transfection solution: same as ½MS liquid medium.
6. PEG-Ca^{2+} solution: Dissolve 4 g of PEG 4000 (final 40%, w/v), 5 mL of 0.8 M mannitol (final 0.4 M), 0.5 mL of 2 M $CaCl_2$ (final 0.1 M), then add ~1 mL water to adjust to a final volume of 10 mL. The solution can be vortexed to speed up the dissolution and sterilized by passing through a 0.22 μm syringe filter with a PES membrane or MCE membrane. PEG-Ca^{2+} solution must be freshly prepared before protoplast transfection (*see* **Note 4**).
7. Callus initiation medium: Add 10 μL of 1 mg/mL NAA (final 1 μg/mL) and 3 μL of 1 mg/mL kinetin (0.3 μg/mL) into 10 mL of ½MS liquid medium containing 0.4 M mannitol and 3% sucrose, pH 5.7. Filter sterilize the solution.
8. Shoot-inducing medium (liquid): For making 500 mL, dissolve 1.1 g of commercial MS stock powder (final ½ x) and 15 g of sucrose (final 3%) in water, add 50 μL of 1 mg/mL TDZ (final 0.1 μg/mL). Adjust pH to 5.7 with 1 M KOH and final volume to 500 mL. Sterilize the medium by autoclaving.
9. Shoot-inducing medium (solid): In a 500 mL shoot-inducing liquid medium, add 5 g Bacto Agar (final concentration: 1%; *see* **Note 5**). Sterilize by autoclaving. Cool down the medium to 50 °C and pour to cell culture dishes (Φ90 mm × H15 mm); each dish should contain a 25 mL medium.
10. Seed germination/root-inducing medium: To prepare 500 mL, dissolve 1.1 g of commercial MS stock powder (final

½ x) and 15 g of sucrose (final 3%) in water. Adjust pH to 5.7 with 1 M KOH and final volume to 500 mL. Add 5 g Bacto Agar (final conc. 1%; *see* **Note 5**). Sterilize by autoclaving. Cool down the medium to 50 °C before portioning medium into test tubes (Φ20 mm × H150 mm) or wide mouth glass bottles with caps.

3 Methods

All processes must be carried out in a sterile laminar flow hood.

3.1 Explant Preparation

1. Surface sterilize tobacco seeds with 75% ethanol for 1 min in a 15 mL centrifuge tube.
2. Remove the ethanol using a pipette.
3. Add 10 mL bleach solution. Place the tube in an ultrasonicator for 15 min.
4. Remove the sterilant using a pipette.
5. Wash the seeds with sterilized water five times.
6. Keep the seeds in 10 mL water for around 30 min to facilitate better germination.
7. Place about 25 sterilized seeds evenly into a wide mouth glass bottle containing a germination medium using a pipette.
8. Maintain seeds in a growth chamber at 26 °C with a light density of 75 μmol/m^2/s and a 12 h light/12 h dark photoperiod.
9. Perform protoplast isolation and transfection after 3 weeks of seed germination.
10. Four weeks after the seed sowing, propagate and re-juvenilize plants by collecting the top branches (using scalpel and forceps) and subculturing the explants to a fresh germination medium. Perform this activity every month.
11. Three weeks after the subculture, the explants can be used as the material for protoplast isolation and transfection.

3.2 Protoplast Isolation

1. Collect 5–7 pieces of 3–4-week-old, fully expanded tobacco leaves, which have been aseptically grown in a germination medium (*see* **Notes 6** and **7**).
2. Place these leaves in a 6 cm sterile glass Petri dish containing 10 mL digestion solution. Soak the leaves in digestion solution.
3. In the digestion solution, cut the leaves into 0.5 cm wide strips using a surgical blade without crushing the tissue.
4. Seal the dish with Parafilm. Incubate the dish at room temperature (24–26 °C) in the dark overnight (16–18 h).

5. On day 2, place the dish on a benchtop orbital shaker. Gently shake the dish at 70 rpm for 10–30 min. The enzyme solution should turn green after gentle swirling, which indicates the release of protoplasts.
6. Check for the release of protoplasts in the enzyme solution under the microscope. The size of *N. tabacum* protoplasts is approximately 30–60 μm (*see* **Note 8**).
7. Carefully filter the enzyme solution/protoplasts through a 40 mm nylon mesh to a new, sterile 6 cm glass dish to separate undigested leaf residues from the protoplast suspension. Discard the undigested leaf residues (*see* **Note 8**).
8. Slowly add an equal volume (approximately 10 mL) of W5 solution into the protoplast suspension to terminate the cell wall digestion reaction.
9. Transfer the solution to four glass screw cap culture tubes (4–5 mL each; *see* **Note 1**).
10. Centrifuge the solution at 360 × *g* with a swing-out rotor at 24 °C for 3 min. Remove as much supernatant as possible.
11. Slowly and gently resuspend the protoplasts with 5 mL of W5 solution for each tube (*see* **Note 9**).
12. Centrifuge at 360 × *g* for 3 min as described in **step 10**.
13. Remove the supernatant. Add 3 mL of 20% sucrose solution and resuspend the protoplast pellet. Carefully mix the content by gentle swirling and tapping the tube.
14. Centrifuge at 490 × *g* for 3 min.
15. Carefully transfer the dark green, intact, and healthy protoplasts suspended on the surface of the sucrose solution into a new glass screw cap culture tube (*see* **Note 10**).
16. Add twice the cell volume of W5 solution to suspend the protoplasts.
17. Centrifuge at 360 × *g* for 3 min.
18. Repeat **steps 10** and **11** twice.
19. Take 10 μL of well-mixed protoplast suspension and load it onto a hemocytometer to measure the protoplast density and yield.
20. Resuspend. Intact and healthy protoplasts will be at 2×10^5/mL in W5 solution after counting cells under the microscope (100×).

3.3 Protoplast Transfection

1. Once the protoplast density is determined, centrifuge the tube at 360 × *g* for 3 min.
2. Remove the W5 solution supernatant as much as possible without touching the protoplast pellet (*see* **Note 11**).

3. Resuspend the cells with transfection solution and adjust the cell density to 3×10^5/mL using a hemocytometer (*see* **Note 12**).
4. Add 40 μL of DNA (20–40 μg) to a glass screw cap culture tube (*see* **Note 13**).
5. Slowly transfer 400 μL (cell density approx. 1.2×10^5) of protoplasts into the tube and mix carefully.
6. Add the same volume (440 μL) of PEG-Ca^{2+} solution.
7. Carefully tap the wall of the tube to mix the protoplasts with the PEG-Ca^{2+} solution completely.
8. Incubate the transfection mixture at room temperature (24–26 °C) for up to 30 min.
9. Add 3 mL of W5 solution. Mix well by gently rocking or inverting the tube to terminate the transfection reaction.
10. Centrifuge at 360 × *g* for 3 min.
11. Remove the supernatant, resuspend, and wash protoplasts gently with 3 mL of W5 solution.
12. Centrifuge at 360 × *g* for 3 min.

3.4 Protoplast Regeneration

1. After centrifuging, remove the supernatant of transfected protoplasts. Add 2 mL of callus initiation medium to resuspend the transfected protoplasts.
2. Transfer the protoplast suspension into a cell culture dish (Φ35 mm × H10 mm). Seal the dish with Parafilm. Incubate in the dark for 3–4 weeks at 24–26 °C (*see* **Note 14**).
3. Four weeks after the incubation in callus initiation medium, the protoplasts can be regenerated into 2 mm calli. Tilt the culture dish 5–10 degrees and remove the supernatant (*see* **Note 15**).
4. Add a 2–3 mL liquid shoot-inducing medium into the culture dish and suspend the calli by pipetting.
5. Transfer the calli and medium to a 9 cm culture dish. Add a liquid shoot-inducing medium to a final volume of 10 mL.
6. Seal the dish with Parafilm. Incubate the calli at 26 °C under a light density of 75 μmol/m^2/s, with a 12 light/12 dark photoperiod.
7. After 4 weeks, green calli can be observed in a liquid shoot-inducing medium and regenerated into 5 mm diameter calli.
8. Pick up green calli using tweezers and transfer to a solid shoot-inducing medium. Repeat **step 6**.
9. Subculture green calli every 4 weeks using a solid shoot-inducing medium. After subculturing one to two times, 100–500 shoot clusters of 1 cm regenerative buds and leaves can be obtained.

10. Transplant the regenerating shoots to root-inducing medium. Adventitious roots will grow after 1 month.
11. Transfer the rooted plantlet to a pot of peat moss, vermiculite, and perlite at a ratio of 1:1:1.
12. Grow the plants in a greenhouse or growth chamber at 26 °C, with a light density of 70 μmol/m^2/s and a 12 h light/12 h dark photoperiod.

3.5 Evaluate CRISPR Reagents in Protoplasts or Similar Topics

3.5.1 Transfected Protoplast Efficiency Testing at the Initial Stage

1. Transfected protoplasts can be used for transient analysis of CRISPR-mediated targeted mutagenesis. Four days after transfection, use 500 μL of pooled protoplasts in callus initiation medium for genomic DNA extraction using a DNA extraction kit.
2. Use a NanoDrop to measure DNA quality and quantity. DNA concentration should be approx. 25–100 ng/μL in sterile H_2O (*see* **Note 16**).
3. Amplify the target site DNA by polymerase chain reaction. Use 1 μL protoplast genomic DNA as template and mix with 1 μL (final 10 μM) forward primer, 1 μL (final 10 μM) reverse primer (*see* **Note 17**), 4 μL 5× PCR Taq mixture, and 13 μL H_2O in a final volume of 20 μL (*see* **Note 18**).
4. PCR conditions: 94 °C for 5 min, denature double-stranded DNA for 35 cycles at 94 °C for 30 s, anneal primers at 55 °C for 30 s (*see* **Note 19**), extend DNA at 72 °C for 30 s, followed by 72 °C for 3 min (*see* **Note 20**).
5. Perform electrophoresis on the PCR product to confirm that the size is similar to that expected.
6. Sequence the PCR product using the Sanger method (*see* **Note 21**). If the targeted site was edited by the CRISPR reagent in the protoplast genomic DNA, one can expect multiple peaks in sequence chromatograph to be seen near 3 bp upstream of the protospacer adjacent motif (PAM) region compared to the non-transfected control. These edited protoplasts can be used for regeneration and further analysis.

3.5.2 Single Protoplast Genotyping

Although the editing events can be assessed using Sanger sequencing of genomic DNA extracted from transfected and pooled protoplasts, the editing efficiency cannot be evaluated this way. Single protoplast analysis can be used to determine the editing efficiency.

1. Pick 10 μL transfected protoplasts in callus initiation medium and adjust to a final concentration of 1 protoplast/μL measured using the hemocytometer with callus initiation medium.

2. Pipette 1 μL callus initiation medium into a glass slide and confirm that a single protoplast is isolated under an optical microscope.
3. Pick up this single protoplast and put it into the 20 μL PCR mixture of the first round Nested PCR containing the first pair of primers to amplify the target site DNA. PCR conditions: 94 °C for 5 min, denature the double-stranded DNA for 20 cycles at 94 °C for 30 s, anneal primers at 55 °C for 30 s, extend DNA at 72 °C for 30 s, followed by 72 °C for 3 min (*see* **Notes 19**, **20**, and **22**).
4. Use 1 μL PCR product as a template and add into the 20 μL PCR mixture containing the second pair of primers for second round Nested PCR. PCR conditions: 94 °C for 5 min, denature the double-stranded DNA for 20 cycles at 94 °C for 30 s, anneal primers at 55 °C for 30 s, extend DNA at 72 °C for 30 s, followed by 72 °C for 3 min (*see* **Notes 19**, **20**, and **22**).
5. Perform electrophoresis on the PCR product to confirm the size, then followed by Sanger sequencing.
6. Sequences with deletions or insertions near the PAM indicate that gene editing occurred.

3.5.3 Genotyping of Regenerated Plants

1. Use 0.2 g regenerated plant leaves for DNA extraction.
2. Amplify target site DNA by PCR reaction following steps described in Subheading 3.5.1.
3. Determine the target sequences of the regenerated plants with non-edited and homozygous edited alleles (contain similar edited sequences in all alleles) using Sanger sequencing analysis. Analyze multiple sequencing results of biallelic and heterozygous regenerated plants using Poly Peak Parser website [37].
4. In the case that the multiple signals cannot be resolved in silico to determine the edited sequences, further analysis can be performed using T/A cloning and sequencing method.
5. Use T4 ligase and ligation buffer to ligate PCR products and linearized destination TA vector that contains LacZ fragment, and adjust to a final volume of 10 μL. A typical molar ratio of insert PCR product to destination vector is 3:1.
6. Set PCR product and vector with ligase mixture at 4 °C overnight.
7. Introduce ligation mixture (vector with PCR product) into bacterium competent cells such as *E. coli* (e.g., strain DH5α) and spread in a LB solid plate with 30 μL of X-gal solution, 30 μL of IPTG solution, and an appropriate antibiotic, e.g., ampicillin (*see* **Note 23**).
8. Incubate the plate at 37 °C overnight.

9. Spread up to 100 μL of transformed *E. coli* cells onto the LB agar plates using a sterile spreader.
10. Blue and white colonies appear on the LB plate surface. Select white colonies that are likely to contain the PCR product (*see* **Notes 24** and **25**).
11. Pick up white single colonies and perform PCR analysis as described in Subheading 3.5.1.
12. Grow the edited plants to maturity in greenhouse using the growth conditions described in **step 12** of Subheading 3.4.
13. Harvest the seeds for progeny analysis. Determine the genotypes of progeny as described in Subheading 3.5.1.

4 Notes

1. Use round-bottomed tube centrifuges in experimental procedures for protoplast isolation and transfection.
2. Cellulose R-10 and Macerozyme R-10: Yakult Pharmaceutical Industry, Japan. Store at 4 °C. We found that the quality of the cellulose and macerozyme source is critical to achieving high protoplast isolation efficiency.
3. W5 solution: In our laboratory, we dispense 200 mL at a time and divide the solution into four bottles. For large volumes, 0.22 μm Stericup Vacuum Filters can be used for sterilization.
4. PEG 4000: Catalog no. 95904, BioUltra, Sigma-Aldrich, USA. We found that the quality and purity of PEG source is critical to achieving high transfection efficiency. The higher the purity has, the higher the transfection efficiency. PEG-Ca^{2+} solution should be freshly prepared at least 1 h before transfection. Freshly prepared PEG-Ca^{2+} solution can improve the protoplast transfection efficiency [32]. PEG-Ca^{2+} solution cannot be sterilized using an autoclave and should be filtered using a 0.22 μm syringe sterilization filter, PES membrane or MCE membrane.
5. Bacto Agar: The weight required per liter of agar varies from company to company. But the amount added is usually 7–10 g/L depending on different brands of a product.
6. Do not use yellowed leaves; the selection of healthy leaves is a very important factor in protoplast experiments.
7. A good preparation yields approximately 10^6 protoplasts per 0.2–0.25 g fresh weight (approximately 5–7 leaves digested in 10 mL of enzyme solution).

8. Operate all the protoplast processes gently. It is not necessary to completely digest all the leaves in enzyme solution. The protoplasts are fragile.
9. Handle protoplasts with regular pipettes and pipette tips but without touching the protoplast pellet.
10. The total volume of the protoplast suspension can range from 0.5 to 1 mL depending on the operator's experience.
11. The less W5 remaining, the higher transfection efficiency. W5 supernatant can reduce transfection efficiency.
12. Before the PEG-Ca^{2+} transfection procedure, replace the W5 supernatant with transfection buffer.
13. Extract plasmid DNA within 2 weeks to increase the transfection. Plasmid DNA of 5–10 kb in size dissolved in water will achieve over 70% transfection rate, and plasmid DNA less than 15 kb in size will achieve 50% transfection rate. Protoplast-plasmid DNA transfection can be successfully performed with 18 kb in size.
14. If protoplasts are derived from healthy leaf materials, most protoplasts should remain intact throughout the isolation, transfection, culture, and harvesting procedures.
15. Subculture on time.
16. DNA purity is measured as 260/280 ratio ranging from 1.80 to 2.00.
17. Most commonly primers are ordered from an oligo-synthesis company, which synthesizes and ships them as a lyophilized powder. The researcher then needs to reconstitute their primers in liquid, normally sterile ultrapure water. To make a 100 μM stock of any primer, add a number of microliters of ultrapure water equal to the number of nanomoles of DNA times 10.
18. *N. tabacum* is allotetraploid; therefore, there are two subgenomes, S and T. For PCR primer design, specific primers for the different subgenomes are important.
19. The annealing temperature varies depending on the GC content of the primer pairs.
20. Extend DNA step: The Taq polymerase has an optimal temperature around 70–75 °C so this step enables the DNA polymerase to synthesize and elongate the new target DNA strand accurately and rapidly. Set the extension step at 30 s to 2 min per kilobase of product depending on whether you are using a polymerase with proofreading capabilities.
21. PCR products need to be purified before Sanger sequencing. Because Sanger sequencing is a highly accurate technique to read DNA sequence base by base, it is very important to purify

PCR products so that unincorporated primers and dNTPs will not interfere with results. This step could be performed by sequencing company that processes your samples or by yourself using protocols such as ethanol precipitation, bead, or column-based purification.

22. The first pair of primers is designed to anneal to sequences upstream of the second pair of primers, whereas the second pair of primers is situated internally or nested with respect to the first pair of primers. The first pair of primers also called "outer primers" amplify a large fragment of the gene which is used as a template in the second round of PCR that targets a smaller region of the amplicon using the second pair of primers also known as "inner primers or nested primers.".
23. Leave the plates to dry in a laminar flow chamber with lids slightly open before spreading transformed *E. coli* cells onto the LB agar plates.
24. X-gal is used as a visual indicator and convenient method of distinguishing a successful cloning product from other unsuccessful ones. This technique is called blue/white screening. The blue/white screening is only a screening procedure; it is not a selection technique.
25. If the color difference between the blue and white colonies after overnight incubation is not obvious, you can leave the plate at 4 °C for 2–4 h to increase the recognition.

Acknowledgments

We thank Ms. Miranda Loney and Plant Editors for English editing. We would like to thank Prof. Kan Wang for valuable comments and suggestions. This research was supported by Academia Sinica, Innovative Translational Agricultural Research Administrative Office (AS-KPQ-107-ITAR-10; AS-KPQ-108-ITAR-10; AS-KPQ-109-ITAR-10), and the Ministry of Science and Technology (105-2313-B-001-007-MY3; 108-2313-B-001 -011 -; 109-2313-B-001 -011 -; 110-2313-B-001-006-MY3), Taiwan.

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Chapter 5

CRISPR DNA- and RNP-Mediated Genome Editing via *Nicotiana benthamiana* Protoplast Transformation and Regeneration

Raviraj Banakar, Krishan M. Rai, and Feng Zhang

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated system) has become the multipurpose tool to manipulate plant genome via their programmable sequence recognition, binding, and cleavage activities. Efficient plant genome modification often requires robust plant transformation. For most plant species, the CRISPR/Cas reagents are delivered into plants as plasmids by *Agrobacterium*-mediated T-DNA transfer or biolistic approaches. However, these methods are generally inefficient, heavily genotype dependent, and low throughput. Among the alternative plant transformation approaches, the protoplast-based transformation holds the potential to directly deliver DNA, RNA, or protein molecules into plant cells in an efficient and high-throughput manner. Here, we presented a robust and simplified protocol for protoplast-based DNA/ribonucleoprotein (RNP)-mediated genome editing in the model species *Nicotiana benthamiana*. Using this protocol, we have achieved the gene editing efficiency at 30–60% in protoplasts and 50–80% in regenerated calli and plants. The edited protoplasts can be readily regenerated without selection agents owing to highly efficient DNA or preassembled RNP transformation frequency. Lastly, this protocol utilized an improved culture media regime to overcome the complex media composition used in the previous studies. It offers quick turnaround time and higher throughput to facilitate the development of new genetic engineering technologies and holds the promise to combine with other genetic and genomic tools for fundamental and translational plant research.

Key words CRISPR, DNA-free gene editing, Polyethylene glycol, Ribonucleoprotein, Transient transformation

1 Introduction

The prokaryotic adaptive immune system clustered regularly interspaced short palindromic repeats (CRISPR) helps attain immunity against invading bacteriophages by performing cleavage of their DNA [1, 2]. The very same system was later adopted to edit the genome of many species including plants [3–10]. Among the CRISPR systems, CRISPR-Cas9 and CRISPR-Cas12a are two

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_5,

major systems exploited to enable plant gene editing in different genomic contexts [3, 6, 11]. These CRISPR-Cas systems have two broad components, Cas nucleases and guide RNA that traverse through the genome by means of unwinding the DNA due to helicase function of Cas proteins [12]. Cas9 targets NGG protospacer adjacent motif (PAM), while Cas12a prefers for TTTV PAM. Once complementary sequence in the genome is recognized by the guide RNA sequence, complementary RNA/DNA hybridization takes place to trigger DNA strand breaks (DSBs) via the Cas nuclease activity, which initiates the subsequent gene editing process [13, 14].

Upon the formation of DSBs, distinct DNA repair pathways are employed to introduce intended mutations, such as deletions, insertions, and point mutations, into the targeted sequences. The error-prone end joining DNA repair pathways have been demonstrated as the dominant pathways to introduce small insertions/deletions (indels) at the CRISPR/Cas cleavage sites in an imprecise manner. On the other hand, the homology-directed repair (HDR) pathway can be exploited to introduce precise modifications from donor templates [15–17]. Because of the low-frequency nature of the HDR pathway, significant efforts have been invested to improve precise gene editing through different approaches, including the DNA repair pathway optimization, cell cycle manipulation, donor template modifications, and more recently CRISPR/Cas repurposing. For example, the new base editing and prime editing technologies fuse catalytically inactive or nickase Cas9 fused with either a deaminase or reverse transcriptase domain to introduce precise point mutations or insertions/deletions through a modified guide RNA donor [18, 19]. Although these new improvements have been demonstrated in few plant species recently, the efficiency of precise gene editing is in general still much lower than that in animal systems. This is largely attributed to the low efficiency and throughput in plant transformation and regeneration [20].

Efficient plant genome editing is ultimately dependent on robust plant transformation. For most plant species, the CRISPR/Cas reagents are delivered into plants as plasmids by *Agrobacterium*-mediated T-DNA transfer or biolistic approaches [7, 21, 22]. However, these methods are generally inefficient, heavily genotype dependent, and low throughput, and thus have been a major bottleneck in achieving the precision and large-scale gene editing in plants. Moreover, CRISPR reagents delivered as plasmid molecules often result in random integration of DNA fragments at cleavage sites as well as elsewhere in the genome [22, 23]. Therefore, more efficient, precise, and high-throughput transformation methods are greatly needed to facilitate the development of new plant gene editing technology and minimize the unintended modifications.

Among the alternative plant transformation approaches, the protoplast-based transformation holds the potential to directly deliver DNA, RNA, or protein molecules into plant cells in an efficient and high-throughput manner [24, 25]. In particular, CRISPR/Cas DNA or RNPs can be delivered to protoplasts by the polyethylene glycol (PEG)-mediated or electroporation approaches, and thus avoid random insertions of DNA constructs [11, 23].

Here, we presented a robust and simplified protocol for protoplast-based DNA/RNP-mediated genome editing in the model species *Nicotiana benthamiana* (Fig. 1). This protocol covered the step-by-step procedures for plant growth, leaf digestion, protoplast isolation, extraction, purification, transformation with

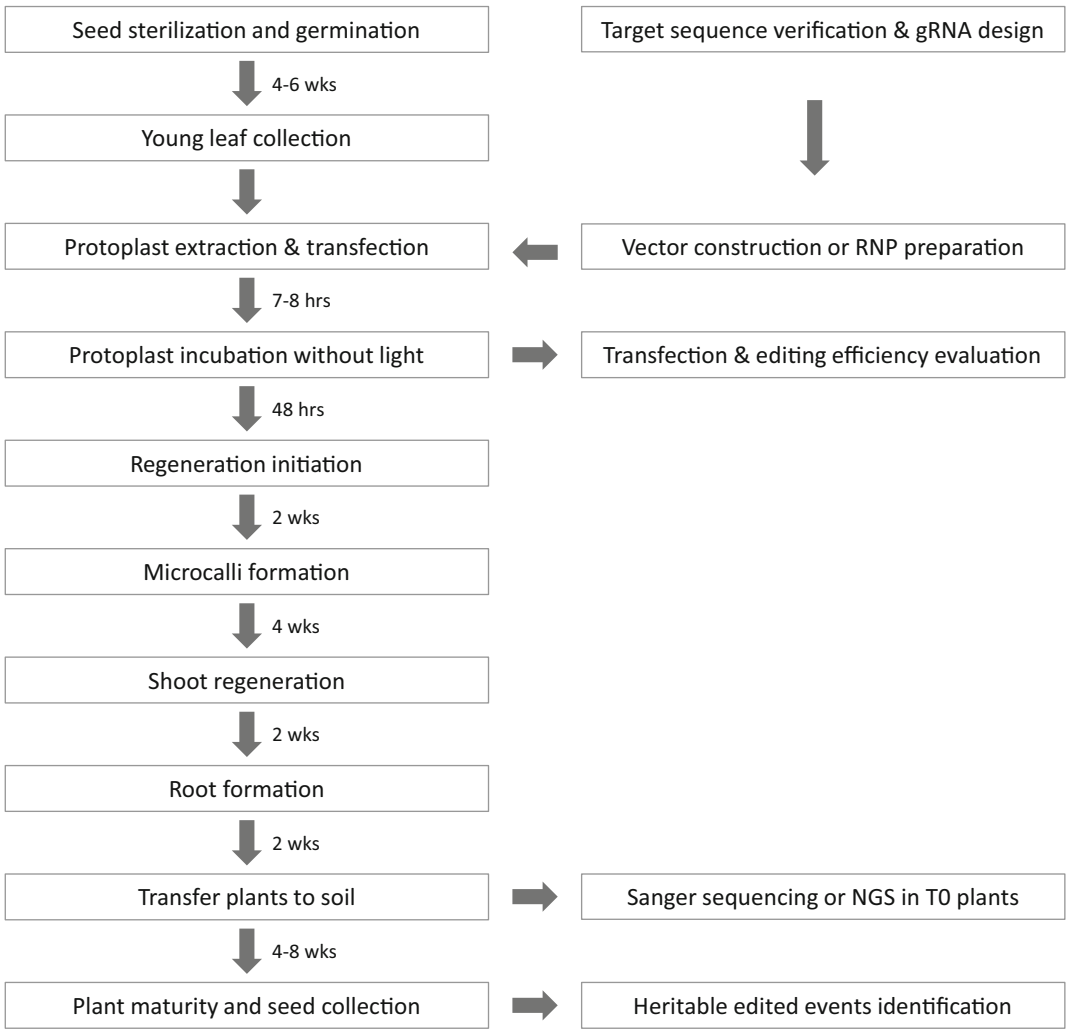

Fig. 1 Illustration of the overall processes and methodology for the protoplast-based genome editing using CRISPR/Cas DNA or RNP in *Nicotiana benthamiana*

DNA/proteins, protoplast culturing, calli, and whole plant regeneration. This process could take up to 60–70 days with transient gene editing efficiency at 30–60% in protoplast cells and at 80% in the regenerated calli and plants. In addition, this protocol utilized an improved culture media regime to overcome the complex media composition used in the previous studies [26, 27]. It offers quick turnaround time and higher throughput to facilitate the development of new genetic engineering technologies and holds the promise to combine with other genetic and genomic tools for fundamental and translational plant research [28, 29].

2 Materials

2.1 Plant Materials

1. *Nicotiana benthamiana* wild-type and GFP16C line: A single copy GFP expressing line developed by Dr. David Baulcombe at John Innes Centre, Norwich, UK [30, 31]. This is a line generated through *Agrobacterium*-mediated transformation with T-DNA containing mGFP5-ER driven by CaMV 35S promoter and transcriptional termination carried by *Agrobacterium tumefaciens* nopaline synthase (*nos*) terminator. In addition, T-DNA also contains the neomycin phosphotransferase II (*npt II*) gene for kanamycin resistance [32].

2.2 Chemicals and Stock Solutions

All stock solutions are prepared using double distilled water (ddH_2O), filtered through 0.22 μm Stericup or syringe, unless otherwise specified.

1. Cellulase R-10: Cellulase R-10 was purchased from the commercial vendor (Yakult Pharmaceutical Industry Co., Ltd., Japan) and stored in 4 °C under dark conditions (*see* **Note 1**).
2. Macerozyme R-10: Macerozyme R-10 was purchased from the commercial vendor (Kanematsu USA Inc.) and stored in 4 °C under dark conditions (*see* **Note 1**).
3. D-Mannitol (0.8 M): To prepare 500 mL, 72.87 g is dissolved in 450 mL ddH_2O (*see* **Note 2**). Adjust final volume to 500 mL with ddH_2O. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
4. MES (0.2 M, pH 5.7): Dissolve 21.35 g of 4-morpholineethanesulfonic acid ($C_6H_{13}O_4NS.H_2O$) in 450 mL ddH_2O, adjust pH to 5.7 with 1 M KOH, adjust the volume to 500 mL with ddH_2O. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
5. KCl (2 M): Dissolve 74.55 g of potassium chloride in 450 mL ddH_2O, adjust the volume to 500 mL with ddH_2O. Filter sterilize and store at room temperature (22 °C) for up to 6 months.

6. $CaCl_2$ (1 M): Dissolve 73.51 g of $CaCl_2$ $2H_2O$ in 450 mL ddH_2O, adjust the volume to 500 mL with ddH_2O. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
7. NaCl (2 M): Dissolve 58.44 g of $NaCl_2$ in 450 mL ddH_2O, adjust the volume to 500 mL with ddH_2O. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
8. $MgCl_2$ (2 M): Dissolve 40.67 g of $MgCl_2$ $6H_2O$ in 80 mL ddH_2O, make up the volume to 100 mL. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
9. Glucose (1 M). Dissolve 18 g of glucose in 80 mL ddH_2O, make up the volume to 100 mL. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
10. Sucrose (0.55 M): Dissolve 18.83 g sucrose in 80 mL ddH_2O, make up the volume to 100 mL with ddH_2O. Filter sterilize and store in 4 °C for up to 3 months.
11. BSA (10%): Dissolve 1 g of bovine serum albumin in 8 mL ddH_2O, make up the volume to 10 mL with ddH_2O. Filter sterilize and store in 4 °C for up to 1 month.
12. KOH (1 M): Dissolve 5.61 g of KOH in 80 mL ddH_2O, make up the volume to 100 mL. Filter sterilize and store at room temperature (22 °C) for up to 6 months.
13. BAP (1 mg/mL): 10 mg of 6-benzylaminopurine is weighed in a 1.5 mL centrifuge tube, to which 200 μL of 1 N NaOH is added. Vortex the tube to dissolve completely. Transfer the BAP solution to a tube of 8 mL ddH_2O. Adjust the final volume to 10 mL with ddH_2O. Filter sterilize. Store the solution at 4 °C for 1 month (*see* **Note 3**).
14. NAA (1 mg/mL): Naphthaleneacetic acid stock solution is prepared similar to BAP stock described above.
15. 10X PBS buffer (pH 7.4): In 800 mL ddH_2O, dissolve 14.20 g Na_2HPO_4, 2.45 g KH_2PO_4, 80.07 g NaCl, and 2.01 g KCl. Adjust the pH value to 7.4 with HCl and bring the final volume to 1000 mL. Autoclave and store at room temperature (22 °C) for up to 1 year.

2.3 Solutions and Media

All solutions and media are prepared using double distilled water (ddH_2O), filtered through 0.22 μm Stericup or syringe or autoclaved at 121 °C for 15 min, unless otherwise specified.

1. Digestion media (DM): Digestion media contains 0.45 M mannitol, 5 mM MES (pH 5.7), 0.8% Cellulase R-10, and 0.2% Macerozyme R-10 (*see* **Note 4**). To prepare 100 mL of

digestion media, weigh 0.8 g of Cellulase R-10 and 0.2 g of Macerozyme R-10 in a conical flask with 20 mL ddH_2O. Add 56.25 mL 0.8 M mannitol and 2.5 mL 0.2 M MES, stir well, and incubate in 37 °C water bath. Adjust pH to 5.7 with 1 M KOH. Make up the volume to 100 mL with ddH_2O. Filter sterilize. Immediately use the solution for protoplast extraction (*see* **Note 5**).

2. Washing buffer (WB): Washing buffer contains 0.45 mM mannitol and 10 mM $CaCl_2$. To prepare 300 mL of this solution, add 188.69 mL 0.8 M mannitol and 3 mL 1 M $CaCl_2$. Adjust pH to 5.7 with 1 M KOH. Make up the volume to 300 mL with ddH_2O. Filter sterilize. Store the solution either at room temperature or 4 °C (*see* **Note 5**).
3. MMG buffer: MMG buffer contains 0.4 M mannitol, 4 mM MES (pH 5.7), and 15 mM $MgCl_2$. To prepare 100 mL MMG buffer, add 50 mL 0.8 M mannitol, 2 mL 0.2 M MES, and 0.75 mL 2 M $MgCl_2$. Adjust pH to 5.7 with 1 M KOH and final volume to 100 mL with ddH_2O. Filter sterilize. Store solution either at room temperature or 4 °C (*see* **Note 5**).
4. PEG-calcium transfection media (TM): Transfection media consists of 0.2 M mannitol, 100 mM $CaCl_2$, and 40% PEG. To prepare 10 mL of transfection solution, add 2 mL 0.8 M mannitol, 1 mL 1 M $CaCl_2$, and 4 g PEG, stir well until clear solution is obtained. Adjust pH to 5.7 with 1 M KOH. Make up the volume to 10 mL with ddH_2O. Filter sterilize and use the solution immediately.
5. Protoplast culture nutrient solution (PCN): In 400 mL ddH_2O, add 563 mL of 0.8 M mannitol (final concertation as 0.45 M), add 4.3 g of MS powder with B5 vitamins (Cat# M404, PhytoTechnology Laboratories Inc., USA), 15 g sucrose (final concentration as 1.5%), 0.1 mL of 1 mg/mL BAP and NAA each (final concentration as 0.1 mg/L). Stir well using magnetic stirrer and adjust pH to 5.7 using 1 M KOH. Then bring the volume to 1000 mL with ddH_2O. Filter sterilize and store solution at 4 °C for up to 1 month (*see* **Note 5**).
6. Protoplast culture media (PCM): In 900 mL ddH_2O, dissolve 4.3 g MS with B5 vitamins, add 17 g sucrose (final concentration as 1.7%), 1 mL BAP (final concentration as 1 mg/L), 0.1 mL NAA (final concentration as 0.1 mg/L). Stir well and adjust pH to 5.7. Adjust the volume to 1000 mL with ddH_2O. Add 7 g Agar. Autoclave, cool down to 55 °C, and pour the media into 60 × 20 mm plates in aseptic conditions. Store the plates at 4 °C for up to 3 months.
7. Rooting media (RM): In 900 mL ddH_2O, add 4.3 g MS powder with B5 vitamins and 10 g sucrose (final concentration

as 1%). Stir well and adjust pH to 5.7. Adjust the volume to 1000 mL with ddH_2O and add 7 g Agar. Autoclave, cool down to 55 °C, and pour the media into 60 × 20 mm plates in aseptic conditions. Store the plates at 4 °C for up to 3 months.

8. Germination media (GM): Dissolve 4.3 g MS powder with B5 vitamins, and 10 g sucrose (final concentration as 1%) to 900 mL ddH_2O, stir well, and adjust pH to 5.7. Adjust the volume to 1000 mL with ddH_2O and add 3 g Phytagel (final concentration as 0.3%). Autoclave the media and cool down to 55 °C. Pour the media into 60 × 20 mm plates in aseptic conditions. Store the plates at 4 °C for up to 3 months.
9. IDTE buffer (1× TE solution, pH 7.5): Mix 10 mL 1 M Tris–HCl (pH 8.0; final concentration as 10 mM) and 2 mL 0.5 M EDTA (pH 8.0; final concentration as 1 mM) to 900 mL ddH_2O. Adjust the pH value to 7.5 with HCl and bring the final volume to 1000 mL. Autoclave and store at room temperature (22 °C) for up to 1 year.
10. Seed sterilization solution: Make 2 mL solution by adding 0.2 mL of commercial bleach (sodium hypochlorite to the final concentration as 0.6%) and 10 μL of 20% Tween 20 (final concentration as 0.1%).
11. Leaf sterilization solution: Make 500 mL solution by adding 25 mL commercial bleach (sodium hypochlorite to final concentration as 0.3%) and 2.5 mL 20% Tween 20 (final concentration as 0.1%).

2.4 Plant Growth Fertilizer Solutions

1. Fertilizer stock solution: Dissolve 200 g Peter's 20-10-20 fertilizer in 800 mL of water. Allow to stir for ~5 min, make up the volume to 1 L with water.
2. Fe-EDTA solution: Dissolve 7.45 g of ethylenediaminetetraacetic acid disodium salt dehydrate (Na_2-EDTA) in 800 mL of ddH_2O on a hot magnetic stir plate (50–60 °C) stir plate. Once Na_2-EDTA is dissolved, gradually add 5.52 g ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) into EDTA solution (50–60 °C) until chemical dissolves completely. Make up the volume to 1 L with ddH_2O.
3. Watering solution: Mix 50 mL of fertilizer stock solution and 10 mL of Fe-EDTA solution in 900 mL water, make up the volume to 2 L.

2.5 Other Supplies

1. Pipettes (1–2 μL, 10 μL, 100 μL, 100–1000 μL).
2. Tempo motorized pipette controller.
3. Test tube and centrifuge vial racks.
4. Laminar hood.
5. Autoclave machine.

6. 24-well plates.
7. 6-well plates.
8. Soilless substrate LC1.
9. 0.22 μm syringe and Stericup filtration system.
10. 24-well and 6-well plates.
11. Petri dish, 60 mm × 20 mm.
12. Forceps.
13. Double edge razor blade.
14. Benchtop orbital shaker.
15. 100 μm cell strainer.
16. 5 mL, 15 mL, 50 mL conical tube.
17. 15 mL round bottom tubes.
18. Wide bore 1000 μL pipette tips.
19. 5 mL, 15 mL, and 25 mL serological pipettes with regular tips.
20. Swing bucket rotor centrifuge with 15 mL and 50 mL conical and round bottom tube adapters.
21. Hemocytometer and cover glass.
22. Desktop microcentrifuge with a fixed-angle rotor.
23. Fluorescent microscope (EVOS FL Auto 2 system, Invitrogen Inc.).
24. Microscope (Nikon TE2000-E Inverted Fluorescence Microscope).
25. Parafilm.
26. Micropore wrap.

3 Methods

3.1 Choice of Target Site and Guide RNA Design

1. The target gene sequences can be identified and downloaded from the plant genome sequence websites, such as https://phytozome.jgi.doe.gov/pz/portal.html.
2. If the targeted sequences are not from the reference genome, they need to be verified by PCR and Sanger sequencing to identify any potential sequence polymorphisms.
3. Several online programs, such as CRISPR/RGEN, CHOPCHOP, and CRISPOR, can be used to identify the potential guide RNA targeted sites in the verified sequences (*see* **Note 6**).

1. In a 5 mL LB broth with 100 μg/mL carbenicillin, inoculate *E. coli* strains containing the SpCas9 (or other Cas proteins)

3.2 Plasmid DNA and CRISPR-RNP Preparation

3.2.1 Plasmid DNA Extraction

and guide RNA plasmids [33]. Shake at 250 rpm at 37 °C overnight (i.e., 16–18 h).

2. Transfer 100 μL of overnight grown culture to 250 mL of LB broth containing 100 μg/mL carbenicillin. Shake at 250 rpm at 37 °C overnight (i.e., 16–18 h) with OD_{600} between 3 and 6.
3. Perform plasmid extraction using NucleoBond Xtra Midi or Maxi prep kit (Takara Inc., Japan) according to the manufacturer's instruction.
4. Elute DNA with 100 μL of nuclease-free water.
5. Determine the quantity and quality of the DNA (*see* **Note 7**).
6. Adjust final concentration to 3 μg/μL in IDTE buffer and store the plasmids at −20 °C until further use.

3.2.2 RNP Complex Preparation

1. For RNP delivery, the guide RNA molecules, such as crRNA and tracrRNA, are chemically synthesized and ordered from Integrated DNA Technologies (Coralville, IA, USA) (*see* **Note 8**). The Cas nucleases, such as SpCas9, AsCas12a, and LbCas12a, can be ordered directly from vendors such as Integrated DNA Technologies (Coralville, IA, USA), which provides the Cas9 and Cas12a enzymes at 10 μg/μL concentration (64 μM and 62 μM, respectively).
2. Before protoplast transformation, dissolve crRNA and tracrRNA in 20 μL of nuclease-free IDTE buffer to a concentration of 100 μM of each.
3. To form the guide RNA complex first, mix equal molar crRNAs (200 pmol) and tracrRNA (200 pmol) in 0.2 mL PCR tubes, place tubes at 95 °C for 5 min.
4. Spin down the guide RNA complex for 5 s, place the tube at the room temperature (22 °C) for 10 min. This RNA complex can be used for RNP assembly.
5. To form the RNP complex, mix 2 μL of guide RNA complex (100 μM) with 2 μL of 10 μg/μL of Cas nuclease along with 2 μL of 1× PBS buffer (pH 7.4).
6. Incubate the tube at the room temperature for 10 min. The RNP complex is then ready for protoplast delivery.

3.3 Growing Plants for Explant for the Protoplast Isolation

1. Transfer *Nicotiana benthamiana* seeds to 2 mL round bottom tubes and sterilize the seeds for 2 min in 70% ethanol.
2. Wash seeds with sterile ddH_2O.
3. Sterilize the seeds again in seed sterilization buffer for 10 min.
4. Wash seeds three times with sterile ddH_2O and transfer seeds to germination media (GM).

5. Place 20–30 seeds per plate and allow for germination at 25 °C under 16/8 h of the light and dark cycle with 80–100 μmol/m^2/s light intensity.
6. Fifteen days after germination, transplant 8–12 healthy seedlings to a growth flat with 8-cell insert with the sterilized (autoclaved) potting mixture. Place humi-dome for vigorous seedling establishment to avoid light scorching and transplantation shock.
7. After a week, remove the humi-dome and maintain eight plants per tray.
8. Grow plants for 4 weeks in the growth chamber with 22/20 °C of day/night temperature under 16/8 h of the light and dark cycle with 80–100 μmol/m^2/s light intensity (*see* **Note 9**).
9. Supply 500 mL of nutrient solution per flat every 3 days (*see* **Note 10**).

3.4 Protoplast Isolation (See Note 11)

1. From 1-month-old plants (Fig. 2a), the second and third expanding leaves from the top are clipped off using a razor blade.
2. Immediately place two leaves in a 600 mL beaker with 250 mL ddH_2O and bring the samples to the laminar flow hood using an icebox.
3. Transfer leaves to sterile plates and wash twice with sterile water.
4. Sterilize two leaves with 250 mL leaf sterilization solution for 5 min and then wash them with 250 mL ddH_2O for three times in a 600 mL beaker.
5. Cut two leaves into 0.2 mm strips using a sharp scalpel in a plate containing 15 mL digestion media (DM). Seal the plates with Parafilm and wrap it with an aluminum foil.
6. Allow the digestion first for 3.5 h at 24 °C under the dark on an orbital shaker (25 rpm) and then increase the speed of oscillation to 40 rpm for 30 min.
7. Filter the leaf strips in digestion solution through a 100 μm cell strainer into a 50 mL Falcon tube.
8. Centrifuge the tubes at 100 × *g* for 5 min.
9. Carefully remove the supernatant. Resuspend the pellet in 5 mL of WBN.
10. Transfer the protoplast suspension onto 10 mL of 0.55 M sucrose in a 15 mL tube.
11. Centrifuge for 10 min at 1000 × *g*.
12. Carefully remove the supernatant.

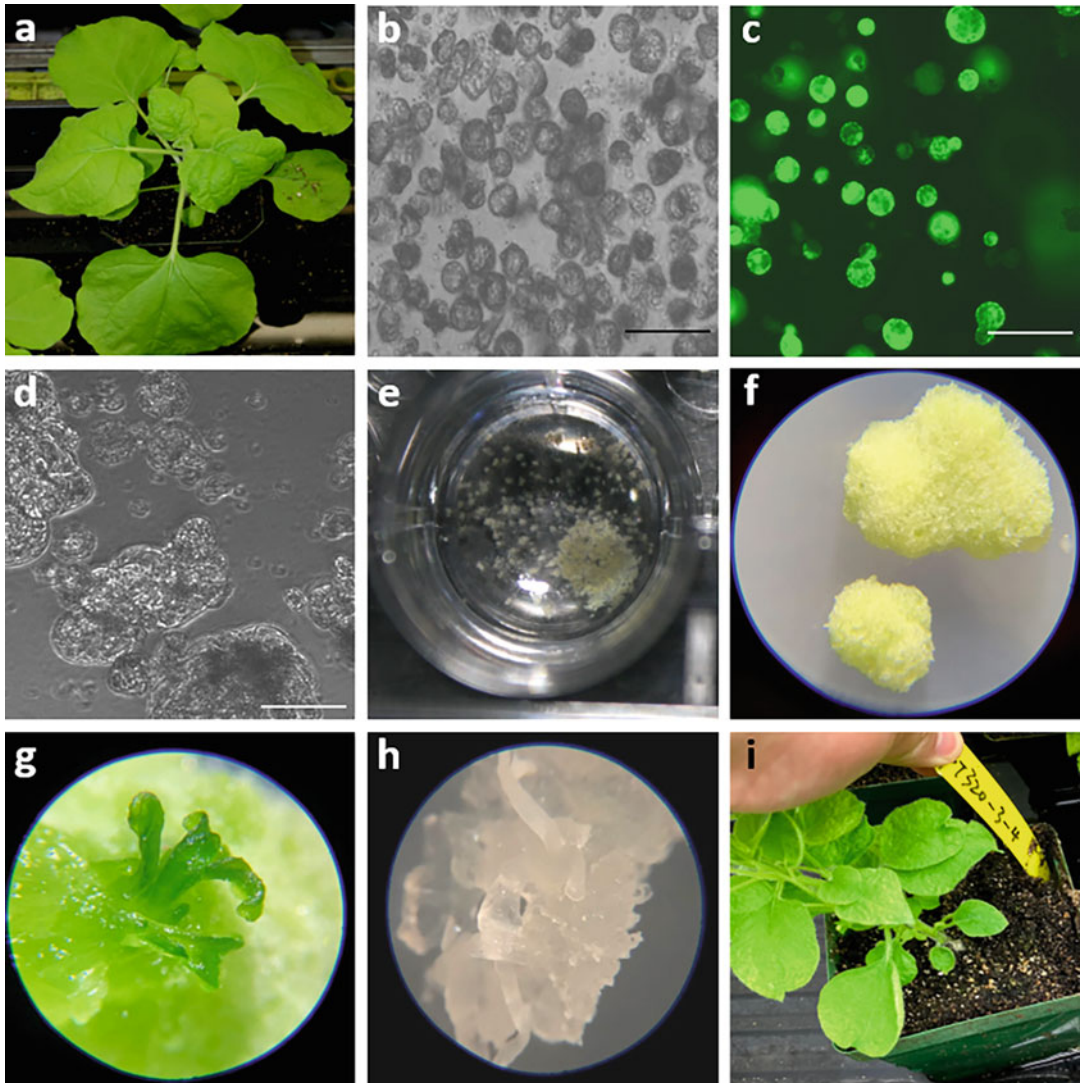

Fig. 2 Illustration of protoplast isolation, transfection, and regeneration. (**a**) The fresh and young leaves collected for protoplast isolation. (**b**) The high-quality mesophyll protoplasts released during digestion. (**c**) The GFP-expressing protoplasts 24 h post transfection. (**d**) The dividing protoplasts after 1 week of regeneration. (**e**) The visible micro-calli ready to be transferred to the solid PCM media. (**f**) The microscopic view of growing calli. (**g**) The regenerating green shoots. (**h**) The regenerating albino shoots resulted from knocking out the *PDS* gene. (**i**) The fully regenerated plants established in growth chamber to set seeds. The scale bars in **b**–**d**, 100 μm; in **f**–**h**, 1 mm

13. Transfer the protoplast-containing interphase layer into a 50 mL Falcon tube containing 5 mL of WBN.
14. Centrifuge the tube at 100 × *g* for 5 min.
15. Remove the supernatant. Resuspend the pellet in 5 mL WBN.
16. Centrifuge the tube at 100 × *g* for 5 min.

17. Remove the supernatant. Resuspend protoplasts in 1 mL of MMG buffer.
18. Estimate protoplast population using hemocytometer. Adjust the final density to 1×10^6/mL in MMG buffer (*see* **Note 11**; Fig. 2b).

3.5 Protoplast Transfection (See Note 12)

1. Transfer 15 μg plasmid DNA carrying CRISPR/Cas transgenes or 6 μL of 20 μg assembled RNP complex prepared in **step 5** of Subheading 3.2. into a 10 mL round bottom vial. Describe the volume of these DNA.
2. Transfer 200 μL of the protoplast suspension (about 2×10^5 protoplasts) into the vial using 1 mL wide bored tips.
3. Transfer an equal volume (205 or 206 μL depending on the plasmid DNA or RNP quantity) of PEG transfection reagent using 1 mL wide bored tips.
4. Immediately mix the suspension well either by slow swirling or by tapping the bottom of the tubes by fingers.
5. Allow the tubes to incubate in dark at the room temperature for 15 min (*see* **Note 13**).
6. Add 0.8 mL of WBN to the transfection tube.
7. Centrifuge the tube down at $100 \times g$ for 5 min.
8. Remove the supernatant. Resuspend the protoplast pellet with 0.8 mL of WBN.
9. Repeat this WBN wash step for two more times (*see* **Note 14**).
10. Resuspend protoplasts in 2 mL of PCN media and transfer protoplasts to a single well in either 12-well plates or 24-well plates.
11. Incubate the transfected protoplasts in dark for 48 h (25 ± 2 °C).
12. Cas nuclease fused with GFP can be visualized 12 h after RNP transfection. Transient GFP expression in DNA-transfected protoplasts can be visualized 2 days after transfection (Fig. 2c).

3.6 Protoplast Culture and Regeneration

1. Forty-eight hours after incubation, transfer 2 mL protoplasts to 6-well plates using the wide bored pipette tips. Add 2 mL of the PCN media.
2. Incubate the transfected protoplasts under a diurnal environment (16/8 h light/dark) with 25 °C and 75 μmol/m^2/s light intensity.
3. Within a week of incubation, the actively dividing micro-calli can be observed under microscope (Fig. 2d).
4. Remove the old media while retaining around 1 mL of it and then add 4 mL of new PCN media.

5. Change the PCN media once a week (*see* **Note 15**).
6. Once 0.1–0.2 mm calli are visible (normally after 17–20 days; Fig. 2e), transfer the calli to protoplast culture media (PCM) using the wide bored tips.
7. Wrap the plates with Parafilm and place them in the growth incubator (16/8 h light/dark) with 25 °C and 75 μmol/m^2/s light intensity.
8. Subculture the calli (Fig. 2f) for two to three times every 2 weeks using forceps. Shoots start to emerge after 2 weeks (Fig. 2g). In our example of knocking out the GFP gene in the GFP16c line, GFP negative calli can be readily observed at this stage before emerging shoots were obtained. These calli will be labelled and regenerated into whole plants (*see* **Note 16**).
9. Transfer healthy-looking shoots to rooting media (RM). Within 15–20 days, the well-established roots can be observed from each shoot (*see* **Note 17**).
10. Within 2–3 weeks, the rooted shoots are ready to be transferred to the soil. Transfer healthy plants to pots with the sterilized soil and place the pots (Fig. 2i) in trays with the humi-dome covers to maintain humidity.
11. Place the trays with 22/20 °C of day/night temperature under 16/8 h of the light and dark cycle with 80–100 μmol/m^2/s light intensity.
12. Plant care should be taken as described in Subheading 3.3.

3.7 Analysis of Genome Editing Results

After CRISPR/Cas reagents were transfected into protoplasts, the genome editing efficacy can be estimated via several approaches. These approaches can be also applied to identify mutants from regenerated plants.

3.7.1 Enzyme Digestion-Based Assay

Taking advantage of DNA polymorphisms induced by CRISPR/Cas at their targeted sites, these approaches can be largely divided into two categories. In the first category, the gRNA targeted sites contain restriction enzyme (RE) sites overlapping with the CRISPR cleavage regions. The 500–1000 bp targeted regions can be PCR amplified first and then digested by the overlapping restriction enzyme. If CRISPR/Cas-induced mutation occurred at the targeted sites, these mutations would demolish the restriction sites and thus led to RE-resistant sequences. The genome editing efficiency can be estimated by measuring the intensity of RE-resistant PCR band over the susceptible band through electrophoresis. In the second category where the gRNA targeted sites do not contain overlapping RE sites, mismatches between mutations and wild-type sequences can be detected by either single-stranded conformational polymorphism (SSCP) analysis [34] or a class of special

endonucleases, such as CEL-I, which specifically cleave DNA sequences containing mismatches [35] (*see* **Note 18**).

3.7.2 Sequencing-Based Assay

While the enzyme digestion-based assay often provides quick estimates, these approaches are usually not very accurate and do not provide the detailed view of mutation profiles. To achieve these goals, next generation sequencing (NGS) is usually used as the golden standard for the mutagenesis efficacy analysis. In this method, the CRISPR targeted region (less than 500 bp) is first amplified with 50 ng of template DNA, forward and reverse primers. PCR amplicons are then sequenced on an Illumina MiSeq instrument (v2 chemistry, 250 bp paired end reads) (Illumina, San Diego, CA, USA). Resulting NGS data is analyzed for genome editing with CRISPResso 2.0 [36]. At each target site, mutation efficiency was calculated as the percentage of total reads containing an indel within the specified window around the cut site (*see* **Note 19**).

4 Notes

1. The quality of cellulase and macerozyme are very critical. We have compared several vendors and found that the enzymes from Yakult Pharmaceutical Industry Co., Ltd. worked in the highest efficiency. These enzymes are currently distributed through Kanematsu USA Inc. They are highly light and temperature sensitive; therefore, storing them in 4 °C under dark conditions is highly advised.
2. Use of double deionized water is advised as this water is free of most trace metals. Protoplasts are sensitive to metal ions; hence, care should be taken to use metal-free water.
3. Both BAP and NAA can be degraded at 4 °C over 1 month. Storing at −20 °C is not advised as these molecules can precipitate and accumulate in the bottom of the vial.
4. Although digestion media stored in 4 °C and −20 °C can be used for protoplast preparation, the best results are obtained from the freshly made solution.
5. The solution stored at 4 °C must be brought to room temperature without any precipitation when being used in the experiment.
6. Most online programs provide the efficiency and specificity scores for different CRISPR systems. These scores are not very accurate for plants most of the time. Our common practice is to pick the target sites with the lowest off-targeting scores first.

7. The plasmids can be quantified with the nanodrop instrument. We usually use 15 μg of plasmids per transformation. For the better quality and consistency, the plasmids should be stored in small aliquots depending on your transformation setup to avoid frequent thaw/freeze cycles.
8. For the Cas9 guide RNA synthesis, two options are available at Integrated DNA Technologies Inc. In option 1, two-part RNA molecules, crRNA and tracrRNA, can be synthesized and ordered separately. They can be annealed first to form the gRNA complex for RNP assembly. In option 2, a single guide RNA (sgRNA) molecule can be synthesized directly. The Cas12a guide RNA is a single guide RNA molecule that does not require a tracrRNA.
9. High level of light intensity could lead to less protoplast yield and transfection efficiency. We found that 80–100 μmol/m^2/s light intensity usually provides the optimal plant growth condition.
10. Before preparing the nutrient solution, Fe-EDTA solution should be free from precipitation. Proper plant care is extremely important for protoplast quality. Care should also be taken not to over- or under-fertilize plants during the plant growth. Nutrient, water stress, or any abiotic and biotic stresses can have serious impacts on protoplast extraction.
11. After the protoplasts were suspended in 1 mL MMG buffer, the protoplast yield can be estimated by following the equation:

    Protoplast density (number per mL) = average number of protoplasts in $25^2 \times 10^4$. The detailed instruction can be found at https://www.hemocytometer.org.
12. Protoplast isolation and transformation should be carried out under the aseptic condition.
13. PEG is toxic to protoplasts. No more than 15 min of PEG treatment is recommended.
14. Gentle pipetting is recommended to keep the protoplast pellet intact.
15. Care should be given to avoid the evaporation of the PCN liquid media.
16. In this stage, mutation-carrying calli can be identified and followed into whole plant regeneration. In our examples with CRISPR/Cas9 plasmid delivery, the albino calli or shoots, which carry CRISPR-mediated knockout of the *PDS* (*Phytoene Desaturase*) gene, a gene that encodes the rate-limiting enzyme in carotenoid synthesis, can be identified at this stage (Fig. 2h). In the example with CRISPR/Cas RNP delivery, the GFP negative calli that carry the GFP gene knockout can be

identified. In both examples, more than 20% calli were found carrying biallelic gene knockout mutations. For the mutations that do not have visible phenotypes, genotyping approaches described in Subheading 3.7 can be used to identify mutation-containing calli or plants. Screening of 30–50 calli is usually sufficient to recover enough number of mutation-carrying calli and plants. Because no selection is used during regeneration, the regenerated plants could be chimeric with multiple genotypes in a single primary plant (E0). We found that the majority of mutant-carrying E0 plants produce heritable mutant plants in the progenies (E1). Following 5–10 E0 plants to the next generation is usually sufficient to obtain heritable and stable E1 mutant plants.

17. When grown on the rooting media, the plantlets could start to push up the plate lid. If happens, simply replace the lids with the bottom side of fresh plates and seal the plates with the micropore tapes.
18. The sensitivity of the enzyme digestion-based assays is usually around 5%. If the mutation rate is less than 5%, it cannot be detected by this type of assays reliably.
19. When performing NGS sample library preparation, different vendors may require different adapter sequences or protocol. Please follow the vendors' instruction.

Acknowledgments

The authors would like to thank Chunfang Wang, Trevor Weiss, Meredith Song, McKenna Quandth, and Kaisa Bornhoft for their assistance. We would like to thank Dr. Kan Wang for the critical reading of the manuscript. This project was partially supported by National Science Foundation Plant Genome Research Program Grants 2040218 to F.Z.

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Chapter 6

Protoplast Isolation and Transfection in Rice

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Abstract

Rice (*Oryza sativa*) is an important cereal crop and a model monocot plant for biology research. The reliable system of foreign DNA transformation and expression is a valuable strategy for basic research and molecular breeding application in rice. The *Agrobacterium tumefaciens*-mediated foreign DNA transformation system was a powerful tool for genetic research. However, it needs a long period to obtain the stable transformants for further analysis and the transformation rate limits in some organism. Protoplasts are plant cells without a cell wall, and it is much easier for foreign DNA transformation and expression. It has been widely applied in transient expression. Here, we describe a simple method for efficient protoplast isolation and transfection in rice.

Key words Dark-grown seedling, DNA transformation, *Oryza sativa*, Polyethylene glycol, Protoplast, Transient expression

1 Introduction

Rice (*Oryza sativa*) is not only the main food for half of the world's population but also an important model monocot plant for biology research. The research on rice gene function revelation and molecular breeding application requires the reliable foreign DNA transformation. The most commonly used method was *Agrobacterium tumefaciens*-mediated foreign DNA transformation, and it had been widely adopted in plants such as rice [1, 2]. The standard procedure for rice stable transformation has four stages: callus induction for 1 week, *Agrobacterium* infection and co-cultivation, selection for 15 days, and regeneration for 2 weeks or longer until the individual plant regenerated. It will take almost 3 months to obtain the transformants. Although the fast transformation method was established [3], it still needs a long period for further research. For some assays such as protein distribution analysis, BiFC, and protein-protein interaction analysis [4], it will take too much time for further analysis through the *Agrobacterium*-mediated stable

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_6,

transformation. For a rapid check of genome editing tool's efficiency, the traditional T-DNA transformation method was also time-consuming and showed limits on mutation efficiency statistics. It needs a huge workload in transformation to obtain the mutants and further for editing profile analysis.

Protoplasts are plant cells without a cell wall, which means the foreign DNA can easily be transformed into plant cell, and it can be used as a transient expression system [5]. The protoplast transfection needs far less days than *Agrobacterium tumefaciens*-mediated T-DNA transformation [6]. Through this method, the result is reliable and vivid. The protoplasts could be checked directly through the microscope; it will be more intuitive for experiment with fluorescence protein. One protoplast sample contains 4×10^5 cells, and it is enough for the CRISPR-Cas system-related experiments such as DNA cleavage analysis [7], base editing assay [8], prime editing assay, and gene regulation assay [9].

Here, we describe a method for efficient protoplast isolation and transfection in rice. It needs 11 days for plant preparation and 2 days for protoplast extraction, transfection, and culture. According to this method, 60 rice seeds from beginning can obtain ~2×10^7 protoplasts. The transfection rates are ~90%. Genome editing (including base editing [10] and prime editing [11]), gene regulation, and protein distribution analysis in rice could be carried out effectively based on this method.

2 Materials

Prepare all the solutions using sterile double-deionized water (sdH_2O) and analytical grade reagents at room temperature (RT, 22–25 °C).

2.1 Plant Material and DNA Construct

1. Seeds of *Oryza sativa* L. japonica. cv. Nipponbare: We got the rice seeds originally from China National Rice Research Institute and planted for seed propagation in the field every year.
2. Plasmid DNA: pZHZ113 (11.5 kb), a construct carrying both the selectable marker gene *hptII* (resistant to hygromycin) and reporter gene enhanced *gfp* (green fluorescent protein) driven by maize ubiquitin gene promoters and *Arabidopsis* heat shock protein terminator [12].

2.2 Solutions and Media

1. Sodium hypochlorite solution (20% (v/v)): For 100 mL, add 20 mL of commercial bleach (6% sodium hypochlorite) to 80 mL of sdH_2O. Add three drops of Tween 20 and mix.
2. MS medium (pH 5.8): To make 1000 mL, add 4.3 g Murashige and Skoog medium (Sigma-Aldrich, M5524) solid by 7 g Agar (Sigma-Aldrich, A1296). The medium was autoclaved at

121 °C for 30 min. For each bottle (*see* Subheading 2.3), a 100 mL medium is needed.

3. Protoplast isolation buffer: 0.6 M D-mannitol, 20 mM 2-morpholinoethanesulfonic acid (MES), 1.5 g Cellulase R10 (Yakult, MX7352), 0.75 g Macerozyme R10 (Yakult, MX7351), 10 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin (BSA), adjust pH to 5.8 with 1 N KOH. Filter sterilize using a 0.22 μm filter. Aliquot the buffer into a 15 mL sterile conic bottom centrifuge tube with 10 mL each and store at −20 °C for up to 6 months.
4. MMG buffer: 0.4 M D-mannitol, 4 mM MES, 15 mM $MgCl_2$, adjust pH to 5.8 with 1 N KOH. Filter sterilize using a 0.22 μm filter. Aliquot the buffer into a 15 mL sterile conic bottom centrifuge tube with 10 mL each and store at −20 °C for up to 1 year.
5. PEG (polyethylene glycol) buffer: 40% (w/v) PEG4000, 0.2 M D-mannitol, 100 mM $CaCl_2$, adjust pH to 5.8 with 1 N KOH. Filter sterilize using a 0.22 μm filter. Aliquot the buffer into a 15 mL clear conic bottom centrifuge tube with 10 mL each and store at −20 °C for up to 1 year.
6. W5 solution: 2 mM MES, 5 mM KCl, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM glucose, adjust pH to 5.8 with 1 N KOH. Filter sterilize using a 0.45 μm vacuum filter. Store at 4 °C for up to 6 months.

2.3 Other Supplies

1. Single edge razor blade, autoclaved at 121 °C for 30 min.
2. Falcon® 100 mm TC-treated cell culture dish (Catalog: 353003).
3. Falcon® 40 μm cell strainer (Catalog: 352340).
4. 200 μL pipette tips and 1000 μL pipette tips, cut off the top 3 mm with a single edge razor blade, autoclaved at 121 °C for 30 min.
5. 5000 μL pipette tips, autoclaved at 121 °C for 30 min.
6. Plant culture bottle, 6.5 cm × 10.5 cm, autoclaved at 121 °C for 30 min.
7. 0.22 μm MILLEX®GP filter unit (Millipore, catalog: SLHVP33R), 0.45 μm vacuum filter.
8. 75% (v/v) ethanol.
9. Tween 20.
10. Centrifuge 5424 R, 5910 R (Eppendorf).
11. 2 mL round bottom microfuge tubes, 15 mL, 50 mL conic bottom centrifuge tube, autoclaved at 121 °C for 30 min.
12. 2–20 μL, 100–1000 μL, and 500–5000 μL pipette.

13. Olympus CX23 biological microscope.
14. Olympus IX53 Routine inverted microscope.

3 Methods

3.1 Plant Preparation (10–12 Days)

1. Place 60 dehusked mature Nipponbare seeds in a 50 mL conic bottom centrifuge tube.
2. Wash the seeds with 40 mL 75% ethanol for 1 min.
3. Add 40 mL of 20% sodium hypochlorite solution to the tube. Sterilize for 15 min on a shaker with 180 rpm.
4. Discard the sterilization solution and repeat **step 3** once (totally sterilize two times).
5. Rinse the seeds with 40 mL sdH_2O two times, 1 min each wash.
6. Place the seeds on sterilized filter papers in a clean hood and let them air-dry for 10 min.
7. Place about 30 seeds in a culture bottle containing MS medium. Seeds should be distributed evenly and embedded fully in the medium with embryo side facing up.
8. Place another sterile bottle on top of the bottle of seeds in a neck-to-neck fashion. Seal the bottleneck part with Parafilm.
9. Incubate the seeds at 28 °C in the dark for about 10–12 days (*see* **Note 1**).

3.2 Protoplast Isolation (6–8 h)

1. Thaw the protoplast isolation buffer, MMG buffer, PEG buffer at RT. Ensure the solutions are thoroughly dissolved and homogeneous.
2. In a Petri dish, cut the shoot and leaf (~150 mm long; Fig. 1a) into ~0.5 mm strips using a sterile sharp razor blade (*see* **Note 2**).
3. Quickly transfer the strips with the help of the razor blade to another Petri dish containing 10 mL protoplast isolation buffer (*see* **Note 3**). Ensure the strips are immersed into the buffer by repeatedly pipetting the buffer slowly.
4. Place the Petri dish with the leaf strips and buffer in a vacuum desiccator. Vacuum-infiltrate under a pressure of 15–20 inHg (0.05–0.07 MPa) for 30 min (*see* **Note 4**).
5. Seal the Petri dish by Parafilm and incubate the mixture in the dark at RT for 6–8 h (*see* **Note 5**) with gentle shaking (60–80 rpm). Then shake the dish at 100 rpm for at last 10 min to facilitate the release of the protoplasts.
6. Pre-wet a 40 μm cell strainer with 2 mL of W5 buffer in a new Petri dish (*see* **Note 6**).

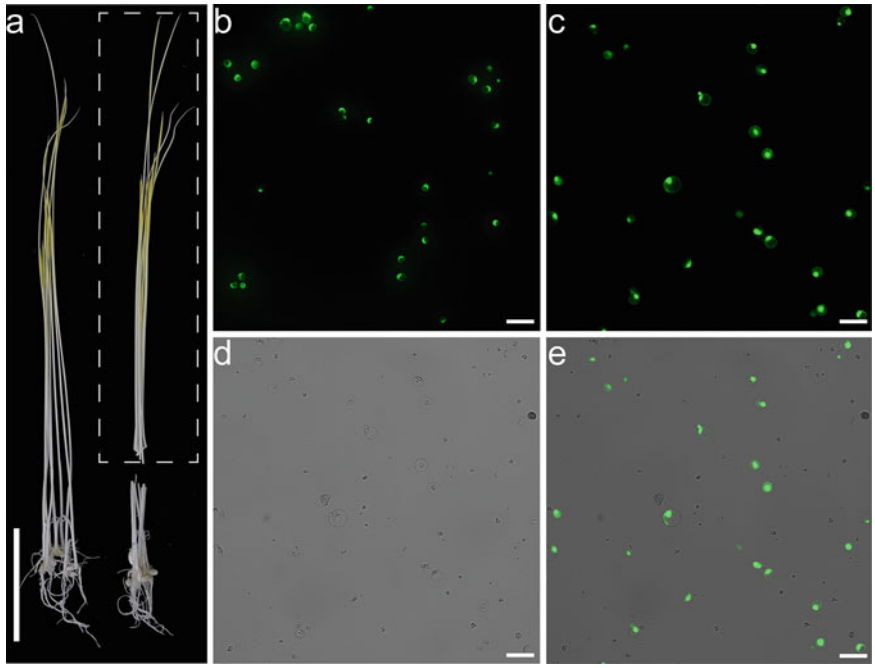

Fig. 1 Rice protoplast extraction, quality check, and GFP transformation. (**a**) Image for rice seedlings grown for 10–12 days after germination under dark condition. The recommended plant parts for protoplast isolation were shown in dashed line box. Scale bar = 50 mm. (**b**) Protoplast quality check by fluorescein diacetate (FDA) stain assay. FDA stains the cytoplasm of live cells. Protoplast transfected with GFP plasmid under fluorescence field (**c**), bright field (**d**), and merge field (**e**). Scale bar (in **b**, **c**, **d**) = 50 μm

7. Gently shake the Petri dish and carefully filter the protoplasts through the cell strainer using a 5 mL pipette (*see* **Note 7**).
8. Add 10 mL W5 solution to the Petri dish containing the digested leaf strips (*see* **Note 8**). Shake the dish at 100 rpm for 1 min to release more protoplasts.
9. Collect the protoplast as described in **step 7** and combine with the protoplast in the Petri dish.
10. Repeat **step 7** once (total rinse two times with W5 buffer).
11. Transfer the protoplast mixture to a new 50 mL conic bottom centrifuge tube (*see* **Note 9**).
12. Centrifuge at 100 × *g*, 5 min at RT. The protoplasts are pelleted at the bottom of the centrifuge tube (*see* **Note 10**).
13. Remove the supernatant carefully by pipetting (*see* **Note 11**).
14. Add 2 mL of W5 solution to the centrifuge tube (*see* **Note 12**). Resuspend the pellet by gently tapping the centrifuge tube.
15. Add another 8 mL of W5 solution to the protoplast suspension. Mix it by gently swirling the tube.
16. Centrifuge at 100 × *g*, 5 min at RT.

17. Remove the supernatant and add 1 mL of W5 solution to resuspend the pellet.
18. Add another 4 mL of W5 solution to the protoplast suspension, and mix it by gently swirling.
19. To count the protoplasts, withdraw 100 μL sample from the centrifuge tube while gently swirling the tube.
20. Place a cover slide over hemocytometer counting chambers. Transfer 6 μL protoplast samples to the hemocytometer, and count the number using the Olympus CX23 biological microscope under 40× objective lens. Count the total protoplast at 5 squares. Repeat this step three times to make the average number (N). The total protoplast number (M) was calculated with the hemocytometer operation instructions: $M = N \times 5 \times 10^4 \times$ volume of protoplast suspension (*see* **Note 13**).
21. Centrifuge at 100 × *g*, 2 min at RT, remove the supernatant, and adjust the protoplast density to 2×10^6/mL by MMG buffer.
22. The protoplast quality can be checked by fluorescein diacetate (FDA) stain assay (*see* Fig. 1b).
23. By following these procedures, the typical yield of rice protoplasts is approximately ~2×10^7 protoplasts per 60 rice seedlings.

3.3 Protoplast Transfection and Culture (2 Days; See *Note 14)*

1. Add 30 μL plasmid DNA (1 μg/μL; *see* **Note 15**) to the bottom of a 2 mL microfuge tube.
2. Gently swirl the protoplast centrifuge tube and carefully transfer 200 μL protoplast (~4×10^5 protoplast) into the tube.
3. Slowly add 230 μL PEG buffer to the tube, mix quickly but gently.
4. Incubate the mixture for 30 min in the dark at RT.
5. Add 900 μL W5 solution to the mixture, stop the reaction by gently inverting the tube ten times until it is completely mixed.
6. Centrifuge at 250 × *g*, 5 min at RT.
7. Carefully remove the supernatant (~600 μL) using a pipette.
8. Add 1 mL W5 solution and carefully resuspend the protoplast by a pipette.
9. Transfer the protoplast suspension into a 12-well cell culture plate, seal the plate using Parafilm. Cover the plate with foil paper and incubate at 32 °C for 48 h (*see* **Note 16**).
10. Collect the protoplast after 48 h incubation for further analysis. For green fluorescence protein transfection, it can be checked under inverted fluorescence microscope with wavelength 490–500 nm laser line (*see* Fig. 1c–e).

11. By following these procedures, the rice protoplast transfection rates can reach ~90%.

4 Notes

1. The culture temperature influenced the plant growth and the plant condition, which directly impact the protoplasts' quality. Please check the plant growth condition frequently; make sure the seedlings are grown healthily. When seedlings reach to the top of the upper bottle wall (around 16–18 cm tall), they can be harvested for protoplast isolation. For fluorescence protein expression, it is recommended that the rice seedlings be grown in the dark to minimize autofluorescence from chlorophyll.
2. The seedlings used should be healthy and strong. The seedlings should be 10–12 days old. Longer incubation time will decrease the typical yield of healthy protoplasts. Please make sure that the seedlings are not contaminated with bacteria or fungi. Microorganism contamination will drastically reduce the yield of protoplast and transfection rate.
3. Please be quick in transferring the strips to avoid the water loss that can negatively impact the quality of protoplasts. Use the side of the razor blade as a scoop for transferring the strips.
4. Please gently shake the vacuum desiccator every 15 min to ensure the proper mix of strips with buffer.
5. The incubation time can be shortened to 4–6 h if less than 12 transfection experiments are planned.
6. Please make sure the cell strainer is well pre-wetted by repeated pipetting ten times.
7. Please be gentle when transferring the protoplasts. This step is critical for obtaining healthy protoplasts.
8. Carefully rinse the strips to make sure it mixes well.
9. Please be careful and let the protoplast suspension slowly flow into the centrifuge tube bottom alongside the centrifuge tube wall.
10. Please be gentle when taking the protoplasts' tube out from the centrifuge to avoid agitation of the tube that may lead to potential resuspension of protoplast pellet.
11. Be careful when removing the supernatant and do not let the pellet resuspend. It is okay to leave some buffer in the tube.
12. Please be very slow when pipetting the W5 buffer to the tube. Letting the buffer flow slowly along the tube wall is recommended.

13. The formula is suitable for the normal 25 smaller central squares' model.
14. Please remain this step under the dark condition. This step under light will negatively impact the quality of protoplasts and leads to low transfection efficiency.
15. The high-quality plasmid DNA is essential for protoplast transfection. High-quality plasmid DNA extraction kit is recommended. The plasmid DNA could be dissolved in sdH_2O or elution buffer following the manufacturer's guidance.
16. For rice protoplast, the 32 °C is enough for most assays, and the protoplast shape is good after 48 h. Higher incubation temperature (>32 °C) is not recommended.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (award no. 31771486, 32072045, and 31960423) and the State Key Laboratory of Rice Biology (20200205).

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Chapter 7

Protoplast Isolation and Transfection in Maize

Monique R. Coy, Shane E. Abbitt, and Mary J. Frank

Abstract

Protoplast-based transient gene expression platforms can be used to study a range of questions concerning gene regulation. Crucial to the success of these studies is the isolation of large quantities of healthy protoplasts from the tissue of interest. Herein, we describe protocols for isolating and transfecting maize mesophyll protoplasts for gene expression studies. The isolation protocol yields approximately $1.8–1.9 \times 10^7$ protoplasts with 80–90% viability from 6 g of etiolated leaf tissue, and the polyethylene glycol-mediated transfection protocol results in 55–58% transfection efficiency. The transfection protocol describes the use of a dual-expression vector that carries the coding sequence for two fluorescent proteins (FPs), one driven by a constitutive promoter for normalization for transfection efficiency and the other driven by the construct of interest. The use of a dual-FP expression vector eliminates the need for co-transfection and separate steps for enzymatic/substrate processing as required for luciferase-based assays. These protocols have been tested on leaf tissue from the maize genotypes B73 and PHR03 and, as written, can be completed in 24 h.

Key words Dual-expression vector, Fluorescent protein, Leaf, Mesophyll, Polyethylene glycol, Transient, *Zea maize*

1 Introduction

Protoplast-based transient gene expression studies are fast, sensitive, and versatile and can be used to study a wide range of gene regulatory mechanisms, including those at the transcriptomic level. In the simplest design, a protoplast-based platform to address transcript studies is comprised of protoplasts and a plasmid vector with a promoter of interest driving the expression of a reporter gene. More complex designs can be employed to address a number of experimental questions regarding gene expression, for example, the effect of *cis*-regulatory elements on promoter activity [1], the identification of motifs involved in cell signaling [2], and the investigation of transcription factor/promoter interactions [3]. In

Monique R. Coy and Shane E. Abbitt contributed equally with all other contributors.

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_7,

principle, once normalized, the magnitude of the signal from the reporter is directly correlated to the level of gene expression. In protoplast assays, two types of reporters are commonly used, luciferase or fluorescent proteins (FP), such as eGFP. The use of a fluorescent protein offers the advantage of a signal that is captured directly and in situ, and thus, unlike luciferase, requires no downstream processing. The use of a second plasmid to normalize for transfection efficiency is common; however, the use of such an approach leaves the possibility of differential uptake of plasmid by the cells, leading to uneven molar uptake. The inclusion of a coding sequence (CDS) for a second FP under the control of a constitutively expressed promoter on the same vector as the reporter FP allows for normalization for transfection efficiency, thus avoiding the need for co-transfection of a second plasmid [4, 5].

Described in this chapter are protocols to isolate and transfect maize mesophyll protoplasts using a dual-FP expression vector for transient gene expression studies. These protocols were adapted from previously published protocols [6–8] and have been tested on leaf tissue from the maize genotypes B73 and PHR03. The isolation protocol yields approximately 1.8–1.9 $\times$ 10^7 protoplasts from 6 g of leaf tissue with 80–90% viability. The transfection protocol makes use of polyethylene glycol (PEG) in the presence of excess calcium [9], thus eliminating the need for an electroporator, with expected transfection efficiencies of 55–58%. The dual-expression vector described here carries the CDS for ZsGreen [10] as the reporter and TagRFP [11] for normalization (Fig. 1). However, other FPs can be substituted, provided that the spectra of each are separate enough for individual quantification. As noted above, the promoter/regulatory construct driving ZsGreen can be modified as needed to address a wide range of experimental questions regarding gene regulation, with the promoter included here as only

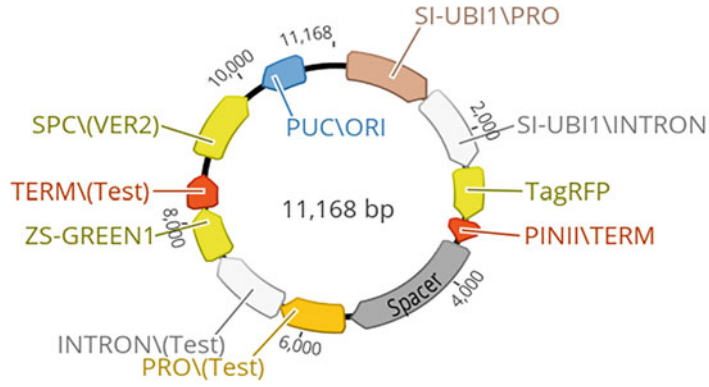

Fig. 1 Generalized dual fluorescent protein vector for protoplast transfection. 2.2 kilobase (kb) vector backbone was from previously described pLGS1 vector (GenBank MN585216.1). Image was created using Geneious Prime

an example. The promoter driving the reporter gene for normalization should drive measurable expression levels of the reporter gene in the protoplasts. The entire process, from isolation to quantification, can be completed within 24 h.

2 Materials

2.1 Plant Materials and DNA Construct

1. Maize seeds: Seeds can be sourced from USDA Germplasm Resources Information Network (GRIN). B73 and PHR03 genotypes have been tested with this protocol, but others may be suitable (*see* **Note 1**).
2. Dual-FP expression vector (10–15 kb): A construct that contains a FP CDS driven by a constitutive promoter such as *Setaria italica* ubiquitin (SI-UBI) and a second FP CDS driven by the construct of interest. A generalized example is shown in Fig. 1 (*see* **Note 2**).

2.2 Stock Solutions

Ultrapure water (18.2 MΩ cm) is used in preparation of all solutions, including working solutions (*see* **Note 3**). Recommended storage conditions for stock reagents are based on our usage, and may differ based on how quickly the reagents are consumed (*see* **Note 4**).

1. D-Mannitol (1 M): Prepare the day before protoplast isolation. To a 500 mL beaker, add 170 mL dH_2O and heat on high in a microwave for 1 min. While water is being heated, weigh out 36.4 g D-mannitol into a weigh boat. Remove the beaker from the microwave, add a stir bar, and place on a stir plate set on medium-low. Add the D-mannitol in batches, allowing it to dissolve between additions and taking care not to get mannitol on the sides of the beaker. Allow stirring to continue for 30 min. Remove from the stir plate, cover tightly with aluminum foil, and allow it to cool to room temperature (r.t., 21–23 °C). Once cool, pour the solution into a graduated cylinder and bring up to 200 mL with dH_2O and mix by inversion. Filter sterilize (0.45 μm). Protect from sunlight and store at r.t. for up to 1 week (*see* **Note 5**).
2. MES-KOH (0.5 M, pH 5.7): In 80 mL dH_2O, dissolve 9.8 g anhydrous 2-(N-morpholino)ethanesulfonic acid (MES). Adjust the solution to pH 5.7 with 10 N KOH and bring up to 100 mL. Filter sterilize (0.45 μm) and store at r.t. in the dark for 1 month or 4 °C for up to 6 months (*see* **Note 6**).
3. NaCl (5 M): Add 29.2 g of sodium chloride (NaCl) to 80 mL hot dH_2O stirring on a hot plate and cover with a piece of aluminum foil. Continue stirring until all NaCl has dissolved. Remove from the hot plate and allow to cool to r.t. Bring up to 100 mL and sterilize by autoclaving. Store at r.t. for up to 1 year (*see* **Note 7**).

4. $MgCl_2$ (1 M): In 70 mL of dH_2O, add 20.3 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and stir on a stir plate until dissolved. Bring up the volume to 100 mL and sterilize by autoclaving. Store at r.t. for up to 3 months (*see* **Notes 8** and **9**).
5. $CaCl_2$ (1 M): In 80 mL dH_2O, add 14.7 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) and stir on a stir plate until dissolved. Bring volume up to 100 mL and sterilize by autoclaving. Store at r.t. for up to 6 months.
6. KCl (1 M): In 80 mL dH_2O, add 7.5 g of potassium chloride (KCl). Bring up to 100 mL of dH_2O and sterilize by autoclaving. Keep at r.t. for up to 6 months.

2.3 Working Solutions

Prepare all working solutions the day of protoplast isolation and transfection and discard after use.

1. Digestion solution: In a 100 mL bottle with a screw cap, combine 30 mL 1 M D-mannitol (final 0.6 M) and 1 mL 0.5 M MES-KOH (final 10 mM). Close the bottle with the cap and heat in the microwave for 20 s. After heating, add a stir bar, then place bottle on a hot plate set at 70 °C and stir on medium-low. Remove the cap, add 0.75 g Cellulase "ONOZUKA" RS (final 1.5% w/v) and 0.15 g *Rhizopus* sp. pectinase (final 0.3% w/v). Replace the cap and allow to stir for 10 min with periodic shaking to submerge the enzymes, taking care to not get them stuck to the side of the bottle. Turn off the stir plate, remove from heat, add 18 mL of ultrapure water and 0.05 g BSA (final 0.1% w/v). Replace the cap and stir on cold stir plate until dissolved. Add 50 µL of 1 M $CaCl_2$ (final 1 mM) and 16 µL β-mercaptoethanol (final 5 mM). Stir for 1 min and filter sterilize with a 0.45 µm cellulose acetate (CA) filter unit (*see* **Notes 10**–**15**).
2. MMg solution: To a 100 mL beaker with a stir bar, add 22.62 mL ultrapure water, 36 mL of 1 M D-mannitol (final 0.6 M), 0.9 mL 1 M $MgCl_2$ (final 15 mM), and 0.48 mL 0.5 M MES-KOH (pH 5.7; final 4 mM). Stir briefly on a stir plate and sterilize with a 0.45 µm filter unit.
3. W5 solution: To a 100 mL beaker with a stir bar, add 41.76 mL of ultrapure water, 1.54 mL 5 M NaCl (final 154 mM), 6.25 mL 1 M $CaCl_2$ (final 125 mM), 0.25 mL 1 M KCl (final 5 mM), 0.2 mL 0.5 M MES-KOH (pH 5.7; 2 mM final). Stir briefly on a stir plate and sterilize with a 0.45 µm filter unit.
4. WI solution: To a 100 mL beaker with a stir bar, add 9.4 mL ultrapure water, 40 mL 1 M D-mannitol (final 0.8 mM), 0.2 mL 1 M KCl (final 4 mM), and 0.4 mL 0.5 M MES-KOH (pH 5.7; 4 mM). Stir briefly on a stir plate and sterilize with a 0.45 µm filter unit.

5. 40% PEG: To a 50 mL plastic conical tube, add 4 g PEG 4000 (final 40% w/v), 6.5 mL 1 M D-mannitol (final 0.6 M), and 1 mL 1 M $CaCl_2$ (final 0.1 M). Mix by inversion and place in a 50 °C oven until needed. Filter sterilize (0.45 μm) (*see* **Notes 16** and **17**).

2.4 Supplies

1. Plastic flats with 96 cell inserts.
2. Plastic 6″ domes.
3. Sun Gro Fafard® Germinating Mix, or other appropriate substrate.
4. Scissors.
5. Scalpel (#11).
6. Small cutting board.
7. Petri dishes (100 mm).
8. Vacuum chamber with hoses (e.g., Bel-Art 42025).
9. House vacuum.
10. 50 mL conical tubes.
11. Steriflip filter device (0.45 μm; Millipore SE1M003M00).
12. Filtration bottle units with cellulose nitrate (CN) membranes (0.45 μm).
13. Filtration bottle units with cellulose acetate (CA) membranes (0.45 μm).
14. 200 μL wide bore sterile pipette tips (e.g., Axygen Cat# T205WBCRS).
15. Ice and ice bucket.
16. 2 mL microfuge round-bottom tubes.
17. Trypan blue (0.4%).
18. Hemocytometer or an automated cell counter (e.g., Invitrogen Countess).
19. 96-well, non-coated plates (e.g., Greiner Sensoplates).

2.5 Equipment

1. Orbital incubation shaker with temperature and variable speed control.
2. Refrigerated tabletop centrifuge with swinging bucket rotor that can accommodate 50 mL conical tubes and 2 mL microfuge tubes (e.g., Beckman Coulter Allegra X-15R with SX4750 SB rotor).
3. Instrument to measure fluorescent signal (e.g., Biotek Synergy Plate Reader, BioTek Cytation™ 5 or similar).
4. Growth chamber(s) in which light, temperature, and humidity are programmable.

3 Methods

3.1 Plant Material Preparation

1. Fill a 96-cell flat with Sun Gro Fafard® Germinating Mix (or other appropriate substrate).
2. Saturate the soil with water and dibble the substrate approximately ½ cm in each cell with the round end of a Sharpie pen or other similarly shaped object.
3. Drop a single maize seed into each dimple and cover the flat with a thin layer of substrate, making sure to adequately cover the seed.
4. Mist the top of the substrate with water until saturated and cover with a dome.
5. Place the domed flat in a growth chamber set at 25 °C and 55% RH and propagate for 3 days under constant light.
6. Remove the dome and continue to grow the seedlings for an additional 3–4 days in dark conditions at 25 °C and 55% RH until the first "true leaf" is fully developed (*see* **Note 18** and Fig. 2a).

3.2 Protoplast Isolation

1. Harvest the first true leaf by cutting the plant at the base (Fig. 2b).
2. Hold the plant between your fingers near the base and pull the leaf out to separate it from the cotyledon (Fig. 2c). Discard the cotyledon.
3. Remove the tip and the base of the leaf so that you are taking a ~10 cm section of the leaf (Fig. 2d).
4. Collect 6 g of leaf tissue (Fig. 2e) for each 25 mL batch of digestion solution. The yield from 6 g of leaf tissue will provide enough protoplasts for ~80–100 transfection aliquots (200,000 protoplasts each).
5. Gather the leaves into a small bundle and align the bottom edges. Using a new scalpel, slice leaf tissue into 0.5–1 mm slices. Slice the tissue as cleanly and finely as possible being careful to slice and not chop or crush the tissue (Fig. 2f and g).
6. Distribute the slices in Petri dishes containing the digestion solution. Mix the material into the solution making sure to submerge the tissue. Approximately 12.5 mL of digestion solution should cover the clippings from 3 g of tissue. Use multiple Petri dishes for larger batches to ensure that the leaf clippings are covered by the digestion solution (Fig. 3a).
7. Place the plates in a vacuum chamber under house vacuum for 30 min (Fig. 3b). Release the vacuum slowly to prevent damage to the protoplasts. The release of the vacuum should take ~5 min.

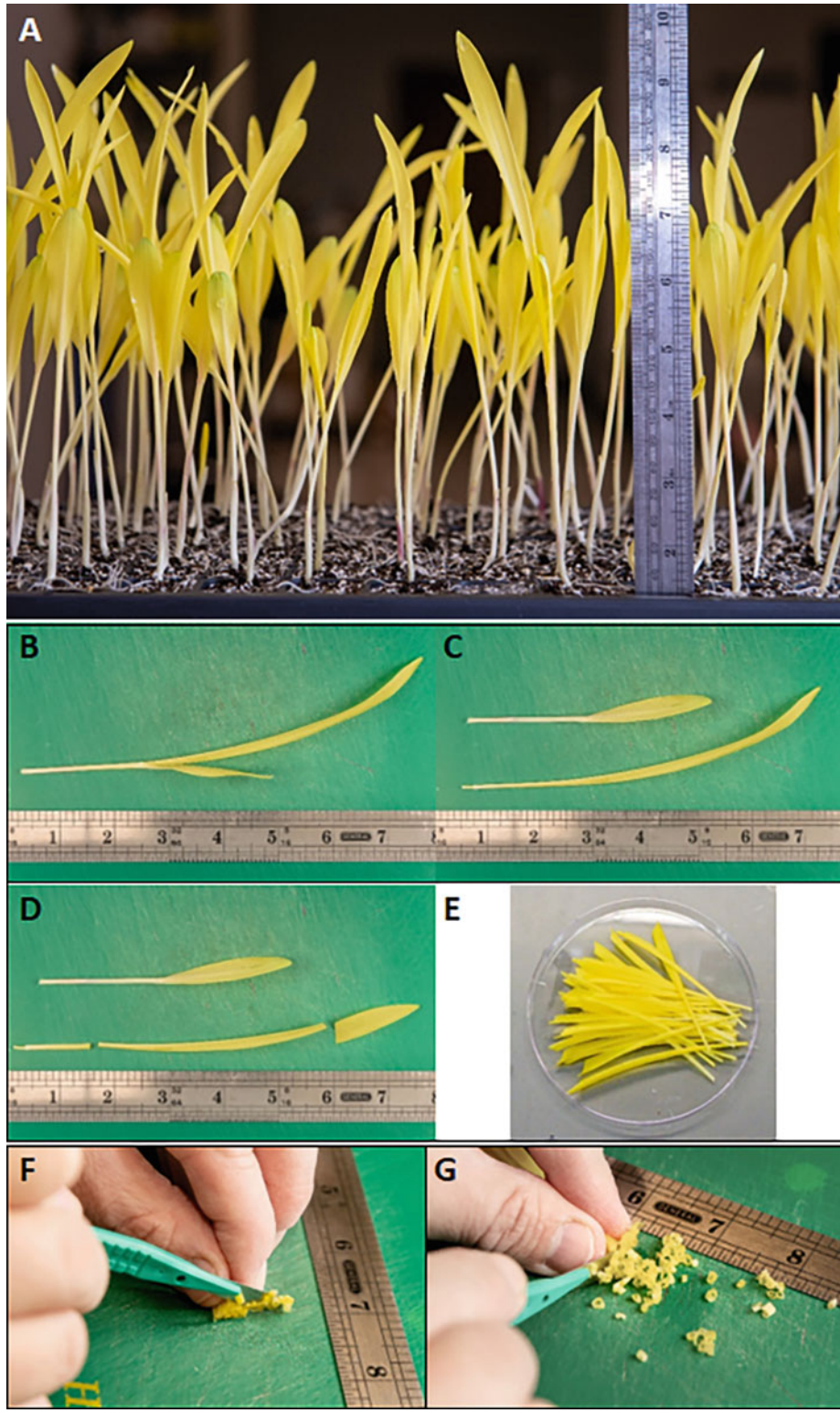

Fig. 2 Plant material for protoplast isolation. (**a**) Seedlings ready for protoplast isolation. (**b**) Leaf material harvest. (**c**) Separation of cotyledon from first leaf. (**d**) Middle section of first leaf used for protoplast isolation. (**e**) Example of amount of tissue needed for protoplast isolation. (**f**, **g**) Slicing the leaf material for digestion

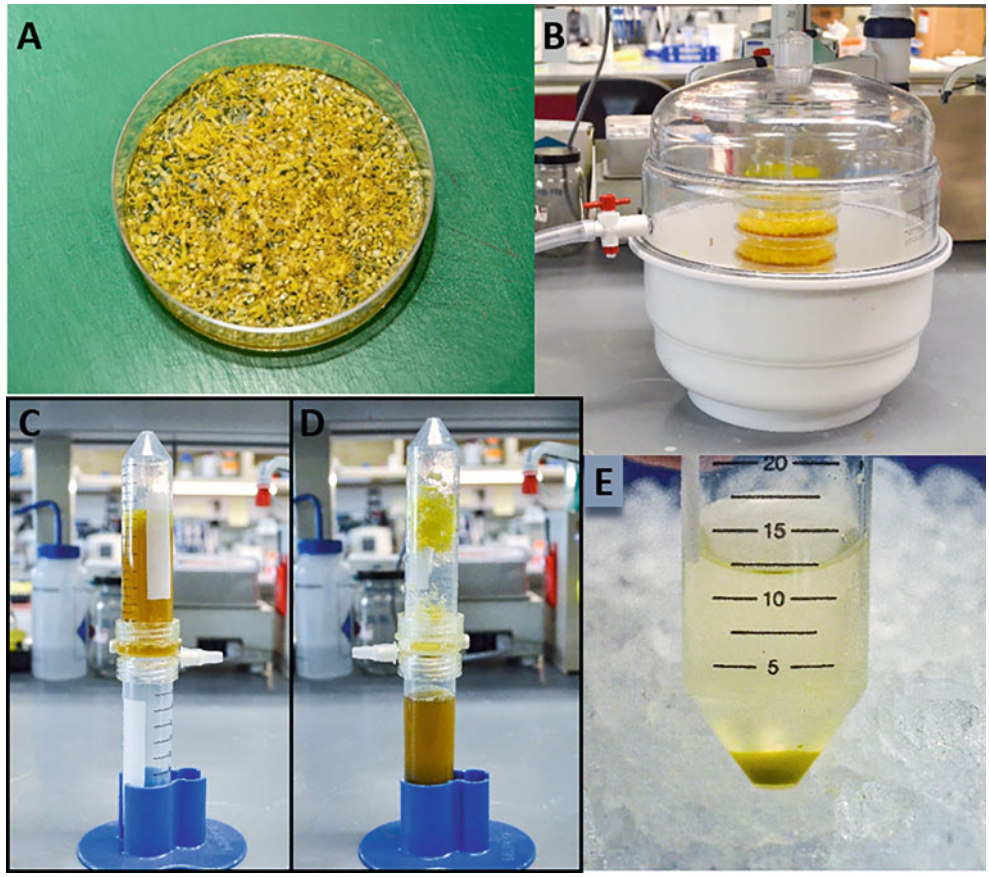

Fig. 3 Protoplast isolation. (**a**) Leaf clippings submerged in digestion solution. (**b**) Vacuum infiltration chamber. (**c**) Steriflip setup before filtration. (**d**) Steriflip setup after filtration. (**e**) Protoplast pellet after washing

8. Place the Petri dishes into an orbital shaking incubator set to 28 °C. Incubate for ~2 h at 40 rpm. The digestion solution will turn yellow as the protoplasts are released.
9. After the 2 h incubation, increase the speed to 80 rpm for 5 min to further release the protoplasts.
10. Remove the plates from the shaking incubator and **gently** pour the protoplast solution into 50 mL conical tubes. Protoplasts are fragile, and it is imperative to be very gentle in all manipulations from this point forward (*see* **Note 19**).
11. Attach a 0.45 μm Steriflip filter device (Millipore) to the top of the 50 mL conical tube and invert slowly.
12. Hold tubes at a 45 ° angle and attach a vacuum hose to the Steriflip port.

13. **Gently** apply the minimal amount of vacuum pressure required to pull protoplasts through the filter. Elevated levels of vacuum pressure will damage the protoplasts (Fig. 3c and d) (*see* **Note 20**).
14. Keep protoplasts on ice from this point forward.

3.3 Wash the Protoplasts

1. Divide the protoplast solution into multiple 50 mL conical tubes so that no tube contains more than 25 mL of solution.
2. Balance the tubes using MMg solution.
3. Centrifuge the protoplasts at 100 × *g* for 2 min at 4 °C using a swinging bucket rotor (*see* **Note 21**).
4. Remove the supernatant, leaving ~5 mL without disturbing the pellet.
5. Wash the protoplasts by adding 10 mL of the MMg solution and gently rock the tube to resuspend the protoplast pellet.
6. Repeat the wash **steps** (**2**–**5**) three times. The final pellet from 6 g of tissue will resemble the pellet shown in Fig. 3e.
7. If there are multiple tubes of protoplasts, they can be combined into one or two tubes during the wash steps, taking care that each tube contains no more than 25 mL of solution.

3.4 Quantification of Protoplast Yield

1. Remove the supernatant down to the pellet, taking care not to disturb it.
2. Add ~1 mL of the MMg solution and resuspend the protoplast pellet by gentle rocking.
3. Obtain a count of the protoplasts by using either a hemocytometer or an automated cell counter (e.g., Invitrogen Countess). *See* Fig. 4 for the expected condition of the protoplasts after isolation (*see* **Note 22**).
4. In a 2.0 mL round-bottom microfuge tube, add 20 μL of trypan blue (0.4%) to 20 μL of protoplast suspension. Mix by gently flicking the tube, then transfer a portion of the mixture to the counting slide chamber (*see* **Note 23**).
5. Calculate the protoplast concentration and viability.
   % viable cells = [1.00 – (Number of dead cells ÷ Number of total cells)] × 100. Typical protoplast isolations produce >80% cell viability.
6. Add MMg to the protoplasts such that the resulting concentration will be 10,000 cells/μL.

3.5 Transfection

1. Combine 160 μL of MMg solution, 40 μL of plasmid DNA (0.125 pmol), and 20 μL of protoplasts (200,000 total) to a 2 mL round-bottom tube. Combine 160 μL of MMg solution and 40 μL of plasmid DNA (0.125 pmol) in a 2 mL round-

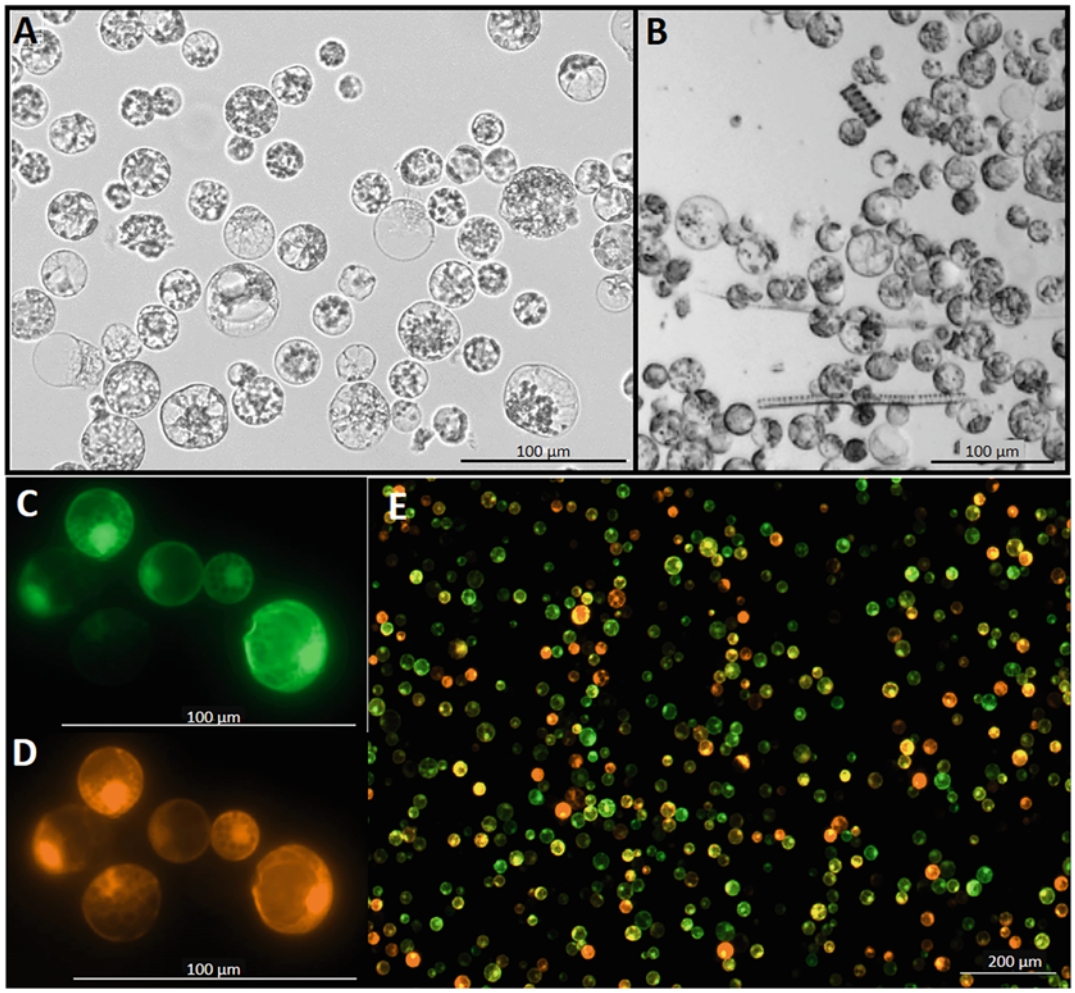

Fig. 4 Maize protoplasts resting in MMg solution after isolation steps. (**a**) Example of a good protoplast preparation. (**b**) Example of a protoplast preparation with cellular debris. (**c**) ZsGreen expression in maize protoplasts. (**d**) TagRFP expression in maize protoplasts. (**e**) ZsGreen and TagRFP expression in maize protoplasts

bottomed tube, and mix by gentle vortexing. After mixing, add 20 µL of protoplasts (200,000 total). Close the cap and gently swirl the tube to mix (*see* **Notes 24–27**).

2. The volume of MMg solution can be adjusted to account for different concentrations of protoplasts and/or plasmid DNA. The total volume of MMg, DNA, and protoplasts should equal 220 µL and with at least 154 µL MMg.
3. Add 220 µL of 40% PEG (cooled to r.t.) and invert the tubes three to four times gently to mix (*see* **Note 28**).
4. Incubate the mixture for 15 min at r.t.

5. Stop the transfection by adding 1 mL of W5 solution and mix by gently inverting the tube four to five times (*see* **Note 29**).
6. Centrifuge in a swinging bucket rotor at 100 × *g* for 2 min at r. t.
7. Remove all the supernatant by vacuum aspiration and gently resuspend the protoplasts in 500 μL WI solution.
8. Transfer 100 μL of the suspensions to the wells of a glass-bottom 96-well plate. Other plate configurations (e.g., 24 or 48 wells) can be used. Scale as appropriate (*see* **Note 30**).
9. Incubate the plate overnight (~16 h) at 28 °C in the dark (*see* **Note 31**).
10. Collect data from the assay ~16–20 h post-transfection using instrumentation capable of capturing fluorescent signals at the cellular level (Fig. 4). The protoplasts will remain viable for 3–4 days (*see* **Notes 32–34**).

4 Notes

1. This protocol has been tested with leaf tissue from the maize genotypes B73 and PHR03. It may be suitable for other genotypes but also may require slight modifications. The concentration of the osmoticum is the most likely constituent to require optimization. If they are wrinkled after incubation, this may indicate that mannitol concentrations are too high and need to be lowered. If bursting is observed, this indicates that concentrations are too low and should be raised.
2. Because the protocol describes the use of etiolated tissue, some light-responsive promoters (e.g., *rbcS*) may not drive expected expression levels.
3. Water impurities can significantly impact protoplast yield, stability, and viability, as well as transfection rates. If a filtration system (e.g., Milli-Q or similar) is not available for purifying water to a resistance of 18.2 MΩ cm, it is advised to purchase molecular grade water for making reagents.
4. We routinely conduct protoplast experiments and rarely store stock reagents for long periods. Thus, recommended storage temperatures and durations are based on this usage. If experiments are infrequent or sporadic, it is recommended to aliquot stock preparations of $CaCl_2$, $MgCl_2$, and KCl and store at −20 °C until needed.
5. Mannitol stock solution should be made fresh for each isolation.
6. Proper pH of all solutions is critical for success across the entire process. It is advised to check the pH of the 0.5 M MES-KOH stock prior to use to make sure it has not drifted. If so, discard

and make fresh – do not attempt to adjust with acid or base. If stored at 4 °C, allow to come to r.t. before checking pH.

7. Making 5 M NaCl is tedious and time-consuming. Alternatively, it can be purchased as a molecular grade reagent, which ensures it is the expected molarity and is typically inexpensive.
8. $MgCl_2$ is highly hygroscopic, and over time the crystals will become saturated in H_2O. Once this occurs, discard the chemical properly. To mitigate this waste, consider purchasing the chemical in smaller bottles (e.g., 100 g) and store with the lid tightly secured.
9. Check stock solutions of 1 M $MgCl_2$ prior to use and discard if microbial growth is present. After preparation, consider dispensing into 10 mL aliquots, keeping one at r.t. and storing the others at −20 °C until needed.
10. Bring any refrigerated or frozen reagents to r.t. before opening vials, especially enzymes. This will reduce condensation buildup within the bottles, which can degrade reagents.
11. β-Mercaptoethanol is an "acute toxin." Avoid oral and dermal contact as well as inhalation. β-Mercaptoethanol should be added in a fume hood.
12. Cellulase is classified as "Respiratory Sensitization (Category 1)" and a known allergen. Avoid inhalation by weighing the powder in a hood.
13. Heat is used in preparing the digestion solution not only to help dissolve the enzymes but also to aid in inactivating DNases and proteases in the enzyme preparations [8].
14. It is not uncommon for enzymes to not completely dissolve in solution. Some particulates can remain and after filtration. The volume will typically be 45–48 mL in total.
15. Filtration with a membrane with low protein binding properties, e.g., cellulose acetate (CA), is recommended.
16. Sourcing PEG 4000 from Sigma, catalog # 81240, is recommended [8].
17. No water is added to the preparation of 40% PEG, and the final volume will be between 10 and 11 mL.
18. Seeds are watered upon planting, then as needed to keep the soil moist. The plants should be ~20 cm tall, and the leaves will be yellow at this time (Fig. 2a).
19. The protoplasts are now fragile. Be very gentle in all movements when handling protoplasts from this point forward. Use 200 μL wide bore sterile pipette tips when pipetting protoplasts and use slow, deliberate pipetting technique.
20. The "post-digestion" filtration process should take ~60 s for ~45 mL of the solution to completely pass through the filter. Refer to Fig. 3c and d for additional details.

21. When centrifuging protoplasts, limit the volume in the 50 mL tube to ~25 mL. Use additional tubes to distribute volumes as needed. Greater volumes can lead to incomplete sedimentation and result in lower protoplast yields.
22. After the protoplast isolation steps, healthy protoplasts are round (Fig. 4a). Observation of some cellular debris is normal, such as dead protoplasts or "spring-like" structures as shown in Fig. 4b. This level of debris will not interfere with transfection. However, increased amounts of debris indicate that the filtration step post-digestion was performed using excessive vacuum pressure.
23. Long exposures to trypan blue can reduce protoplast viability. Complete counting within 3 min after the dye has been added to the protoplasts. Living cells will exclude the dye and appear clear, while dead cells will take up the dye and appear blue.
24. To convert from μg to pmol, use the formula below, or the Promega Biomath calculation tools (https://www.promega.com/resources/tools/biomath/):

$$\text{pmol of plasmid DNA} = \mu\text{g of plasmid DNA} \times \frac{\text{pmol}}{660\,\text{pg}} \times \frac{10^6\,\text{pg}}{1\,\mu\text{g}} \times \frac{1}{N}$$

N is the number of nucleotides in the plasmid.

25. Protoplast viability will decrease as the amount of plasmid DNA increases. Limit the total DNA load to 3–5 pmol. Plasmid DNA concentrations above 5 pmol in the above-listed volumes will result in the preciptation of DNA in the presence of PEG, causing the protoplasts to coagulate and die. If combining more than one plasmid, adjust amounts of each so that the total is below 5 pmol.
26. Endotoxin levels in the plasmid preparation can affect results. Propagate the bacteria in media such as 2X YT or LB. The use of rich media such as terrific broth can lead to the production of higher endotoxin levels which lowers protoplast viability.
27. Highly purified and concentrated plasmid DNA (>800 ng/μL) is key for successful transfections. It is highly recommended to use a plasmid prep kit with endotoxin reducing steps such as the Qiagen Plasmid Plus or Invitrogen PureLink Fast Low-Endotoxin kit.
28. The use of an automated repeat pipettor to dispense the 40% PEG allows for the transfection of up to 24 tubes at one time. Work quickly, but consistently, and dispense the PEG against the inside of the tube. Use a second rack to cover the tray of tubes and invert all 24 at one time to mix the transfection reaction.
29. When adding W5 and WI to the microfuge tubes, take care not to "blow out" the contents within the tube, especially if using an automated repeat pipettor.

30. Use non-coated plates for plating protoplasts. We prefer to use the Greiner Sensoplates which are black plates with a glass bottom because they facilitate image capture by reducing reflections and image distortion.
31. Incubation of the plates in the light has not been tested.
32. The user will need to consult the technical manual that accompanies the instrumentation and conduct preliminary experiments to determine the optimal exposure time, light intensity, etc., for each protein fluorophore.
33. If data capture will be conducted at the cellular level, only cells expressing the normalization FP (e.g., TagRFP) fall within a size of 16–55 μm, and circularity of equal or greater than 0.49 should be quantified.
34. Transfection efficiency was determined by collecting both bright-field and fluorescence images of ZsGreen expression using 20× objective with the BioTek Cytation™ 5. The ZsGreen fluorescent protein was driven by maize ubiquitin promoter. The total number of protoplasts along with protoplasts expressing ZsGreen (green cells) was counted. Transfection efficiency was calculated by dividing the number of green cells by the total number of cells and then multiplying by 100. A minimum of 290 protoplasts was counted for each genotype.

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Chapter 8

Isolation and Transfection of Maize Endosperm Protoplasts

Yufeng Hu and Yubi Huang

Abstract

Endosperm of cereal crops is the main component of its grain. Improvement of endosperm traits will bolster grain yield and quality. Functional analysis of endosperm trait-related genes often requires the use of an endosperm cell system. Here, we describe a protocol for the isolation and transfection of maize endosperm cell protoplast. The endosperm protoplast system can be used for several molecular studies including protein subcellular localization, protein-protein interaction by bimolecular fluorescence complementation (BiFC), protein immunoblotting, transient gene expression, and regulatory analyses by qRT-PCR.

Key words Immature seed, Protein immunoblotting, Protein-protein interaction, Protein subcellular localization, Transcriptional regulatory analyses, Transient system, *Zea mays*

1 Introduction

The cereal endosperm is a storage tissue which serves as an important source of nutrients for humans and animals. It can also be used as industrial raw materials. Improvement of endosperm traits is usually beneficial to crop yield or grain quality. The endosperm tissue is a highly differentiated organ, which contains specific sub-organelles and amyloplasts [1, 2]. Moreover, many genes including endosperm development-related genes [3], storage protein genes [4], starch synthesis-related genes, and their regulatory genes [5] affect endosperm characteristics. Most of these genes are specifically expressed in endosperm cells. Therefore, functional analyses of these genes or their regulatory networks require endosperm cell-based system. Generally, the use of mutants or transgenic plants is currently the main approach for the functional analysis of endosperm trait-related genes. Such approaches have resulted in various advances made in the functional analysis of genes related to endosperm and by far have provided good scientific foundations for the improvement of cereal endosperm traits [6–9]. However, the generation of mutants or genetically modified plants is difficult and time-consuming for most cereal crops.

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_8,

Development of simple and efficient cell-based systems is particularly important for rapid analysis of gene functions. Some decades ago, an endosperm suspension cell system was developed for the functional analysis of endosperm trait-related genes [10]. The endosperm suspension cells are generated from endosperm callus through multi-generation of suspension culture, which is also laborious and time-consuming. Another useful technique for analyzing the functions of endosperm trait-related genes is the use of a particle bombardment system to deliver DNA into developing endosperm cells [11–13]. One of the limitations of this system is that only one target gene can be analyzed for a single transformation experiment. The system is also laborious and requires a lot of experience and expertise to achieve reasonable efficiency.

Recently, we have developed a maize endosperm protoplast (EP) system, which can quickly and easily analyze the function of the target gene in endosperm cells. In addition to being economical and simple, the EP system can be used to transiently analyze a vast number of genes and their regulatory networks simultaneously in one transfection system. We present the details of the protocols here, which include selection of immature seed for endosperm protoplast isolation, plasmid DNA preparation, and protoplast isolation and transfection with foreign genes. Using this protocol, we can achieve a protoplast yield of ~2 × 10^6 cells/mL from 30 to 40 pieces of endosperm and transfection rates in the range of 63–68%. The maize EP system can be efficiently employed in several molecular experiments including protein subcellular localization, protein-protein interaction by bimolecular fluorescence complementation (BiFC), protein immunoblotting, transient gene expression, and regulatory analyses by qRT-PCR.

2 Materials

2.1 Plant Material and Bacterial Strain

1. Maize (*Zea mays* L.) inbred line (Mo17): Planted and grown under the recommended agronomic guidelines and self-pollinated with pollination date properly recorded.
2. *Escherichia coli* strain DH5α: For gene cloning and maxi-plasmid preparation.

2.2 DNA Constructs

1. pBI221-GFP (5.7 kb [14]): The CaMV 35S promoter and *GUS* gene in the pBI221 vector were replaced with maize ubiquitin promoter and *eGFP* gene, respectively.
2. pBI221-ZmMYB14 (6.1 kb [15]): The *eGFP* gene in the pBI221-GFP vector was replaced with the *ZmMYB14* gene.
3. pCAMBIA2300-eGFP (10.3 kb): The eGFP gene regulated by CaMV 35S promoter and NOS terminator was inserted within the MCS site of the pCAMBIA2300 vector [15].

4. pCAMBIA2300-ZmBt1-eGFP (11.6 kb [15]): The coding sequence of *ZmBt1* was inserted upstream of the eGFP gene in the pCAMBIA2300-eGFP vector.
5. pSAT6-cEYFP-C1-B-O2 (E3108-O2) (5.4 kb): The coding sequence of *O2* was fused to the downstream of the C-terminal of the *eGFP* gene in the pSAT6-cEYFP-C1-B vector [14, 16].
6. pSAT6-nEYFP-C1-PBF1 (E2884-PBF1) (5.4 kb): The coding sequence of PBF1 was fused to the downstream of the N-terminal of the *eGFP* gene in the pSAT6-nEYFP-C1 vector [14, 16].

2.3 Stock Solutions

All stock and working solutions are prepared using double distilled water (ddH_2O) and analytical grade reagents. All solutions are sterilized using a 0.45 μm filter system. The solutions are stored at 4 °C unless otherwise specified. Room temperature refers to temperature between 22 and 25 °C.

1. MES (0.2 M, pH 5.7): In ~90 mL ddH_2O, add 3.9 g 4-morpholineethanesulfonic acid (MES), dissolve the chemical thoroughly, and adjust the pH of the solution to 5.7 with 5 N NaOH. Add additional ddH_2O to a final volume of 100 mL.
2. $CaCl_2$ (1 M): In ~90 mL ddH_2O, add 14.7 g $CaCl_2{\cdot}2H_2O$, dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 100 mL.
3. KCl (2 M): In ~90 mL ddH_2O, add 14.91 g KCl and dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 100 mL.
4. NaCl (5 M): In ~90 mL ddH_2O, add 292.2 g NaCl, dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 100 mL.
5. $MgCl_2$ (1 M): In ~90 mL ddH_2O, add 20.3 g $MgCl_2{\cdot}6H_2O$, dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 100 mL.
6. BSA (10%): Dissolve 1 g of bovine serum albumin (BSA) in ~9 mL ddH_2O, and add additional ddH_2O to a final volume of 10 mL.
7. D-Mannitol (0.8 M): In ~90 mL ddH_2O, add 14.57 g D-mannitol and dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 100 mL.
8. EDTA (0.5 M, pH 8.0): In ~90 mL ddH_2O, add 18.61 g ethylenediaminetetraacetic acid disodium salt ($Na_2EDTA{\cdot}2H_2O$), dissolve the chemical thoroughly, and adjust the pH of the solution to 8.0 with 5 N NaOH. Add additional ddH_2O to a final volume of 100 mL.

9. SDS (10% (w/v)): Dissolve 10 g of sodium dodecyl sulfate (SDS) thoroughly in ~90 mL hot ddH_2O, cool to room temperature, and add additional ddH_2O to a final volume of 100 mL.
10. LiCl (4 M): In ~45 mL ddH_2O, add 8.478 g LiCl, dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 50 mL.
11. NaAC (3 M): In ~45 mL ddH_2O, add 20.412 g NaAC·$3H_2O$, dissolve the chemical thoroughly, and add additional ddH_2O to a final volume of 50 mL.
12. Tris–HCl (1 M, pH 6.8): In ~45 mL ddH_2O, add 6.055 g Tris-base, dissolve the chemical thoroughly, and adjust the pH of the solution to 6.8 with 5 N HCl. Add additional ddH_2O to a final volume of 50 mL.

2.4 Working Solutions and Media

1. 1/2 MS solid medium: In ~190 mL ddH_2O, add 0.443 g Murashige and Skoog (MS) basal medium and 13.69 g glucose, and dissolve the chemical thoroughly. Adjust the pH of the solution to 5.8 with 1 N KOH and add additional ddH_2O to a final volume of 200 mL. Then add 1.8 g Phytoagar. Autoclave and pour ~15 mL into 9 cm × 1.5 cm Petri dishes after the medium has cooled down to ~55 °C.
2. LB liquid medium: In ~490 mL ddH_2O, dissolve 5 g tryptone, 2.5 g yeast extract, and 5 g NaCl. Adjust the pH of the solution to 7.2 with 1 N NaOH and final volume to 500 mL. Autoclave.
3. LB solid medium: Same as LB liquid medium but add 7.5 g Phytoagar in 500 mL. Autoclave, then pour ~15 mL into 9 cm × 1.5 cm Petri dishes after the medium has cooled down to ~55 °C.
4. Plasmid DNA Maxi-Preparation solution I: In 490 mL ddH_2O, add 10 mL of 0.5 M EDTA (pH 8.0).
5. Plasmid DNA Maxi-Preparation solution II: In 440 mL ddH_2O, add 10 mL of 5 M NaOH and 50 mL of 10% SDS. Prepare freshly before use.
6. Plasmid DNA Maxi-Preparation solution III: Dissolve 147 g potassium acetate in 450 mL ddH_2O, then add 57.5 mL acetic acid. Adjust final volume to 500 mL.
7. 10× TE buffer: To make 20 mL, add 10 mL of 1 M Tris–HCl (pH 6.8), 4 mL of 0.5 M EDTA (pH 8.0) in 6 mL ddH_2O.
8. PEG-Ca^{2+} solution: In ~45 mL ddH_2O, dissolve 6.5 g polyethylene glycol (PEG) 8000 and 4.675 g NaCl. Add additional ddH_2O to a final volume of 50 mL.
9. Enzyme solution: Add ~8 mL ddH_2O to a 10 mL round-bottomed tube, then add 0.15 g Cellulase R-10, 0.075 g

Macerozyme R-10, 0.728 g D-mannitol, and 0.5 mL of 0.2 M MES (pH 5.7), mix vigorously for 30 s by vortexing. Place the tube in a 55 °C water bath for 10 min. After cooling the solution to room temperature, add 100 μL of 10% BSA, 100 μL of 1 M $CaCl_2$, and adjust the volume to 10 mL with ddH_2O (*see* **Note 1**).

10. PEG-Ca^{2+} transfection solution: Mix 2.5 mL of 0.8 M of D-mannitol, 1 mL of 1 M $CaCl_2$ and 6 mL ddH_2O in a 10 mL round-bottomed tube, then add 4 g PEG 4000 and stir to dissolve. Adjust the volume with ddH_2O to 10 mL (*see* **Note 2**).
11. W5 solution: In 800 mL ddH_2O, add 30.8 mL of 5 M NaCl, 125 mL of 1 M $CaCl_2$, 2.5 mL of 2 M KCl, and 10 mL of 0.2 M MES (pH 5.7). Adjust the pH to 5.8 with 1 N KOH. Adjust the volume to 1 L with ddH_2O and filter sterilize. The solution can be kept at room temperature for 2 months.
12. MMG solution: In 950 mL dH_2O, add 7.5 mL of 2 M $MgCl_2$, 20 mL of 0.2 M MES (pH 5.7), and 72.868 g D-mannitol, and stir to dissolve. Adjust the pH to 5.6 with KOH. Add ddH_2O to a final volume of 1 L and filter sterilize. MMG solution can be kept at room temperature for 3 months.

2.5 Supplies and Equipment

1. Electronic analytical balance.
2. pH meter.
3. Vortex mixer.
4. Thermal cycler.
5. Autoclave.
6. Thermostat water bath.
7. Thermostatic incubator.
8. Vacuum concentrator.
9. Centrifuge with swing bucket rotor.
10. Real-time PCR detection system.
11. Scientific Fluoroskan Ascent FL (Thermo Fisher Scientific, CN).
12. Gel imaging and documentation system.
13. Ultra-microspectrophotometer.
14. Ordinary optical microscope.
15. Inverted fluorescence microscopy.
16. Laser confocal microscopy.
17. 2 mL centrifugal tube.
18. 10 mL and 50 mL centrifugal tube.
19. 100 μm nylon mesh screen.

20. 0.45 μm disposable needle filter.
21. Cell scrapers.
22. Disposable Petri dish (90 × 15 mm).

3 Methods

3.1 Preparation of Plasmid DNA

1. Pick and culture a single transformed *E. coli* cell in a 400–500 mL LB liquid broth overnight at 37 °C with shaking, 200 rpm (*see* **Notes 3** and **4**).
2. Transfer the culture into a centrifugal tube and collect pelleted cells by centrifugation at 4500 × *g* for 15 min. Discard the supernatant and turn the tube upside down on an absorbent paper for 2–3 min, to absorb the residual liquid (*see* **Note 5**).
3. Add 20 mL solution I, and completely resuspend the cell pellet by mixing on a vortex mixer. Afterwards, add 40 mL freshly prepared solution II, and mix gently by inverting the tube back and forth several times (*see* **Note 6**). Thereafter, quickly add 15 mL solution III, and mix gently by inverting the tube back and forth for five to six times.
4. Centrifuge at 4200 × *g*, 4 °C for 10 min.
5. Filter the supernatant through a double-layer gauze cloth into a sterilized beaker.
6. Add isopropyl alcohol (same volume as that of the supernatant). Mix by inverting the tube back and forth several times.
7. Repeat **step 4**. Discard the supernatant.
8. Gently wash the pellet by adding 2 mL absolute or 95% ethanol (*see* **Note 7**) along the wall. Carefully remove ethanol with a pipette. Air-dry for 5–10 min.
9. Resuspend and dissolve the pellet in 2 mL solution I. Divide and transfer the liquid into two 2 mL Eppendorf tubes.
10. Add the equal volume of 4 M LiCl and mix thoroughly. Spin at 10,000 × *g* for 10 min.
11. Transfer the supernatant into a 2 mL Eppendorf tube (1 mL per tube, i.e., 4 tubes total). Add 1 mL isopropyl alcohol to each tube and mix.
12. Centrifuge at 12,000 × *g* for 10 min. Discard the supernatant.
13. Rinse the pellet in each tube with 1 mL 95% ethanol. Transfer and combine the ethanol and DNA pellets into a 2 mL Eppendorf tube.
14. Centrifuge at 12,000 × *g* for 5 min. Discard the supernatant. Air-dry for about 5–10 min.
15. Add 495 μL 1× TE to dissolve DNA pellet thoroughly.

16. Add 5 μL RNase and promptly place in water bath at 37 °C for 30 min.
17. Add 500 μL PEG/NaCl, mix completely, and keep at room temperature for 10 min.
18. Centrifuge at 12,000 × *g* for 5 min. Discard the supernatant.
19. Add 800 μL 1× TE to dissolve DNA pellet completely. Let the tube sit on the bench for 10–20 min.
20. Add 400 μL Phenol:Chloroform:Isoamyl alcohol (25:24:1) to a DNA tube. Mix the content thoroughly and let the tube sit on the bench for 3 min.
21. Centrifuge at 10,000 × *g* for 10 min.
22. Carefully transfer the supernatant (about 800 μL) into a new 2 mL Eppendorf tube.
23. Add 200 μL 3 M NaOAc. Fill the tube with absolute ethanol to 2 mL mark. Mix thoroughly and let the tube sit on the bench for 20 min.
24. Centrifuge at 12,000 × *g* for 5 min. Discard the supernatant.
25. Wash the DNA pellet with 2 mL 70% ethanol.
26. Centrifuge at 12,000 × *g* for 2 min. Discard the supernatant. Air-dry for 5–10 min.
27. Repeat **steps 24** and **25**. Air-dry the DNA pellet by inverting the tube on an absorbent paper.
28. Dissolve with 200–500 μL double-deionized water.
29. Measure the concentration and store at 4 °C.

3.2 Endosperm Dissection and Digestion

1. Plant and grow maize (*Zea mays* L.) inbred line under recommended agronomic guidelines. Self-pollinate and harvest ears at 7–9 days after pollination (DAP) (*see* **Note 8**).
2. Remove the husk of the ear (Fig. 1a). Choose the kernels that grow at the middle section of the ear (*see* **Note 9**).
3. Carefully cut off one-third of the kernel crown using a sharp razor blade.
4. Use a pair of forceps to dissect the endosperm from the kernel (Fig. 1b and c) and place them on a Petri dish containing 1/2 MS medium (Fig. 1d) for 30–60 min (*see* **Note 10**).
5. Cut the endosperms in half with a sharp razor blade (*see* **Note 11**).
6. Transfer 30–40 endosperms into a conical flask containing 10 mL freshly prepared enzyme solution (Fig. 1d; *see* **Notes 12** and **13**). Ensure the endosperms are submerged in the enzyme solution.

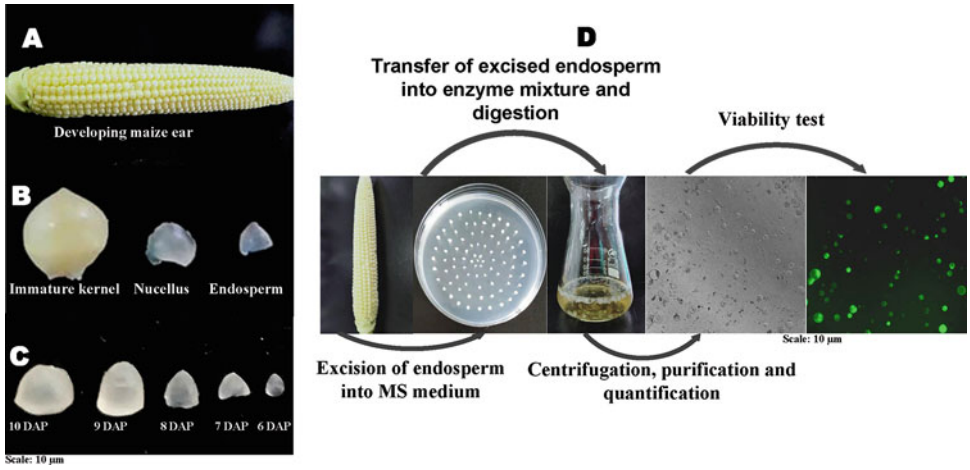

Fig. 1 Immature ear and kernels at different stages and flow chart of major procedures involved in endosperm protoplast isolation. (**a**) Developing ear at 8 DAP. (**b**) Developing kernel, nucellus, and excised endosperm at 8 DAP. (**c**) Developing endosperms at 6–10 DAP. (**d**) Schematic diagram of protoplast isolation. Bar = 10 μm. (Image **d** is reproduced from [14])

7. Place the flask on an orbital shaker (50 rpm) in a dark room at room temperature for 4–6 h (*see* **Note 14**). Turbid enzyme solution and softened endosperm mass indicate the release of protoplasts.
8. Check for the release of protoplasts in the solution under microscope (*see* Fig. 1d); the size of maize endosperm protoplast is approximately 40–80 μm.

3.3 Protoplast Isolation

During the entire process of protoplast experiment, each step should be gently handled to prevent the rupture of protoplast. Protoplasts are fragile; they must be handled with care. Wide bore pipette tips must be used for all steps described in this section.

1. Add 10 mL of W5 (same volume as the enzyme solution) into the conical flask along the wall. Gently swirl the flask to mix and dilute the enzyme/protoplast solution.
2. Prewet a sterile 100 μm nylon mesh (*see* **Note 15**) with W5 solution and place it on the top of a 50 mL round-bottomed centrifuge tube.
3. Carefully filter the enzyme/protoplast suspension through the nylon mesh into the tube. Use the wide bore pipette tips.
4. Add 5 mL of W5 to the flask and gently swirl the flask to release more protoplasts.
5. Repeat **steps 3** and **4** several times until the endosperm tissue suspension is clear. Discard the undigested endosperm tissues.
6. Centrifuge the 50 mL round-bottomed tube at 110 × *g* for 3 min to pellet the protoplasts.

7. Remove the majority of the supernatant with pipette tips but retain at least 1 mL at the bottom. Gently tap the tube to resuspend the protoplast pellet.
8. Carefully add moderate volume of W5 solution along the tube wall to suspend the protoplast (*see* **Note 16**).
9. Repeat **step 6**. Remove the supernatant.
10. Carefully add 1 mL W5 solution to resuspend the protoplast pellet.
11. Keep the protoplasts on ice for 30 min. The cell numbers of the resuspended protoplasts can be counted under microscope using a hemocytometer during this period.
12. Centrifuge the tube at 110 × *g* for 2 min to pellet the protoplasts.
13. Remove the supernatant as much as possible.
14. Add moderate volume of MMG solution to make the final cell density at 2×10^6 protoplasts/mL.

3.4 Protoplast Yield Quantification

1. Aliquot 5 μL protoplast suspension to 0.1 mm^3 hemocytometer and observe under normal optical microscope.
2. Count protoplast cells within each of the five grids (four grids at the corners with one central grid).
3. Repeat this with three independent samples and take the average.
4. Compute the protoplasm yield (PY) using the formula **PY (cells/mL)** = total number of protoplasts (in the 5 grids) × 5 × 10^4 × (protoplast volume/2) (*see* **Note 17**).

3.5 Protoplast Viability Determination

1. Prepare fluorescein diacetate (FDA) solution with acetone (5 mg/mL).
2. Add FDA solution into protoplast suspension (25 μL FDA/mL).
3. Incubate for 20 min in the dark at room temperature.
4. Observe under the fluorescence microscope: the active protoplast cell emits yellow-green fluorescence, while the nonviable cell does not glow.
5. Select five microscopic fields of view for observation and photography. Repeat with three independent samples.
6. Calculate the total number of protoplast cells and fluorescent cells in the view.
7. Compute protoplast viability rate as percentage of numbers of fluorescent protoplasts in total protoplast cells.

3.6 Transfection

Wide bore pipette tips must be used for all steps described in this section. Protoplasts are fragile; they must be handled with care.

1. In a 2 mL round-bottomed microfuge tube, add 10 μL plasmid (10–20 μg, 5–10 kb in size) and 100 μL protoplast (2×10^5/mL) (*see* **Note 18**).
2. Mix thoroughly by gently pipetting up and down the suspension (*see* **Note 19**).
3. Slowly add 110 μL PEG-Ca^{2+} solution and mix the transfection mixture gently and completely with pipette tips. Incubate the transfection mixture at room temperature for 15 min.
4. Add 440 μL W5 buffer into the transfection mixture, gently invert the tube, and mix the content completely to terminate the transfection.
5. Centrifuge the tube at 110 × *g* for 2 min. Remove the supernatant with pipette tips.
6. Carefully add 1 mL W5 buffer along the tube wall to resuspend the protoplasts.
7. Repeat **steps 4** and **5**.
8. Check for cell integrity under microscope (*see* Subheading 3.4).
9. Place the transfection tube horizontally. Incubate protoplasts at 26 °C in the dark for 12–16 h (*see* examples in Subheadings 3.8, 3.9, and 3.10).
10. Centrifuge the transfection tube at 110 × *g* for 2 min. Remove the supernatant with pipette tips to harvest protoplasts.

3.7 Transfection Rate Determination

1. Aliquot 40–50 μL W5 into a transparent well plate.
2. Add 5–10 μL GFP transfected protoplast with wide bore pipette tips.
3. Observe under a laser confocal scanning microscope (GFP excitation and emission wavelengths of 488/507 nm). Successfully transfected protoplast cells emit green fluorescence due to the expression of GFP, whereas the non-transfected cells remain dark.
4. Select five fields of view for visualization and photography. Repeat with three independent samples.
5. Count the number of total protoplasts and fluorescent cells in the view. Calculate transfection efficiency as percentage of numbers of GFP expressing protoplasts in total protoplast cells.

3.8 Example 1: Analysis of Protein Subcellular Localization – ZmBT1 Localization in Endosperm Protoplast System [14]

Analysis of subcellular localization of a protein provides valuable information on the protein specific location and interpretation of its function in a cell [17]. Protoplast, a cell-based system, is typically an ideal and efficient system to study protein subcellular localization. Here, we used a maize endosperm protoplast (MEP) system to confirm the localization of ZmBT1 protein at the amyloplast envelope membrane of maize endosperm cells [18], using green fluorescent protein (GFP) as a fluorescent marker. ZmBT1, a gene involved in the ADP-Glc transport into endosperm plastids, plays critical roles in the biosynthesis of starch [19]. In molecular studies, GFP has become a standard marker in gene expression study and protein targeting in intact cells, because of its ability to produce an extremely visible green fluorescence under high-resolution microscopy [20].

1. To investigate the subcellar localization of ZmBT1, 10 μg of ZmBT1-GFP fusion expression vector, pCAMBIA2300-ZmBT1-GFP, was transfected with 100 μL protoplast suspension (1.5×10^6 cells in MMG solution; *see* Subheading 3.3). Similarly, 10 μg GFP vector, pCAMBIA2300-GFP, was also transfected with 100 μL protoplast suspension and used as the control.
2. The transfected protoplast cells were incubated in the dark at 26 °C for 12–16 h.
3. Then, 40–50 μL W5 was aliquoted into a transparent well plate, and 5–10 μL transfected protoplast was added with wide bore pipette tips.
4. Protein localization was visualized and photographed under the laser confocal scanning microscope.

As shown in Fig. 2, the protoplast transfected with pCAMBIA2300-GFP shows fluorescent signals in the nucleus, cytosol, and cell membrane (Fig. 2a), whereas the protoplast cell transfected with pCAMBIA2300-ZmBT1-GFP produced green fluorescent signal only at the amyloplast envelope membranes (Fig. 2b), confirming the localization of ZmBT1 protein.

3.9 Example 2: Analysis of Protein-Protein Interaction by BiFC Assay in Endosperm Protoplast System [14]

Protein-protein interaction can be investigated by several methods including bimolecular fluorescence complementation (BiFC). We examined the interaction of PBF1 and O2 proteins by BiFC in a MEP system. PBF1 and O2 are endosperm-specific transcription factors and cooperatively associated with metabolism of storage materials (starch and zein proteins) in maize endosperm [21].

1. The binary-molecular expression vectors containing fluorescent fusion proteins; 10 μg of pSAT6-nEYFP-C1-PBF1 and 10 μg of pSAT6-cEYFP-C1-B-O2 were co-transfected with 100 μL protoplast suspension (1.5×10^6 cells in MMG solution; *see* Subheading 3.3).

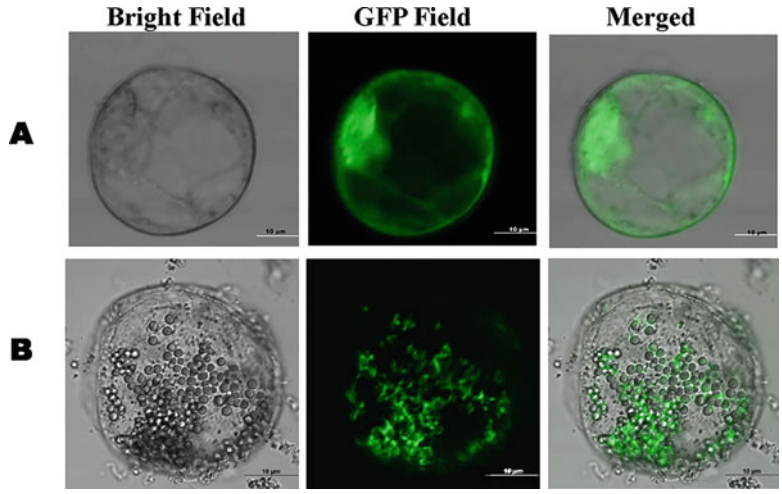

Fig. 2 Subcellular localization ZmBT1 in a maize endosperm protoplast system. (**a**) Protoplast transfected with pCAMBIA2300-GFP (control) showing the localization of GFP in the nucleus. (**b**) Protoplast transfected with ZmBT1-GFP fusion protein vector, pCAMBIA2300-ZmBT1-GFP, showing the localization of BT1-GFP fusion protein in amyloplast envelope membranes. Bar = 10 μm. (Reproduced from [14])

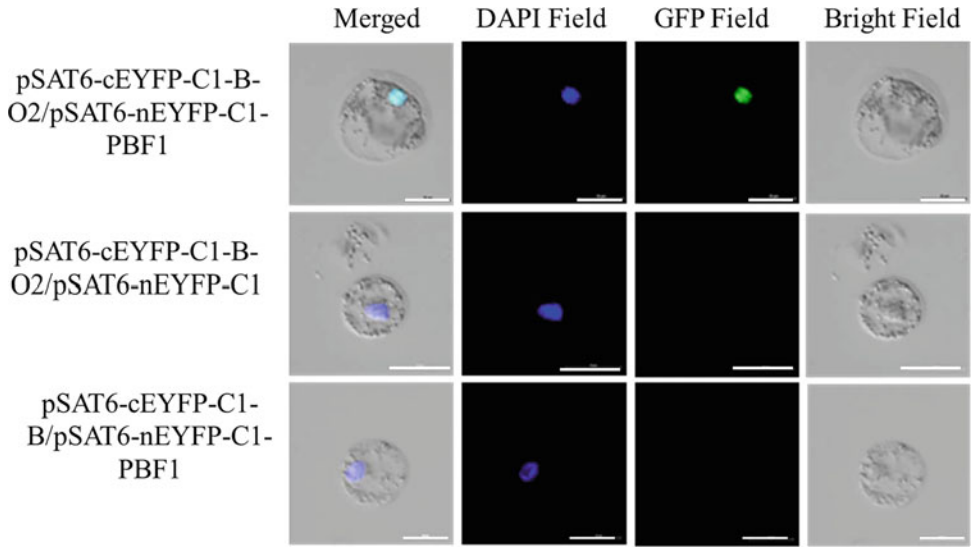

Fig. 3 Interaction of PBF1 and O2 by BiFC in maize endosperm protoplast cells. The detection of GFP in the nucleus indicates nuclear localization. Bars = 10 μm. (Reproduced from [14])

2. For negative controls, two sets of construct pairs were used (Fig. 3). They were co-transfected similarly as described in **step 1**.
3. The transfected protoplast cells were incubated in the dark at 26 °C for 12–16 h.
4. For nuclear staining, 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) was incubated with transfected protoplast cells for 5 min.

5. The interaction of pSAT6-nEYFP-C1-PBF1 and pSAT6-cEYFP-C1-B-O2 was visualized and photographed under a laser confocal scanning microscope (DAPI excitation and emission wavelengths of 358/461 nm), following DAPI staining.

Under the confocal microscope, all the protoplast systems containing the different pairs of constructs pSAT6-cEYFP-C1-B-O2 and pSAT6-nEYFP-C1-PBF1, pSAT6-cEYFP-C1-B-O2 and pSAT6-nEYFP-C1, and pSAT6-cEYFP-C1-B and pSAT6-nEYFP-C1-PBF1 showed blue fluorescent DAPI-stained DNAs in the nuclei (Fig. 3). In the protoplast system co-transfected with pSAT6-cEYFP-C1-B-O2 and pSAT6-nEYFP-C1-PBF1, some protoplasts emitted green fluorescent signals (Fig. 3), indicating the nuclear interaction of the two proteins.

3.10 Example 3: Transcriptional Activity and Transient Expression Analysis of ZmMYB14 in Endosperm Protoplast System [14]

Transcription factors are key regulators of gene expression at the transcriptional level [22]. *ZmMYB14* is a transcription factor, which has been reported to positively regulate the promoter activity of *ZmBt1*, a starch biosynthesis-related gene [15]. Here, we utilized the MEP system to transiently express *ZmMYB14* and analyze its transcriptional regulatory influence on the expression of *ZmBt1*.

1. The expression vector pBI221-ZmMYB14, containing the coding sequence of ZmMYB14, and empty vector, pBI221 (control), were independently transfected with endosperm protoplast suspension and incubated (*see* Subheading 3.6).
2. The transfected cells were harvested by centrifugation 13,000 × *g* for 1 min at 4 °C. The supernatant was discarded.
3. RNA was extracted from the harvested cells using TRIzol extraction kit protocol. The isolated RNA was confirmed by gel electrophoresis (Fig. 4a).
4. cDNA was generated from 1 μg RNA by reverse transcription following the PrimeScript RT reagent kit.
5. The cDNA was diluted by tenfold and was used for RT-qPCR assays with a CFX96 Real-Time System. The maize TXN gene was used as internal control [23], and the RT-qPCR was performed in triplicates.
6. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

ZmMYB14 was successfully overexpressed in the maize EP system, and its overexpression positively activated the activity of *ZmBt1* promoter, as shown in Fig. 4b. The results were consistent with the previous study [15]. This work validates the utility of the maize EP system in molecular studies.

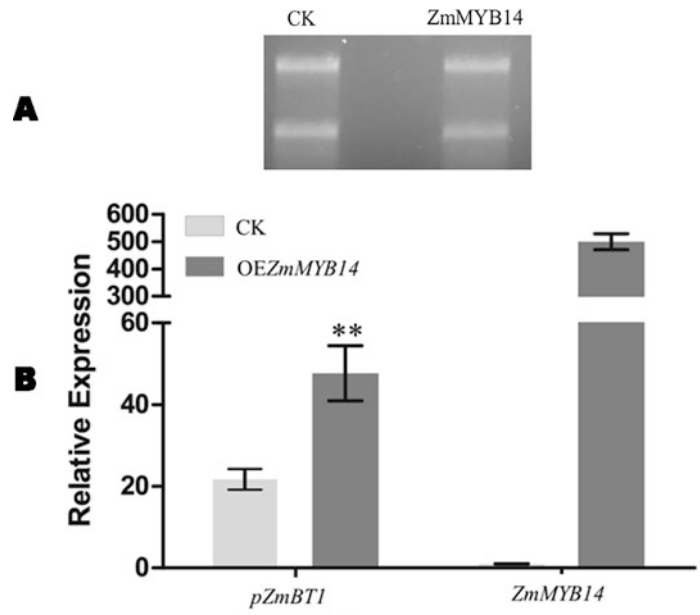

Fig. 4 (**a**) RNA detection through gel electrophoresis. (**b**) Transient expression of ZmMYB14 and its direct activation of *pZmBt1* promoter activity. CK = empty vector pBI221 control. (Reproduced from [14])

4 Notes

1. Enzyme solution should be freshly prepared when it is needed. Cellulase R-10 and Macerozyme R-10 are recommended from Yakult Pharmaceutical Ind. Co., Ltd., Japan.
2. The PEG-Ca^{2+} solution should be freshly prepared for transfection. The volume to be prepared is determined by the sample size; each sample requires 110 μL PEG-Ca^{2+} solution. During preparation, add the required amount of $CaCl_2$ mannitol, and PEG 4000, then add minimal ddH_2O and allow to dissolve completely, before adjusting to final volume with ddH_2O.
3. For plasmid DNA preparation, the amount of LB liquid broth used is determined according to the final amount of DNA required, typically 400–500 mL.
4. In order to obtain more active bacteria, *E. coli* should be activated in advance, and fresh single clone should be selected for culturing in LB liquid broth, at least for 12–15 h (preferably overnight).
5. After harvesting the bacteria by centrifugation and supernatant discarded, the centrifugal tube is flipped over the absorbent paper and drained.

6. During plasmid preparation, the time interval between addition of solution II and solution III should not be more than 5 min. These steps should be handled gently and quickly.
7. The ethanol (95%) used for plasmid DNA preparation is HPLC grade.
8. The age of collected ears is dependent on the inbred line genotype and temperature of cultivation period. Generally, 8 DAP is the most appropriate day for many inbred lines; the 8 DAP endosperm is colorless and transparent and has no obvious starch or protein accumulation in it. It is recommended that the ears are collected at the day of experiment, or they can be stored with their husks still intact, at 4 °C for 1 or 2 days.
9. The developing kernels at the top or bottom sections of the ear may vary in their stages of development. Therefore, they are not suitable for protoplasts and can affect the transfection efficiency.
10. Immediately the endosperm is excised from the developing kernels; it should be placed in 1/2 MS medium to maintain optimum moisture and osmotic conditions of the endosperm. The MS medium helps to preserve the moisture content and viability of the endosperm cells.
11. Each endosperm should be carefully cut into two-halves with a sharp surgical blade while preventing damage to the endosperm as much as possible. New surgical blade or razor blade sterilized with 95% ethanol and air-dried can be used.
12. The volume of enzymatic solution can be adjusted according to the quantity of endosperm. For 10 mL of enzymatic solution, 30–40 g of endosperm is appropriate.
13. When preparing the enzyme solution, the enzyme mixture should be placed in water bath at 55 °C for 10 min. This is required for activation of protease and also improves the solubility of the enzyme.
14. The enzymatic digestion time can vary depending on the quantity of endosperm mass. More endosperms require longer digestion time. It is not necessary to release all the protoplasts from the tissues. Be gentle with protoplasts, and handle them with wide bore pipette tips, to prevent the mechanical damage of the protoplasts during the transfer process.
15. The nylon mesh is normally kept in 95% ethanol; rinse well with water before use.
16. The volume of W5 solution added depends on the protoplast amount and is usually 1–5 mL. W5 solution can be added several times in small amounts to enhance the suspension of

the protoplasts. Inadequate suspension will reduce the transformation efficiency of the protoplasts.

17. After protoplast isolation, the final density of the protoplast should be adjusted to 1.5–2.0 × 10^6 cells/mL by hemocytometer.
18. For a higher transfection rate, use 3–5 kb plasmid DNA at concentration ≥1000 ng/μL; 260/280: 1.7–1.95 and 260/230 >2.
19. During the transfection process, first add plasmid DNA [10–15 μL (10–15 μg)], then add protoplast suspension and gently finger flick the tube to mix thoroughly. Afterwards, add PEG-Ca^{2+} solution, and then gently tap the tube and mix well.

Acknowledgments *This work was supported by the National Key R&D Program of China (2016YFD0100503) and the National Natural Science Foundation of China (31771809).*

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Chapter 9

Protoplast Isolation from Undifferentiated Maize Seedling Shoot Tissue

James W. Satterlee and Michael J. Scanlon

Abstract

Protoplasts are plant cells that have had their cell walls removed, which allows for a variety of cellular manipulations that are not possible within the context of intact plant tissue. Unfortunately, the removal of cell walls is not trivial and can be sensitive to cell type and cell differentiation state. Here, we describe a modified protoplasting protocol that improves isolation of viable protoplasts from the seedling maize shoot apex.

Key words Fluorescein diacetate (FDA), Protoplast viability, Shoot apical meristem (SAM), Single-cell transcriptomics, *Zea mays*

1 Introduction

Plant cells are surrounded by rigid cellulosic cell walls. This feature limits the ease with which intact plant cells can be experimentally isolated and manipulated [1]. For example, techniques such as plant cell culture, cell transfection, flow cytometry, and single-cell transcriptomic analysis require the generation of protoplasts, plant cells which have had their cell walls removed. A typical approach is to use cell wall-digesting enzymes (e.g., cellulases and pectinases) isolated from plant pathogenic fungi to catalyze cell wall breakdown of macerated or sliced plant tissues of interest. Protoplasts are released into an osmotically stabilized medium (typically made up of mannitol or other metabolically inert compounds), washed to remove cellular debris, and then resuspended for downstream analysis. Protoplasts generated in this fashion are living and can be used immediately or cultured for longer-term purposes. For example, maize protoplasts isolated from embryogenic callus in some inbred lines have been shown to be useful in plant regeneration [2]. Other uses of maize protoplasts include the investigation of signal trans-

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_9,

duction pathways and the dissection of gene expression regulation using transient transformation [3, 4]. In one instance, transient transformation of maize protoplasts was used to overexpress and knock down candidate transcriptional regulators of benzoxazinoids, defensive compounds involved in plant response to stress, to test these genes' control over the benzoxazinoid biosynthesis pathway [5]. Protoplasts isolated from maize tissues have also been useful in the study of developmental biology. Flow cytometric sorting of maize protoplasts expressing fluorescently tagged tissue-specific gene expression markers has been used to isolate specific cell types in maize roots for transcriptional analysis [6]. More recent uses of maize protoplasts have included studying gene expression signatures in developing tissues at the single-cell level. Such analyses have provided insights into the heterogeneous landscapes of gene expression in complex developing tissues such as the maize seedling shoot apex and ear primordium at high spatial resolution [7, 8].

While some plant tissues are readily amenable to protoplast generation, others such as the vegetative shoot apex pose challenges [1]. For example, protoplast isolation from the maize shoot apical meristem (SAM) is complicated by the microscopic size of the tissue and the relatively low viability of protoplasts isolated from these stem cell-enriched tissues. Difficulties associated with protoplasting in the context of these undifferentiated shoot tissues are a major hindrance to studying the developmental genetics of these systems. We sought to improve the efficiency of viable protoplast isolation from undifferentiated seedling maize shoot tissues by modifying the protoplasting medium for downstream use in single-cell transcriptomics [7]. Consistent with previous reports, we found that supplementing the protoplasting medium with L-arginine could stabilize protoplasts during isolation [9]. In addition, further improvements in viability were observed at a near-neutral pH of 7.5 versus the much lower pH of 5.7 used in other methods. This may be due to the higher relative intercellular pH of the undifferentiated tissues in the plant shoot apex, as was shown in *Chenopodium* [10]. Preliminary evidence suggests that these modifications improve the representation of undifferentiated cell types not only in the maize vegetative shoot apex but also in developing ear primordia (David Jackson, personal communication). In addition, this protocol results in higher transcript capture efficiency in downstream single-cell transcriptomic analyses. We incorporate these technical modifications for generating protoplasts from the maize vegetative shoot apex.

In this protocol, we use a biopsy punch to rapidly dissect a core of tissue containing the maize (*Zea mays*) seedling vegetative shoot apex. Fine dissection of excess leaves yields tissue consisting of the SAM and six most recently initiated leaf primordia. The harvested tissue is briefly macerated using a scalpel and then incubated for 2 h

in an enzymatic solution that releases protoplasts by digesting away the cell wall. A series of wash steps following the incubation remove the enzyme solution. Protoplast viability and yield can be assessed with the use fluorescein diacetate viability staining and a hemocytometer under a fluorescent compound microscope. This approach allows the isolation of approximately 70 shoot apices within 1 h, which typically yields ~0.25×10^6 protoplasts after a 2 h incubation in enzyme solution. Dissection of smaller tissues requires increased amounts of input tissue to achieve the same yield. The resulting protoplasts can then be used for downstream purposes, such as single-cell transcriptomic analysis.

2 Materials

2.1 Plant Materials

1. Maize inbred B73 seedlings grown to 2 weeks old in a Percival growth chamber (*see* Subheading 2.3, **item 2**).

2.2 Solutions and Media

1. MOPS (pH 7.5): 250 mM 3-(N-morpholino)propanesulfonic acid. Filter sterilize and store at 4 °C.
2. Arginine stock solution: 250 mM L-arginine HCl, 10 mM MOPS (pH 7.5). Filter sterilize and store at 4 °C.
3. Protoplasting solution: 1.5% Cellulase Onozuka R-10 (Research Products International, Cat. No. C322005.0), 1.5% Cellulase Onozuka RS (Research Products International, Cat. No. C32400-10.0), 1% Macerozyme R-10 (Research Products International, Cat. No. M22010-5.0), 1% Hemicellulase (Sigma-Aldrich, Cat. No. H2125-150KU), 10 mM MOPS (pH 7.5), 10 mM L-arginine HCL, 0.65 M mannitol, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.1% bovine serum albumin (BSA) (*see* **Note 1**).
4. Protoplast wash buffer: 0.65 M mannitol, 10 mM MOPS (pH 7.5), 10 mM L-arginine HCL (*see* **Note 2**).
5. FDA: 5 mg/mL fluorescein diacetate in acetone. Store at −20 °C.

2.3 Supplies and Equipment

1. 72-well potting flats.
2. Percival A100 growth chamber set to 16 h days, a day temperature of 29.4 °C, a night temperature of 23.9 °C, a relative humidity of 50%, and a light intensity of 340 μmol/m^2/s.
3. Potting mix consisting of 1:1 mixture of Turface MVP and LM111.
4. Stericup filtration system (0.22 μm).
5. 1 mm tissue biopsy punch.
6. Steel razor blades.

7. Scalpel and forceps.
8. Dissecting light microscope.
9. 20 mL glass scintillation vials.
10. Shaking warm water bath set to 28 °C.
11. 40 μm Filcon cup-type cell strainers.
12. C-Chip DHC-N01 disposable hemocytometers.
13. Sterile 1.5 mL microcentrifuge tubes.
14. Sterile 15 mL centrifuge tubes.
15. Sterile plastic Pasteur pipettes.
16. Compound fluorescent microscope equipped with a 488 nm excitation filter and a 510 nm emission filter.

3 Methods

3.1 Prepare Plant Material

1. Fill a 72-well tray potting flat with a 1:1 mixture of Turface MVP and LM111 soil.
2. Sow maize inbred B73 seeds approximately ½ inch below the soil surface and water thoroughly.
3. Place the flat in the growth chamber and water daily (*see* **Note 3**).
4. For protoplasting of the maize vegetative shoot apex and/or SAM, harvest seedlings at 2 weeks after sowing. Seedlings should have ~4 fully expanded leaves.

3.2 Dissect Maize Shoot Apex Tissue

1. To dissect the maize SAM and the six most recently initiated primordia, use a razor blade to cut the shoot transversely just above the soil surface.
2. Make another transverse cut approximately 5 mm above the first cut.
3. Using a scalpel, make transverse cuts up the shoot from the base until the visible stem tissue is approximately 1 mm in diameter (*see* **Note 4**; Fig. 1).
4. Place the tissue biopsy punch over the stem and depress into the whorl to its full extent.
5. Rotate the biopsy punch 360° and remove from the tissue.
6. Depress the plunger on the biopsy punch to release the captured tissue (Fig. 1).
7. Under a dissection microscope, use forceps to remove any leaf tissue beyond the sixth primordium.
8. Use a scalpel to briefly macerate the tissue and transfer the tissues to the 5 mL of protoplasting solution in a glass scintillation vial kept at room temperature (22–24 °C).

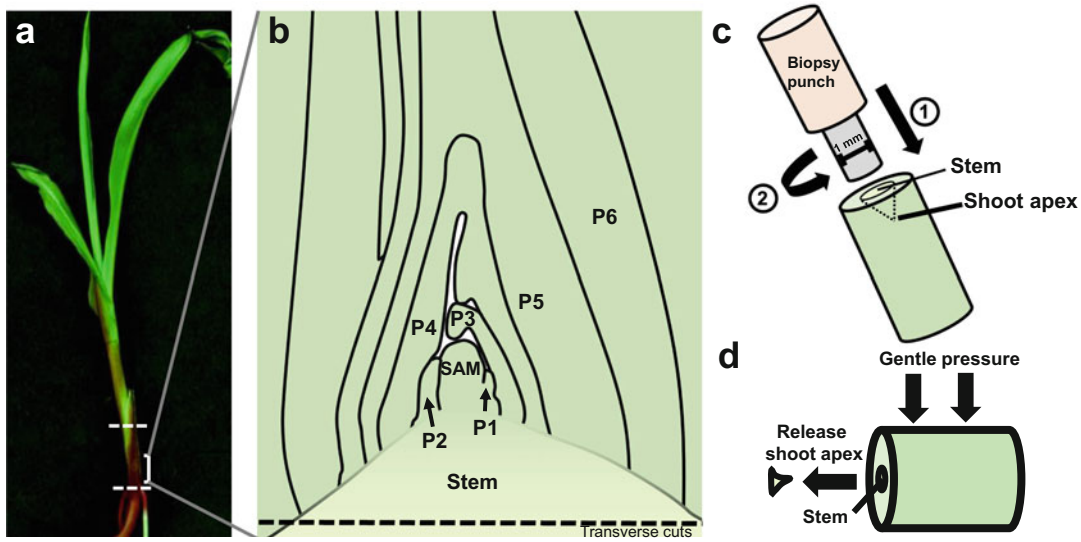

Fig. 1 Dissection of the maize seedling shoot apex. (**a**) A 2-week-old maize seedling. The bracket indicates the location of the shoot apex; dashed lines indicate the locations of the first two transverse cuts used to separate the tissues of interest from the rest of the plant. (**b**) Longitudinal representation of the shoot apex, indicating the location of the shoot apical meristem (SAM) and leaf primordia 1–6. Transverse cuts are made from the bottom upwards towards the SAM until the disk of visible stem tissue at the sample base has a diameter of approximately 1 mm. (**c**) Coring of the shoot apex with a biopsy punch. The biopsy punch is inserted into the disk of visible stem tissue and is (1) depressed until the punch is fully embedded and (2) rotated several times. The released cylinder of tissue is dissected using forceps to remove any undesired leaf tissues beyond P6. (**d**) For isolation of the SAM and earlier leaf primordia, perform transverse cuts until the visible stem radius is <0.5 mm. Apply pressure to the side of the tissue using the blunt edge of a scalpel to release the shoot apex. Dissect away any undesired primordia using forceps

9. Complete harvest of maize tissues within approximately 1 h (*see* **Note 5**). Transfer the vials containing the tissue to a warm water bath set to 28 °C, gently shaking (90 rpm), for 1–2 h (*see* **Note 6**).

3.3 Isolate Protoplasts

1. After 2 h of enzyme digestion, add 5 μL of 5 mg/mL FDA solution to the vial (1:1000 dilution). Mix well and incubate the solution at room temperature in darkness for 5 min.
2. Place a 50 μm cell strainer over a 15 mL conical centrifuge tube.
3. Slowly pour the protoplast suspension through the strainer (*see* **Note 7**).
4. Wash the strainer with 5–10 mL of protoplast wash buffer to collect remaining protoplasts, collecting the flow through in the centrifuge tube.
5. Centrifuge the protoplast solution at 250 × *g* for 3 min at 4 °C.

6. Using a Pasteur pipette, carefully remove the supernatant (*see* **Note 8**).
7. Add 10–15 mL of wash buffer. Resuspend the cells by gently rocking the tube or flicking the tube bottom.
8. Repeat **steps 5**–7 three times, using 10–15 mL of wash buffer each time.
9. Resuspend the cells in 50–100 μL of wash buffer.
10. Take a 3 μL aliquot of the protoplast suspension and dilute in 9 μL of wash buffer inside a 1.5 mL microcentrifuge tube (*see* **Note 9**).

3.4 Determine Cell Density and Quality

1. Load 10 μL of the diluted sample onto a hemocytometer.
2. Place the hemocytometer under a microscope. Image and count the protoplasts under bright field to determine the cell density, taking into account the fourfold dilution performed in **step 10** of Subheading 3.3.
3. If there is an unacceptably high level of cell clumping, pass the cells back through the 50 μm cell strainer and repeat the previous step (*see* **Note 10**).
4. Check cell viability by exciting the sample with ~488 nm wavelength light under a fluorescent stereomicroscope. Viable cells with intact cell membranes will fluoresce at 510 nm (*see* **Note 11**). A standard GFP channel is sufficient to visualize the staining. If desired, overlay bright-field and fluorescent images to calculate the percent cell viability. Representative results are shown in Fig. 2.
5. If the cell concentration is higher than desired, dilute the cells with wash buffer. If the cell concentration is too low, centrifuge for 3 min at 250 × *g* at 4 °C, remove the supernatant, and resuspend in the desired volume of wash buffer.

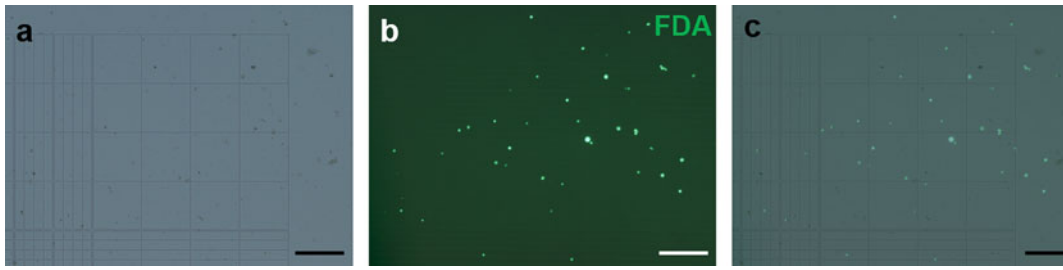

Fig. 2 Representative results of protoplast generation from maize seedling shoot apex tissue. (**a**) Bright-field image of protoplasts in a hemocytometer. (**b**) FDA fluorescence indicates viable cells. (**c**) Overlay of images in (**a**) and (**b**). Scale bars 250 μm

4 Notes

1. Prepare the protoplasting solution fresh on the day which protoplasts will be prepared; 5 mL of protoplasting solution per sample is typically sufficient. Make up 1.5% Cellulase RS, 1.5% Cellulase R-10, 1% Macerozyme R-10, 1% hemicellulose, 0.6 M mannitol, and 10 mM MOPS (pH 7.5) in 2.5 mL water. Incubate the solution at 55 °C for 10 min, mixing by inverting two to three times. Cool to room temperature. Next add 10 mM L-arginine HCl (pH 7.5), 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.1% bovine serum albumin. Bring to 10 mL volume with water. Filter sterilize the protoplasting solution.
2. Prepare the wash buffer during the 1–2 h of enzyme digestion incubation period. Typically, 50 mL of wash buffer is sufficient for a single 5 mL protoplast reaction. Make up 0.65 M mannitol, 10 mM arginine HCL, and 10 mM MOPS (pH 7.5) in 25 mL of water. Bring to volume with water, filter sterilize, and keep chilled at 4 °C or on ice.
3. Ensure that plants are well watered and free from any visible stress (biotic or abiotic) prior to isolating protoplasts. Stressed plants tend to yield fewer protoplasts with lower cellular viability. We have also observed that even after plants have recovered from drought stress, protoplast quality can be poor.
4. If earlier stage primordia and the SAM are desired, use the scalpel to make transverse cuts until the stem diameter is at a smaller size. This size may need to be determined empirically. To obtain the SAM and the two most recently initiated primordia, cut until the stem diameter is approximately 0.5 mm. Apply gentle pressure on the base of the cut shoot with the flat edge of the scalpel. This pressure will draw out the SAM and early primordia that can be readily dissected down to the appropriate primordium number.
5. If cells are to be used in downstream experiments, such as transcriptomic analysis, it is best to minimize the dissection and digestion times as much as possible to avoid adverse effects of these processes on gene expression.
6. If your experimental design requires the protoplasts not be exposed to light, the enzymatic digestion and wash steps can be performed with the suspensions wrapped in aluminum foil to ensure darkness.
7. Pour slowly to prevent overspilling and tap the strainer gently if clogging occurs.
8. Cells should be visible as a pellet at the bottom of the tube. A brown precipitate of protoplasting enzymes may be present but will be removed through additional washes.

9. A dilution of the sample is done before loading onto the hemocytometer to make cell counting more efficient and accurate. If the cell concentration is too high or too low, further or no dilution may be required, respectively.
10. Surface tension may prevent the small volume of cell suspension from passing through the cell strainer. In order to overcome this, place the strainer over the opening of a 1.5 mL microcentrifuge tube, add the cell suspension, and use a brief swinging arm movement to pull the solution through the filter. Alternatively, low-speed centrifugation <50 × *g* can be used.
11. Fluorescein diacetate (FDA) is nonfluorescent but when taken up by cells is converted into fluorescent metabolites by intracellular esterases. The metabolites are hydrophilic rendering them unable to pass through intact cell membranes, resulting in the staining of viable cells.

Acknowledgments

This work was funded by NSF grants IOS-2016021 and IOS-1238142 (to M.J.S.), as well as a Schmittau-Novak small grant (to J.W.S.)

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Chapter 10

Protoplast Isolation and Transfection in Wheat

Guangbin Luo, Boshu Li, and Caixia Gao

Abstract

Wheat is one of the major staple crops around the world. A transient expression system is crucial for gene functional studies in wheat as stable transfection is still difficult in most cultivars. Protoplasts could serve as a versatile transient expression tool in wheat research. Here, we describe protocols for wheat protoplast isolation and transfection that are enabled by cellulase R-10 and macerozyme R-10 containing enzymatic solution and polyethylene glycol-mediated method, respectively. In addition, we show an example of efficiency evaluation of the emerging base editors in wheat protoplasts. These protocols are of wide use in both conventional gene functional analysis and reagent functionality evaluation of genome editing in wheat.

Key words Base editor, Base-editing efficiency, Genome editing, Polyethylene glycol (PEG), *Triticum aestivum*

1 Introduction

Wheat ranks third among staple foods of global importance in annual production [1]. The global wheat production is estimated to 761.5 million tons in 2020 (http://www.fao.org/worldfoodsituation/csdb/en/). Despite the great contribution to human diet and health, stable transformation in major wheat cultivars is still difficult, which hampers the genetic studies in wheat. In addition to conventional breeding, genetic engineering, especially genome editing, is gaining popularity in wheat improvement to feed the ever-fast-growing world population with gradually decreasing arable land. Evaluation of reagents in vivo before the stable transformation is the prerequisite of successful genome editing in wheat. For example, the efficiencies of single guide RNAs (sgRNAs) have to be tested in vivo before delivery into a plant in clustered regularly interspaced short palindromic repeats (CRISPR)

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Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_10,

gene editing [2]. Therefore, the establishment of a transient expression system is conducive to wheat research and improvement.

Plant protoplasts are intact and viable cells with the cellulase wall removed. Without the protection of cell wall, macromolecules, such as DNA, RNA, and proteins, could be efficiently introduced into protoplasts using numerous methods including microinjection, electroporation, and PEG-mediated transfection. As delivered reagents could transiently be expressed or function in an intracellular environment, protoplasts can serve as a multipurpose in vivo experimental platform in plant research. Since the first successful isolation of protoplasts from tomato in 1960, protoplasts have been widely used in gene functional studies (e.g., promoter activity analysis, protein subcellular localization, and protein-protein interaction), single-cell DNA or RNA sequencing, synthetic biosynthesis, and in vivo testing of components in genome editing. To date, isolation and transfection systems have been established in diverse model plant and agronomically important crop species, such as *Arabidopsis* [3], tobacco [4], barley [5], *Brachypodium* [6], grapevine [7], Ma bamboo [8], maize [9], *Panicum virgatum* L. [10], perennial ryegrass [11], pineapple [12], rice [13], soybean [14], and sugarcane [15].

In wheat genome editing, protoplasts isolated from leaf tissue are extensively used to evaluate the cleavage efficiency of sgRNAs in vivo. Shan et al. isolated protoplasts from young wheat seedling leaves using cellulase R-10 and macerozyme R-10 containing enzymatic solution [2]. And they subsequently co-delivered Cas9 and sgRNA expression cassette containing plasmids into protoplasts through PEG-mediated transfection to test the cleavage efficiency of sgRNAs. Similarly, Wang et al. [16] and Cui et al. [17] validated the functionality of CRISPR/Cas9 components in wheat protoplasts through the same cellulase R-10- and macerozyme R-10-mediated isolation and PEG-mediated transfection methods with limited modifications. Using the isolation and transfection methods of Shan et al. [2], Liang et al. [18, 19] transformed CRISPR/Cas9 ribonucleoproteins (RNPs) into wheat protoplasts to estimate the efficiency of RNP complexes. Brandt et al. [20] also introduced RNP complexes into wheat protoplasts via a streamlined protoplast isolation and transfection protocol. In this protocol, the viable protoplasts were selected using a sucrose gradient, while the protoplasts were still isolated using the cellulase R-10- and macerozyme R-10-mediated enzymatic digestion and subsequently transformed through the PEG-mediated method. Using the same isolation and transfection methods of Shan et al. [2], Zong et al. [21] and Li et al. [22] estimated the substitution efficiencies of base editors in wheat protoplasts. Collectively, the cellulase R-10- and macerozyme R-10-mediated enzymatic digestion and PEG-mediated delivery are the dominant isolation and transfection methods of wheat protoplast, respectively.

In this chapter, we present a protocol of protoplast isolation and transfection in wheat. This protocol comprises planting of young wheat seedlings, isolation of protoplasts using cellulase R-10- and macerozyme R-10-mediated enzymatic digestion, PEG-mediated transfection of protoplasts with a green fluorescent protein (GFP) expression cassette containing plasmid, and the evaluation of the transfection efficiency. Using the protocols described here, we can achieve a protoplast yield from each seedling with 5×10^5 cells, which is enough for one plasmid sample transfection. The transfection efficiency is typically >60%. Moreover, we also show an example of efficiency evaluation of the emerging base editors in wheat protoplasts. This protoplast isolation and transfection protocol has excellent performance not only in conventional gene functional analyses but also in the reagent functionality evaluation of genome editing in wheat.

2 Materials

2.1 Plasmids (See Note 1)

1. pJIT163-Ubi-GFP (for transfection): A plasmid in which GFP is driven by the wheat ubiquitin promoter [2] is used as a positive control in this work.
2. pnCas9-PBE (for base editing, Addgene No. #98164): This plasmid contains rat cytidine deaminase APOBEC1, a Cas9 variant (Cas9-D10A nickase, nCas9), and uracil glycosylase inhibitor (UGI) [21]. The codons of the above three proteins have been optimized for cereal plants, and this fusion protein is driven by the maize *Ubiquitin-1* (Ubi-1) gene promoter.
3. pUbi-BFPm (for base editing) [21]: This plasmid comprises the expression cassette of mutant blue fluorescent protein (BFPm) driven by the maize Ubi-1 promoter. The mutation at codon 66 from CAC (histidine) to TAC (tyrosine) can convert BFPm to GFP.
4. pTaU6-BFP-sgRNA (for base editing) [21]: This is the expression plasmid of the sgRNA which is designed to induce the mutation of CAC to TAC at codon 66 in the coding sequence of BFP. TaU6 is the wheat U6 promoter.

2.2 Bacterial Strain and Plant Material

1. Cloning competent *Escherichia coli* (*E. coli*) cell strains, such as 5-alpha and 10-beta competent *E. coli*, for the propagation of pJIT163-Ubi-GFP, pnCas9-PBE, pUbi-BFPm, and pTaU6-BFP-sgRNA (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences).
2. Common wheat cultivar Kenong 199 (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences).

2.3 Stock Solutions and Chemicals

1. Mannitol stock (0.8 M): Add 14.57 g mannitol to 80 mL Milli-Q water. Dissolve the chemical completely. Add additional Milli-Q water to bring to 100 mL before filter sterilization (FS) using a 0.22 μm filter into a sterile container. Store at room temperature (RT, 22–25 °C) for up to 1 month.
2. MES stock (0.2 M, pH 5.7): Add 4.26 g 2-(N-morpholino) ethanesulfonic acid (MES) to 80 mL Milli-Q water. Dissolve the chemical completely. Adjust the pH to 5.7 using 1 M KOH. Add additional Milli-Q water to bring to 100 mL. FS using a 0.22 μm filter into a sterile container. Store at RT for up to 1 month.
3. $CaCl_2$ stock (1 M): Add 14.70 g $CaCl_2{\cdot}2H_2O$ to 80 mL Milli-Q water. Dissolve the chemical completely. Add additional Milli-Q water to bring to 100 mL before FS using a 0.22 μm filter into a sterile container. Store at RT for up to 1 month.
4. $MgCl_2$ stock (1 M): Add 20.33 g $MgCl_2$ to 80 mL Milli-Q water. Dissolve the chemical completely. Add additional Milli-Q water to bring to 100 mL before FS using a 0.22 μm filter into a sterile container. Store at RT for up to 1 month.
5. Bovine serum albumin (BSA).
6. Cellulase R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan).
7. Macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan).
8. Polyethylene glycol 4000 (PEG 4000).

2.4 Working Solutions

1. Mannitol solution (0.6 M): Add 30 mL 0.8 M mannitol stock to a sterile container. Add additional sterilized Milli-Q water to bring to 40 mL.
2. Cell-wall-dissolving enzyme solution: Add 0.75 g cellulase R-10, 0.38 g macerozyme R-10, 5.46 g mannitol, and 0.11 g MES to 30 mL Milli-Q water. Add additional Milli-Q water to bring to 50 mL. Warm the solution in a 55°C water bath for 10 min to dissolve the chemicals completely. After the solution cools down to RT, adjust the pH to 5.7 using 1 M KOH. Add 0.07 g $CaCl_2{\cdot}2H_2O$ and 0.05 g BSA. Dissolve the chemicals completely before FS using a 0.22 μm filter into a sterile container (*see* **Note 2**).
3. W5 solution: Add 4.50 g NaCl, 9.19 g $CaCl_2{\cdot}2H_2O$, 0.19 g KCl, and 0.21 g MES to 400 mL Milli-Q water. Dissolve the chemicals completely. Adjust the pH to 5.7 using 1 M NaOH. Add additional Milli-Q water to bring to 500 mL. FS using a 0.22 μm filter into a sterile container.
4. MMG solution: Pipette 5 mL 0.8 M mannitol stock, 0.15 mL 1 M $MgCl_2$ stock, and 0.2 mL 0.2 M MES stock to a sterile

container. Add additional Milli-Q water to bring to 10 mL before FS using a 0.22 μm filter into a sterile container (*see* **Note 3**).

5. PEG solution: Pipette 1 mL 0.8 M mannitol stock, 0.4 mL 1 M $CaCl_2$ stock to a sterile container. Add 1.6 g PEG 4000. Add additional Milli-Q water to bring to 4 mL before FS using a 0.22 μm filter into a sterile container (*see* **Note 4**).
6. TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.5 Supplies and Equipment

1. 6-in. pot.
2. Micro-dissecting scissors.
3. Single-edge razor blade.
4. Sterile plastic Petri dishes (100 mm × 15 mm).
5. Sterile 40 μm nylon mesh.
6. Sterilized conical flask.
7. Aluminum foil.
8. Vacuum desiccator.
9. Orbital shaker.
10. 0.22 μm syringe filter.
11. 50 mL round-bottom centrifuge tube.
12. 2 mL round-bottom Eppendorf Safe-Lock microcentrifuge tubes.
13. Centrifuge with a swinging bucket rotor.
14. 6-well cell culture plate.
15. Stereo fluorescence microscope.
16. Flow cytometry.
17. QIAGEN Plasmid Midi Kit (Cat. No./ID: 12143).
18. NanoDrop One Spectrophotometer.

3 Methods

3.1 Growth of Wheat Seedlings

1. Seeds of Kenong 199 are sown in a 6-in. pot filled with peat moss (Fig. 1a). The pot is maintained in a greenhouse at 25 °C under 16 h per day of 13.5 μmol/m^2/s light. Seedlings reaching the length of approx. 12 cm are ready for protoplast isolation (*see* **Notes 5**–**7**). This process may take about 10–12 days.

3.2 Isolation of Wheat Leaf Protoplasts

1. Cut leaves at 1 cm from the bottom using a pair of micro-dissecting scissors. Collect 20 cut leaves in a bundle.
2. Crosscut the leaf bundle into 0.5–1.0 mm strips using a single-edge razor blade.

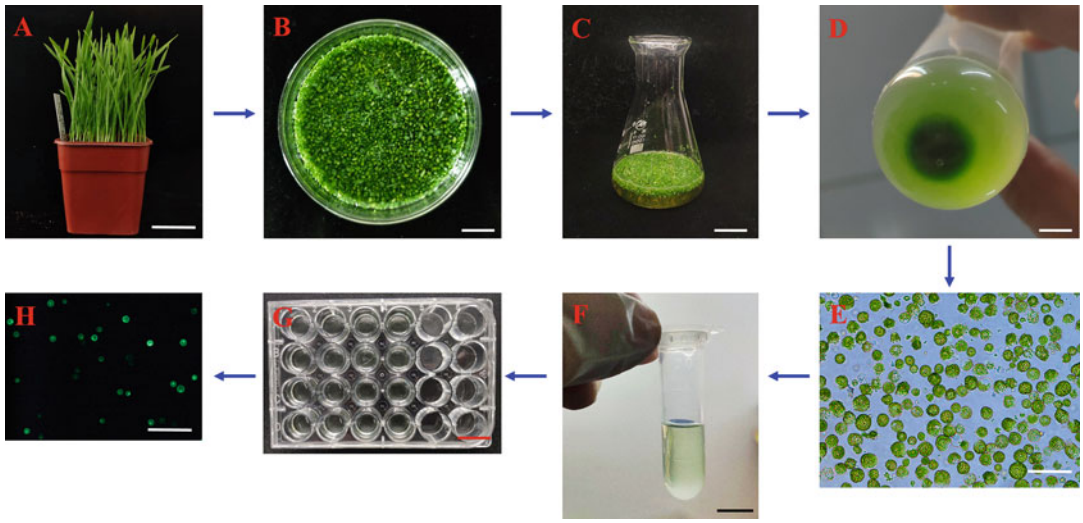

Fig. 1 The procedure of wheat protoplast isolation and transfection. (**a**) Wheat seedlings with a length of approx. 12 cm. Bar = 5 cm. (**b**) Leaf strips in the mannitol solution. Bar = 1.5 cm. (**c**) Leaf strips in the cell-wall-dissolving enzyme solution. Bar = 2 cm. (**d**) Protoplast cells settled to the bottom of the centrifuge tube by gravity. Bar = 0.2 cm. (**e**) The condition and concentration of protoplast cells are checked under a microscope (100×). Bar = 300 μm. (**f**) Protoplast cells in the PEG solution. Bar = 0.5 cm. (**g**) Incubation of transformed protoplast cells in a 24-well plate. Bar = 2 cm. (**h**) The transfection efficiency is estimated by counting the number of cells with GFP fluorescence. All the cells shown in this picture are GFP cells. Bar = 1 mm

3. Move the strips of 40 leaves to a 100 mm × 15 mm Petri dish with 30 mL of mannitol solution (Fig. 1b). Make sure all strips are immersed in the solution completely. Incubate the mixture in the dark at RT for 10 min (*see* **Note 8**).
4. Collect the leaf strips with a sterile 40 μm nylon mesh and gently extrude the residual liquid to prevent changes in osmotic pressure of the cell-wall-dissolving enzyme solution.
5. Transfer the collected leaf strips to a sterilized conical flask containing 50 mL cell-wall-dissolving enzyme solution (Fig. 1c).
6. Wrap the flask with aluminum foil to keep it away from light. Place it in a vacuum desiccator with a full vacuum of approx. 450 mmHg for 30 min (*see* **Note 9**).
7. After vacuum treatment, place the foil-wrapped flask on an orbital shaker (30–50 rpm) for 5–6 h at RT (*see* **Note 10**).
8. Add 30 mL of W5 solution to the flask after enzymatic digestion.
9. Gently tap the flask by fingers for 10 s to help the release of protoplast cells.

10. Filter the mixture through a 40 μm nylon mesh (*see* **Note 11**) into 3–4 50 mL round-bottom centrifuge tubes (*see* **Note 12**).
11. Wash the strips stopped on the nylon mesh one to two times with 20 mL of W5 solution to release more protoplasts and increase the yield.
12. The protoplast suspension in tubes is centrifuged in a swinging bucket rotor at 80 × *g* at RT for 3 min (*see* **Note 13**).
13. Remove the supernatant as much as possible without disrupting the pellet using a pipette.
14. Resuspend the protoplast pellet using 10 mL of W5 solution. Incubate the suspension on ice for 30 min. Intact protoplast cells would settle to the bottom of the centrifuge tube by gravity after this process (Fig. 1d).
15. Remove the supernatant as much as possible without disrupting the pellet using a pipette.
16. Gently resuspend the protoplast pellet with 5 mL of chilled MMG solution (*see* **Note 14**).
17. Check the integrity and concentration of protoplast cells in MMG solution under a microscope (100×) (Fig. 1e). Adjust the concentration to a final value of 2.5×10^6 cells per mL using MMG solution (*see* **Note 15**).

3.3 Example 1: Transfection of GFP Plasmid DNA

1. In a sterilized 2 mL round-bottom microcentrifuge tube, add 10 μg of pJIT163-Ubi-GFP plasmid DNA (*see* **Note 16**).
2. Carefully pipette 200 μL of protoplasts (5×10^5 cells in total) into the tube. Gently tap the bottom of the tube to mix protoplasts and plasmids thoroughly.
3. Slowly add 250 μL of PEG solution to the plasmid and protoplast mixture. Invert the tube gently to mix the solutions thoroughly (Fig. 1f), and then incubate the tube in the dark at RT for 30 min (*see* **Note 17**).
4. Slowly add 880 μL of W5 solution into the tube to stop the PEG-mediated transfection. Invert the tube gently to mix the solutions thoroughly.
5. Centrifuge the mixture in a swinging bucket rotor at 80 × *g* at RT for 3 min (*see* **Note 13**).
6. Remove the supernatant as much as possible without disrupting the pellet using a pipette.
7. Resuspend the protoplast pellet with 1 mL W5 solution by gently inverting the tube.
8. Transfer the protoplast suspension to a single well of the 6-well plate (Fig. 1g) (*see* **Note 18**).

9. Wrap the plate with aluminum foil and incubate it in the dark at RT.
10. After at least 16 h of incubation, the GFP fluorescence can be detected under a fluorescent microscope (Fig. 1h; *see* **Note 19**). The transfection efficiency is calculated by the percentage of protoplast cells with GFP fluorescence in total intact protoplasts (*see* **Note 20**).
11. This protocol can also be used to evaluate CRISPR reagents, such as sgRNAs and RNPs, for wheat genome editing. Details can be found in our previously published protocols [2, 18, 19].

3.4 Example 2: Efficiency Evaluation of Base Editors

1. Perform co-transfection of plasmid DNA (10 μg per construct) of the base-editing vectors pnCas9-PBE, pUbi-BFPm, and pTaU6-BFP-sgRNA (Fig. 2a, b) by following **steps 1–6** described in Subheading 3.3.
2. Resuspend the transfected protoplast pellet with 1 mL W5 solution in a sterile 2 mL round-bottom microcentrifuge tube.
3. Place the tube horizontally and then incubate in the dark at RT for 48–60 h, preferably 48 h.
4. GFP-positive cells are sorted using flow cytometry. The base-editing efficiency is calculated by the percentage of protoplast cells with GFP fluorescence in total intact protoplasts (Fig. 2c).
5. This protocol can also be used to evaluate the base-editing efficiencies of other target genes in wheat. Details can be found in our previous work [21].

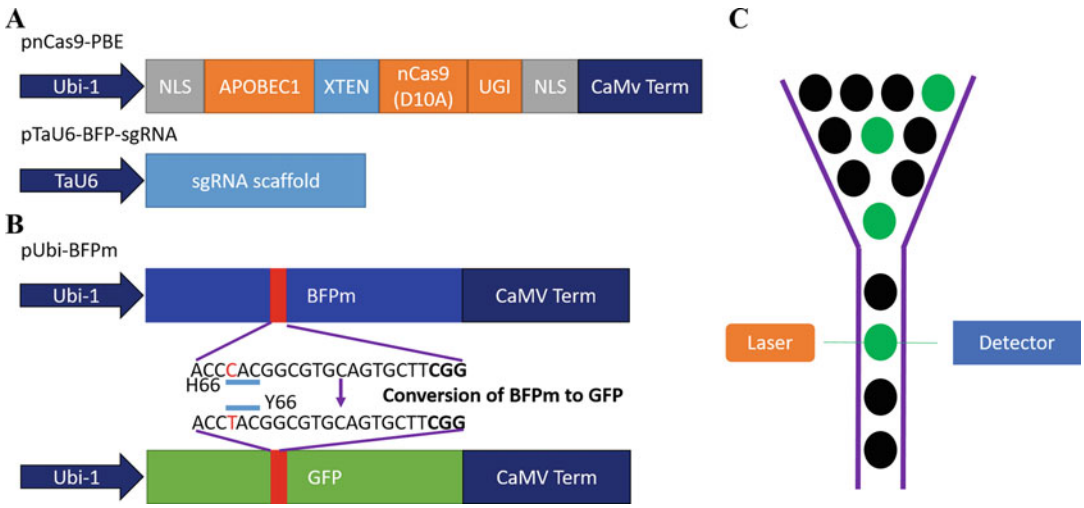

Fig. 2 Efficiency evaluation of base editors. (**a**) Representation of the base-editing vectors pnCas9-PBE and pTaU6-BFP-sgRNA. NLS, nuclear localization signal. XTEN is a short linker. (**b**) The molecular mechanism of BFPm-to-GFP reporter system. The mutation at codon 66 from CAC (H66) to TAC (Y66) can convert BFPm to GFP. CGG in bold type is the PAM site. (**c**) Cell sorting using flow cytometry. Green and black balls indicate GFP cells and non-GFP cells, respectively

4 Notes

1. Plasmid DNAs are extracted and purified using the QIAGEN Plasmid Midi Kit (Cat. No./ID: 12143). The plasmid DNA is dissolved in TE buffer, and the final concentration should be at least 1 μg/μL. These vectors are available from the corresponding author upon request.
2. Cellulases and macerozymes from different manufacturers have incompatible activities. The cellulase R-10 and macerozyme R-10 from Yakult Pharmaceutical Ind. are the best among the enzymes we tried. And the cell-wall-dissolving enzyme solution should be prepared on the same day of protoplast isolation.
3. The MMG solution does not need pH adjusted as the MES is used as a running buffer here. The MMG solution should be prepared on the same day of the transfection.
4. The PEG solution should be prepared on the same day of the transfection.
5. Generally, leaves of one to two seedlings are enough to produce protoplast cells (5×10^5) for a single transfection reaction.
6. Common wheat cultivars with thicker leaves have a higher yield of protoplast cells than the cultivars with thin leaves.
7. To extend the production period for protoplasting, one can consider placing young seedlings in low temperatures (4 °C under 16 h per day of 13.5 μmol/m^2/s light) to slow down the growth. A high yield of protoplast cells could be obtained from seedlings growing in low temperatures for less than 1 month according to our experience, preferably less than 10 days.
8. The mannitol treatment enables quick plasmolysis.
9. The vacuum could help the enzyme solution infiltrate into the strips. Vacuum infiltration is crucial to the high yield of protoplast cells.
10. An orbital shaker helps to prevent cell rupture.
11. The pore size of nylon mesh, which is 40 μm, is essential to let intact protoplast cells pass and stop other particles.
12. To avoid the disruption of protoplast cells, round-bottom centrifuge tubes and pipette tips with blunt ends are preferred in both isolation and transfection of protoplasts. And the swinging bucket rotor could also increase the ratio of intact cells.
13. Set both acceleration and deceleration rates at 3 to avoid the breaking of protoplast cells.

14. It is better to chill the MMG solution in advance to eliminate the heat shock to protoplast cells settled in the W5 solution on ice.
15. Before transfection, a quality check, including the integrity and concentration of protoplast cells, is necessary. The protoplast concentration could be estimated with a hemocytometer or flow cytometry.
16. In the transfection of a single plasmid, at least 10 μg plasmid is needed. In the co-transfection with multiple plasmids, an equal quantity (10 μg) of each plasmid is also preferred despite the different plasmid sizes.
17. The incubation time during the PEG-mediated transfection should be no more than 30 min to avoid the high disruption of protoplast cells.
18. 12-well or 24-well plates can also be used for larger-scale experiments.
19. GFP fluorescence could be observed after incubation in the dark for 16 h. However, for the in vivo testing of sgRNAs, mutations could only be detected after no less than 48 h of incubation. Most transformed cells will break after 96 h of incubation.
20. Always include a positive control plasmid in any transfection experiment for quality control of protoplast isolation and transfection experiments. In our case, a typical successful transfection experiment using pJIT163-Ubi-GFP plasmid DNA should give 60–80% of transfection efficiency.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31788103) and Novo Nordisk Foundation (NovoCrops; project #2019OC53580).

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Chapter 11

Protoplast Isolation, Transformation, and Regeneration for Forage and Turf Grasses

Zeng-Yu Wang and German Spangenberg

Abstract

Forage and turf grasses are widely grown and contribute significantly to sustainable agriculture. This chapter describes a protocol for protoplast transformation and plant regeneration for major forage and turf grass species, including tall fescue, red fescue, meadow fescue, perennial ryegrass, and Italian ryegrass. Embryogenic calli induced from caryopsis were used to establish embryogenic cell suspension cultures. Protoplasts were isolated from embryogenic suspension cultures and used for direct gene transfer. Chimeric genes were introduced into protoplasts by polyethylene glycol treatment. Upon selection with antibiotics or herbicide, resistant calli were obtained and transgenic plants were regenerated from these calli.

Key words Direct gene transfer, Embryogenic suspension cultures, Forage and turf grasses, Genetic transformation, Transgenic plants

1 Introduction

Forage and turf grasses occupy vast acreage on the earth and are of immeasurable value for livestock, wildlife, soil and water conservation, and sport and recreational activities. A large number of grass species are used for forage or turf purposes. The most widely grown species include fescues (*Festuca* spp.), ryegrasses (*Lolium* spp.), bluegrasses (*Poa* spp.), orchardgrass (*Dactylis* spp.), bromegrasses (*Bromus* spp.), bentgrasses (*Agrostis* spp.), bermudagrass (*Cynodon* spp.), switchgrass (*Panicum* spp.), and dallisgrass (*Paspalum* ssp.). Some species, such as tall fescue and perennial ryegrass, can be used either for forage or for turf purpose, depending on how the cultivars were selected.

Tremendous progress has been made in cell culture and genetic transformation of forage and turf grasses in the last three decades. Different genetic modification methods have been established and various agronomical genes have been introduced into these species

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_11,

[1–4]. The first transgenic grass plants were obtained by direct gene transfer to protoplasts [5].

For these monocotyledonous species, totipotent protoplasts are isolated from embryogenic cell suspension cultures. The establishment of morphogenic cell suspensions has proven to be an important prerequisite for the development of a protoplast-based plant regeneration system [6, 7]. Once such a regeneration system is established, cell suspension-derived protoplasts can be used for the generation of transgenic plants and somatic hybrids [1].

The protocol outlined in this chapter is based on our work on tall fescue (*Festuca arundinacea* Schreb., syn *Lolium arundinaceum*), meadow fescue (*F. pratensis* Huds.), red fescue (*F. rubra* L.), perennial ryegrass (*Lolium perenne* L.), and Italian ryegrass (*Lolium multiflorum* Lam.) [1, 5–10]. Mature seeds are used as explants to induce embryogenic calli. Morphogenic cell suspension cultures can be initiated by agitating embryogenic calli in liquid medium. Well-established suspension cultures are composed mostly of proembryogenic cell clusters and groups of small, meristematic cells that contain starch and are rich in cytoplasm [11]. Protoplasts isolated from suspension cultures are treated with plasmid DNA and polyethylene glycol (PEG). Antibiotic or herbicide resistant calli can be obtained after selection. Transgenic plants can be recovered from these calli and grown to maturity. Using this method, one can typically obtain $2–5 \times 10^5$ protoplasts from 1 g of fresh weight suspension cells. Overall transformation frequencies are in the range of $0.5–2 \times 10^{-6}$, that is, one transgenic plant per one million transfected protoplasts.

2 Materials

2.1 Plant Material and DNA Construct

1. Seeds (*see* **Note 1**): Cultivars of tall fescue (cvs. Jesup, Kentucky 31), meadow fescue (cvs. Barmondo, Belimo), red fescue (cvs. Roland, Gondolin), perennial ryegrass (cvs. Citadel, Kelvin), and Italian ryegrass (cvs. Andy, Axis) (*see* **Note 2**).
2. Plasmid DNAs: Plasmid pAB1, bearing the phosphinothricin acetyltransferase gene (*bar*) coding sequence under control of the rice actin 1 promoter, is used for phosphinothricin (PPT) selection [8]. Plasmid pGL2, bearing the hygromycin phosphotransferase gene (*hph*) coding sequence under control of the CaMV35S promoter, is used for hygromycin selection [12]. Plasmid DNA is isolated using a modified cetyltriethylammoniumbromide (CTAB) extraction method [13]. Plasmid DNA is dissolved in water and stored at −20 °C.

2.2 Stock Solutions and Chemicals (See Note 3)

1. Calcium hypochlorite: Prepare fresh 3% (w/v) calcium hypochlorite solution in a glass bottle.
2. Low-melting temperature SeaPlaque agarose (FMC BioProducts, Rockland, ME 04841, USA): Used as gelling agent for protoplast bead-type culture (*see* **Note 4**).
3. Cellulase "Onozuka" R-10 and Macerozyme® R-10: Yakult Pharmaceutical Ind. Co., Ltd., 1–1-19 Higashi Shinbashi Minato-ku Tokyo, 105, Japan (*see* **Note 5**).
4. Pectolyase Y-23: Kikkoman Corporation, 4-13 Koamicho, Nihonbashi, Chuo-ku, Tokyo, 103, Japan (*see* **Note 5**).
5. Calf thymus DNA: Dissolve 10 mg calf thymus DNA (used as carrier DNA) in 5 mL distilled water, and shear the DNA by passage through an 18-G needle to an average size of 5–10 kb.

2.3 Culture Media and Working Solutions (See Note 3)

The composition of the media used at the final concentrations of their individual ingredients is given in Table 1.

1. Suspension culture medium AAF and protoplast culture medium AP2: Modified AA media [14] plus chemicals listed in Table 1.
2. Medium M1, M5, and MSK: Modified Murashige and Skoog (MS) media [15] plus chemicals listed in Table 1.
3. Washing solution WF: 10 mM $CaCl_2$ and 0.6 M mannitol. Adjust pH 5.8 with KOH and autoclave. Store at room temperature.
4. Enzyme solution: 3% (w/v) Cellulase "Onozuka" RS, 1% (w/v) Macerozyme R-10, and 0.1% (w/v) Pectolyase Y-23 dissolved washing solution WF. Spin down to pellet contaminating starch of the enzyme preparations. Adjust pH 5.8 with KOH and filter-sterilize (0.2 μm pore size). Store at 4 °C for no longer than 4 weeks.
5. PEG solution: Dissolve polyethylene glycol (PEG) 6000 in 0.4 M mannitol and 0.1 M $Ca(NO_3)$, adjust pH to 8–9, and autoclave (*see* **Note 6**).

2.4 Supplies and Equipment

1. Sterile filter paper (7 cm diameter).
2. Sterile 6.6 cm diameter (190 mL) plastic culture vessels.
3. Sterile 5.0 cm diameter (175 mL) plastic culture vessels.
4. 6-well culture dishes.
5. Sterile round-bottomed plastic centrifuge tubes with screw cap.
6. Sterile disposable pipettes: 1 mL, 2 mL, and 10 mL.
7. Sterile plastic Petri dishes (diameter 9 cm).
8. Sterile plastic Petri dishes (diameter 3 cm).
9. 100 mL glass beaker.

Table 1
Composition of the media used. From [10] with permission

Media component (Media)	AAF	AP2	MI	M5	MSK
Macroelements (mg/1 final concentration)					
KNO_3			1900	1900	1900
NH_4NO_3			1650	1650	1650
$CaCl_2 \times 2H_2O$	440	440	440	440	440
$MgSO_4$	370	370	370	370	370
KH_2PO_4	170	170	170	170	170
KCl	2950	2950			
Microelements (mg/1 final concentration)					
Na_2EDTA	37.3	37.3	37.3	37.3	37.3
$FeSO_4 \times 7H_2O$	27.8	27.8	27.8	27.8	27.8
H_3BO_3	6.2	6.2	6.2	6.2	6.2
Kl	0.83	0.83	0.83	0.83	0.83
$MnSO_4 \times H_2O$	16.9	16.9	16.9	16.9	16.9
$ZnSO_4 \times 7H_2O$	8.6	8.6	8.6	8.6	8.6
$CuSO_4 \times 5H_2O$	0.025	0.025	0.025	0.025	0.025
$Na_2MoO_4 \times 2H_2O$	0.25	0.25	0.25	0.25	0.25
$CoCl_2 \times 6H_2O$	0.025	0.025	0.025	0.025	0.025
Carbohydrates (g/1 final concentration)					
D(+) Sucrose	20		30	30	30
D(+) Glucose $\times H_2O$		108			
D-Sorbitol	20				
Hormones (mg/1 final concentration)					
2,4-D Kinetin	1.5	1.2	1.5	5.0	0.2
Vitamins (mg/1 final concentration)					
Pyridoxine HCl	1.0	1.0	0.5	0.5	0.5
Thiamine HCl	10.0	10.0	0.1	0.1	0.1
Nicotinic acid	1.0	1.0	0.5	0.5	0.5
Inositol	100	100	100	100	100
Other organics (mg/1 final concentration)					
Glycine	7.5	7.5	2	2	2
L-Glutamine	877	877			
L-Asparagine	266	266			
L-Arginine	174	174			
Casein hydrolysate			400	400	

10. Forceps, scalpel, and blades.
11. Drummond Pipet-Aid and sterile disposable pipettes.
12. Magnetic stirrer and stir bars.
13. Water bath.
14. Rotary shaker.
15. A swing rotor centrifuge or a more specific bench centrifuge with swing-out rotor.
16. Hemocytometer.
17. 280 μm, 100 μm, and 50 μm stainless steel mesh sieves.
18. Spectrophotometer.
19. Stereo microscope.
20. Metro Mix 350 soil (Sun Gro Horticulture, Terrell, TX).

3 Methods

3.1 Sterilization and Callus Induction

1. Immerse seeds in 3% calcium hypochlorite solution in a glass bottle. Put a magnetic stir bar in the bottle and place the bottle on a magnetic stirrer. Agitate for 2 h (*see* **Note 7**).
2. Rinse the seeds three times with sterile dH_2O and leave the seeds in water overnight at 4 °C.
3. Sterilize the seeds again for 30 min in 3% calcium hypochlorite solution the next day, and rinse the seeds three times in sterile dH_2O (*see* **Note 8**).
4. Place about 20 caryopses/seeds per 9 cm culture dish containing M5 medium.
5. Keep dishes, sealed with Parafilm, in the dark at 25 °C for 4–6 weeks; calli are induced.

3.2 Establishment of Embryogenic Cell Suspensions

1. Subculture embryogenic friable yellowish calli, developed from mature seed-derived embryos (and thus representing single genotypes), onto new dishes containing M1 medium. Keep dishes in the dark at 25 °C.
2. Select and transfer single genotype-derived friable, yellowish, embryogenic callus (Fig. 1a) separately into individual wells of 6-well culture dishes containing 3 mL liquid AAF medium/well.
3. Place the 6-well culture dishes at 25 °C in the dark on a rotary shaker at 60 rpm.
4. Subculture weekly by replacing 2/3 of the culture medium with fresh ones for 4 weeks.
5. Transfer single genotype-derived suspension cultures individually into 6 cm culture vessels containing 12 mL AAF medium.

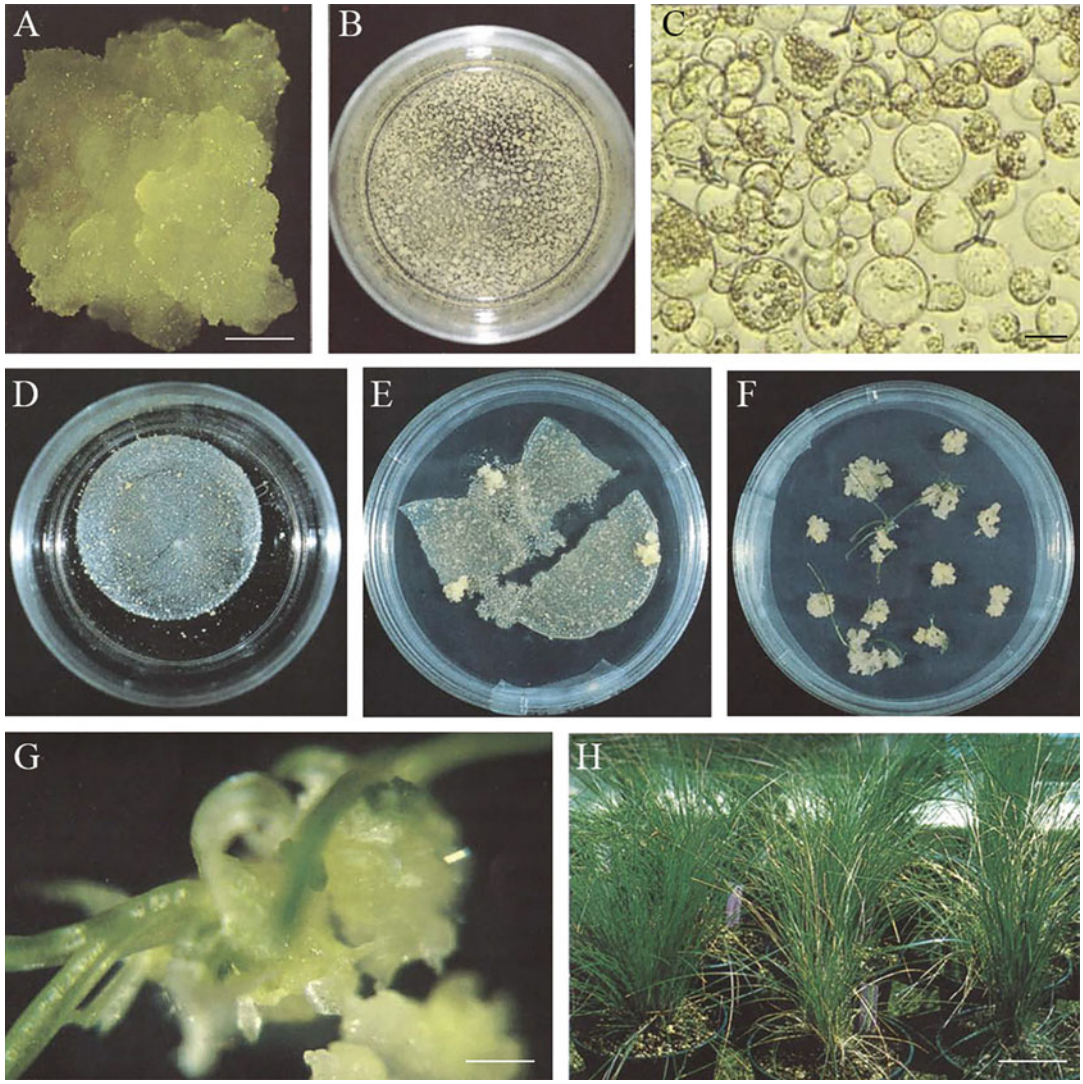

Fig. 1 Protoplast isolation and generation of transgenic plants of red fescue by direct gene transfer to protoplasts. (**a**) Two-month-old mature seed-derived embryogenic callus used for initiation of cell suspensions; scale bar = 0.2 cm. (**b**) Single genotype-derived embryogenic cell suspension used for protoplast isolation. (**c**) Freshly isolated protoplasts from morphogenic cell suspensions; scale bar = 10 μm. (**d**) Bead-type culture of red fescue protoplasts 4 weeks after transformation with the bar gene in 50 mg/L PPT-containing medium. (**e**) Agarose bead with growing PPT resistant colonies transferred onto proliferation medium containing 50 mg/L PPT, 8 weeks after protoplast plating. (**f**) Regeneration of shoots from PPT resistant callus clones of red fescue, 3 months after direct gene transfer to protoplasts. (**g**) Close view of shoots regenerated from resistant callus; scale bar = 0.2 cm. (**h**) Transgenic mature plants potted in soil and growing under greenhouse conditions; scale bar = 8 cm. (Reproduced from ref. 8)

6. Keep suspension cultures under the same culture conditions and subculture them (by replacing 2/3 of the medium with fresh AAF medium) every 7–10 days.
7. Eight weeks later, embryogenic suspensions can be established (Fig. 1b) (*see* **Note 9**).

3.3 Protoplast Isolation

1. Protoplasts can be obtained from growing cell suspension cultures 2–6 months after their initiation. Resuspend 2–4 mL settled volume of these suspension cells, 3–4 days after subculture of the suspension culture, in 12–14 mL enzyme solution.
2. Incubate the protoplast isolation mixture in the dark at 25 °C on a gyratory shaker at 40 rpm for 4 h.
3. After this incubation period, gently agitate the digest and pour through a 280 μm stainless steel mesh sieve placed on a 100 mL sterile glass beaker.
4. Rinse the sieve with 1–2 mL washing solution WF to release more protoplasts.
5. Sequentially pour the filtrate through a 100 μm and a 50 μm stainless steel mesh sieve placed on corresponding 100 mL glass beakers.
6. After each filtering step, rinse the sieve with 1–2 mL washing solution WF and collect these in the glass beaker.
7. Transfer the pooled filtrate into 14 mL round-bottomed sterile plastic centrifuge tubes with screw cap (*see* **Note 10**).
8. Pellet the protoplasts by centrifugation at 70 × *g* for 5 min.
9. Carefully remove the supernatant and resuspend the protoplast pellet in 8–10 mL washing solution WF by gently tilting the capped tube (Fig. 1c).

3.4 Protoplast Transformation

1. Pellet the protoplasts by centrifugation at 70 × *g* for 5 min.
2. Gently resuspend the pellet in WF solution at a density of 2–3 × 10^6 protoplasts/mL. Distribute 0.5 mL protoplasts into each 14 mL round-bottomed tube.
3. Add 30 μl DNA solution containing 10 mg plasmid DNA and 40 mg calf thymus DNA as carrier into each tube, and slowly add (dropwise addition) 150 μl PEG 6000.
4. Incubate the protoplast-DNA-PEG mixture at room temperature for 10–15 min with occasional mixing.
5. Add a total of 10 mL of WF to dilute the mixture in a stepwise fashion. Namely, first add 0.5 mL WF twice, then 1 mL WF twice and 2 mL WF twice, and finally add 3 mL WF once. Tilt and swirl the tube gently after each time adding WF.
6. Pellet the protoplasts by centrifugation at 70 × *g* for 5 min. Carefully remove the supernatant.
7. Resuspend the protoplasts in 1.5 mL AP2 medium, and transfer the protoplast suspension into a 3 cm culture dish.
8. Use a 2 mL pipette, and add 1.5 mL pre-warmed (melt in a microwave oven and keep in a water bath at 42 °C) AP2 medium containing 2.4% (w/v) SeaPlaque agarose to the culture dish. Mix gently and allow to set (*see* **Note 11**).

9. When the medium is solidified (after 1 h), seal the dishes with Parafilm and culture the protoplasts for 24 h in darkness at 25 °C.
10. After this first day in culture, use a spatula to transfer the solidified agarose medium with the embedded protoplasts into a 6 cm culture vessel containing 12 mL liquid protoplast culture medium AP2.
11. When nurse culture is used, add to each culture vessel ca. 200 mg (fresh weight) non-morphogenic fast-growing cells from the corresponding species to be used as nurse cells to the AA medium around the agarose beads (*see* **Note 12**).
12. Place the bead-type culture for 7 days on a rotary shaker at 40 rpm in the dark at 25 °C.
13. After this first week of bead-type culture, wash the agarose beads three times with fresh AP2 medium to completely remove the nurse cells.
14. Add 12 mL fresh AP2 medium to the 6 cm culture vessel.
15. Keep the cultures on a rotary shaker at 40 rpm under dim light (about 10 μE/m^2/s, 16 h day/8 h night) at 25 °C.

3.5 Selection of Stable Transformed Colonies and Recovery of Transgenic Plants

1. After another week of culture, add corresponding selective agent to the vessel. For hygromycin selection, a final concentration of 200 mg/L hygromycin is used; for phosphinothricin selection, a final concentration of 50 mg/L phosphinothricin is used (*see* **Note 13**).
2. After 3–4 weeks of culture under selection conditions, the agarose bead containing visible protoplast-derived colonies or individual colonies growing on the agarose beads (Fig. 1d) can be transferred onto M1 medium containing selective agent (200 mg/L hygromycin or 50 mg/L phosphinothricin) in 9 cm sterile culture dishes. Seal culture dishes with Parafilm and incubate them in the dark at 25 °C for further proliferation of protoplast-derived colonies.
3. After 3–4 weeks, place the growing protoplast-derived calli (Fig. 1e) onto medium MSK in 9 cm culture dishes for plant regeneration. Seal culture dishes with Parafilm and keep them under fluorescent light conditions (16/8 h photoperiod; 40 μE/m^2/s) for 4–5 weeks until regenerating calli are obtained (Fig. 1f and g).
4. Transfer the regenerated shoots to 5.0 cm culture vessels or glass jars containing hormone-free half-strength MS medium.
5. Transfer well-rooted plantlets (Fig. 1g) to 3 × 3 in. (7.6 × 7.6 cm) wells in an 18-well flat (6 × 3 wells) filled with Metro Mix 350 soil (*see* **Note 14**).

6. Grow plantlets under greenhouse conditions (390 μE/m^2/s, 16 h day/8 h night at 24 °C/20 °C) (*see* **Note 15**). Plants can be grown on Ebb-Flo® benches and watered once a day with fertilized water containing 50 ppm N (Peters Professional 20-10-20 General Purpose is used as the water-soluble fertilizer).
7. Transfer the established plants to 6 in. (15 cm) pots filled with soil and grow them under greenhouse conditions (390 μE/m^2/s, 16 h day/8 h night at 24 °C/20 °C) (Fig. 1h).
8. Confirm the transgenic nature of regenerated plants by molecular analysis.

4 Notes

1. Endophyte-free seeds should be used. Older seeds contain less viable endophyte, but the seeds should be viable.
2. Transformation efficiency varies with the cultivar used.
3. All solutions and media should be prepared using sterile distilled water (dH_2O). Room temperature refers to temperature ranging between 22 °C and 25 °C.
4. Must use low melting point agarose to embed protoplasts; regular agarose will not work.
5. Enzymes from these vendors work consistently.
6. The pH of PEG will take several hours or overnight to stabilize in this solution and will drop to pH 6–7 after autoclave.
7. If the seeds are dirty, the first sterilization time can be extended to 2.5 h or even 3 h. Make sure all seeds have good contact with the solution.
8. The majority of seed bracts (lemma and palea) surrounding caryopsis become detached after the second sterilization. The surface sterilization procedure will not remove endophyte in the seeds. If the seeds contain endophyte, then the endophyte should be removed by treatment with high humidity and relatively high temperature.
9. For the establishment of embryogenic cell suspensions, the correct choice of single genotype-derived friable yellowish embryogenic callus is crucial.
10. Throughout the protocol, treat protoplast suspensions gently, pipette smoothly, and avoid strong shaking of tubes. For all centrifugation steps, be sure that the speed builds up slowly.
11. After plating of protoplasts in melted agarose-containing culture medium, do not move culture dish until medium is solidified.

12. Use of nurse cells often improves protoplast plating efficiency, although it is optional. Nurse cells are fast-growing cells that have lost their regeneration potential (non-morphogenic). When a fast-growing cell suspension culture lost its regeneration ability, it can be used as a nurse culture.
13. The endogenous resistance of cells to hygromycin or phosphinothricin selection varies with species; it is better to first test the endogenous resistance level of the target species.
14. Before transfer to soil, rinse the roots with water or remove excessive medium with a damp paper towel.
15. It is beneficial to use humidom for acclimation of the plantlets, although not essential.

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Chapter 12

Efficient Protoplast Isolation and DNA Transfection for Winter Oilseed Crops, Pennycress (*Thlaspi arvense*) and Camelina (*Camelina sativa*)

Minjeong Kang, Keunsub Lee, and Kan Wang

Abstract

Pennycress (*Thlaspi arvense*) and camelina (*Camelina sativa*) are nonfood winter oilseed crops that have the potential to contribute to sustainable biofuel production. However, undesired agronomic traits of pennycress and camelina currently hinder broad cultivation of these plants in the field. Recently, genome editing using the CRISPR-Cas technology has been applied to improve poor agronomic traits such as the weedy phenotype of pennycress and the oxidation susceptible lipid profile of camelina. In these works, the CRISPR reagents were introduced into the plants using the *Agrobacterium*-mediated floral dipping method. For accelerated domestication and value improvements of these winter oilseed crops, DNA-free genome editing platform and easy evaluation method of the CRISPR-Cas reagents are highly desirable. Cell wall-free protoplasts are great material to expand the use of gene engineering tools. In this chapter, we present a step-by-step guide to the mesophyll protoplast isolation from in vitro culture-grown pennycress and soil-grown camelina. The protocol also includes procedures for DNA transfection and protoplast viability test using fluorescein diacetate. With this protocol, we can isolate an average of 6×10^6 cells from pennycress and 3×10^6 cells from camelina per gram of fresh leaf tissues. Using a 7.3 kb plasmid DNA carrying green and red fluorescent protein marker genes, we can achieve an average transfection rate of 40% validated by flow cytometry for both plants.

Key words Flow cytometry, Fluorescein diacetate, GFP, Mesophyll protoplast, Protoplast viability test, RFP

1 Introduction

Biofuels are considered as sustainable alternatives to the fossil fuels, but their production from prominent crops such as corn and soybean often conflicts with the increasing demands for food security [1]. Using nonfood oilseed crops, such as pennycress (*Thlaspi arvense*) and camelina (*Camelina sativa*), can avoid such conflicts as they can potentially be used to produce biodiesel that is comparable to the conventional biodiesels produced from vegetable oils

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_12,

[2–4]. Pennycress and camelina are potential winter oilseed crops, which can be cultivated in between common soybean-corn cropping seasons in the US corn belt, typically from the late fall to late spring next year [5, 6].

Pennycress, also known as stinkweed [7], belongs to the Brassicaceae family and is related to other crops such as rape, canola, and camelina. This weedy plant is native to Europe and has been widespread in the temperate North America region [8]. Despite of its weedy phenotype, pennycress is considered as a potential oilseed crop due to its high oil content in the seeds, which is higher than that of soybeans [9]. Although pennycress seeds are not suitable for human consumption due to the high content of toxic erucic acid [10, 11], pennycress oil can meet the industrial needs of biodiesels because of the low degree of saturated fatty acids, which provides better cold temperature properties than other biodiesels from the food crops [2, 12]. In addition, as a cover crop, pennycress can be beneficial in weed controls and soil erosion managements in the Midwest [13]. Because of its quick maturation time and cold tolerance, pennycress can be planted in fall to grow over the wintertime in the Midwest and harvested before the corn seasons, and thus can provide additional economic values to farmers [14, 15]. Pennycress domestication is underway to overcome its weedy phenotypes, such as strong seed dormancy and extensive pod shattering using the genetics, mutagenesis, and transgenesis approaches [16–18].

Camelina is another winter annual plant in the Brassicaceae family, also known as false flax and gold of pleasure [19]. Camelina is from southeastern Europe and southwestern Asia and has been used as animal feed [20]. With its high oil content and several beneficial agronomic traits, such as drought tolerance, cold tolerance, and short maturation time, camelina is now considered as an alternative energy crop in North America [21–23]. However, camelina oil is not desirable for industrial and fuel uses because the high polyunsaturated fatty acid content makes it more susceptible to oxidation [24]. Therefore, breeding efforts have been taken to improve camelina oil lipid profile using genetic engineering methods [25–28].

Recently, genome editing of pennycress and camelina using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated systems (CRISPR-Cas) has been reported and showed successful trait improvements [18, 25–29]. Currently *Agrobacterium*-mediated floral dip method is used for both plants in delivering genome editing reagents, which requires segregation of the integrated transgenes in the subsequent generations. Moreover, the polyploid genome of camelina makes it challenging to design highly efficient guide RNAs (gRNAs) for a target gene [30]. Thus, it is highly desirable to develop a DNA-free genome editing platform and an easy screening method to identify efficient

gRNA and Cas nuclease combinations to facilitate trait improvements of pennycress and camelina.

Unlike animal cells, somatic fusion or delivering large molecules (DNA, RNA, and proteins) into plant cells faces a difficulty due to the presence of cell wall. Protoplasts, plant cells with the cell walls removed after enzymatic treatment, present a great platform to achieve somatic fusion or large molecule delivery via electroporation or polyethylene glycol (PEG)-mediated transfection [31–33]. Protoplasts have been widely used in many studies including biochemical analysis, developmental biology, and protein subcellular localization [34]. Recently, protoplasts have been successfully used for plant genome editing applications to test the efficiency of the CRISPR-Cas systems and gRNAs [35–39]. Although protoplast isolation and transfection protocols have been established for the major model plants such as *Arabidopsis*, rice, wheat, maize, and soybean [36, 40–42], no efficient protocol is yet available for pennycress and camelina.

Here, we report highly efficient protoplast isolation protocols and demonstrate successful DNA transfection and transient marker gene expressions in the pennycress and camelina. This protocol is modified from the protocol developed by Yoo et al. (2007) [40] for *Arabidopsis* and optimized for pennycress and camelina mesophyll protoplasts. With this protocol, protoplasts can be isolated from both in vitro- and soil-grown plants; however, soil-grown plants may contain microorganisms that require addition of antibiotics to the media when the protoplasts need to be incubated for more than 2 days. We provide step-by-step guides for protoplast isolation and DNA transfection using pennycress and camelina grown under a controlled environment to achieve uniform plant growth. Especially, pennycress was grown in in vitro medium to obtain bacteria-free protoplast. The mesophyll protoplasts isolated from fresh leaves can be immediately used for transfection experiments. This chapter also provides guidance for protoplast viability testing using fluorescein diacetate (FDA) staining [43] and transfection rate evaluation by flow cytometry. Protoplast yield produced using this protocol ranged from 3.6 to 8.0 $\times$ 10^6 cells and 3.1–3.6 $\times$ 10^6 cells per gram of fresh leaf tissue of pennycress and camelina, respectively. An average of 40% transfection rate can be achieved from 4 $\times$ 10^4 protoplasts transfected with 10 μg of a 7.3 kb plasmid DNA. The isolation process takes up to 6 h and the transient fluorescent protein expression reaches a detectable level about 36 h after transfection. Isolated protoplasts will be suitable for many more applications such as gRNA efficiency testing, CRISPR-Cas system optimization, and riboprotein delivery of CRISPR reagents for DNA-free genome editing.

2 Materials

2.1 Plant Materials

1. *Thlaspi arvense* spring type 1371: Original seed stocks were obtained from the CoverCress Inc. (Saint Louis, Missouri, USA). Seeds for the protoplast isolation were propagated in the Iowa State University's greenhouse (Ames, Iowa, USA) in 2019–2020 (*see* **Notes 1** and **2**).
2. *Camelina sativa* var. Suneson: Seeds were purchased from the Experimental Farm Network Seed Store (Philadelphia, Pennsylvania, USA) and propagated in the Iowa State University's greenhouse in 2019–2020 (*see* **Note 2**).

2.2 Chemicals and Stock Solutions

1. Cellulase (Onozuka R-10): CAS 9012-54-8, Research Products International (RPI), Mount Prospect, Illinois, USA (*see* **Note 3**).
2. Macerozyme R-10: CAS 9032-75-1, RPI, Mount Prospect, Illinois, USA (*see* **Note 3**).
3. Mannitol (0.8 M): For 300 mL, add 43.72 g mannitol to 200 mL dH_2O in a flask with a stirring bar. Place the flask on a stir plate with low heat, dissolve the chemical completely, then adjust to the final volume using additional dH_2O (*see* **Note 4**).
4. Calcium chloride (1.0 M): For 300 mL, dissolve 44.1 g $CaCl_2{\cdot}2H_2O$ in 200 mL dH_2O, then adjust to the final volume using additional dH_2O (*see* **Note 4**).
5. Potassium chloride (0.5 M): For 300 mL, dissolve 11.18 g KCl in 200 mL dH_2O, then adjust to the final volume using additional dH_2O (*see* **Note 4**).
6. Sodium chloride (0.5 M): For 300 mL, dissolve 8.76 g $NaCl_2$ in 200 mL dH_2O, then adjust to the final volume using additional dH_2O (*see* **Note 4**).
7. Magnesium chloride (1.0 M): For 300 mL, dissolve 28.56 g $MgCl_2$ in 200 mL dH_2O, then adjust to the final volume using additional dH_2O (*see* **Note 4**).
8. MES buffer (0.2 M, pH 5.7): For 300 mL, dissolve 11.71 g 2-(*N*-morpholino)ethanesulfonic acid in 250 mL dH_2O. Adjust the pH to 5.7 using 10 N NaOH and to the final volume using additional dH_2O (*see* **Note 4**).
9. Bovine serum albumin (BSA) solution (5%): For 10 mL, dissolve 0.5 g BSA in 8 mL of sterile dH_2O, then add additional dH_2O to the final volume. Filter sterilize using a 0.22 μm syringe filter. Aliquot the solution into 2 mL tubes. Store at 4 °C for up to 3 months.
10. Sucrose (0.6 M): For 50 mL, dissolve 10.26 g sucrose in 30 mL dH_2O, then add additional dH_2O to the final volume. Filter

sterilize using a 0.22 μm syringe filter. Store at room temperature (22–25 °C) for up to 3 months.

11. Fluorescein diacetate (FDA) stock (20 mg/mL): CAS 596-09-8. Dissolve 100 mg of FDA in 5 mL of acetone. No sterilization needed. Store in freezer for up to 6 months. Avoid light exposure.

2.3 Working Solutions and Media

1. Enzyme solution: For 10 mL, add 0.15 g Cellulase R-10, 0.04 g Macerozyme R-10, 5 mL of 0.8 M mannitol (final 0.4 M), 1 mL of 0.2 M MES buffer (pH 5.7; final 20 mM), 0.4 mL of 0.5 M KCl (final 20 mM), and 3.3 mL of sterile dH_2O into a sterile 50 mL tube. Vortex to dissolve enzyme completely. Incubate at 50 °C for 10 min and cool it down to room temperature (22–25 °C). Add 100 μL of 1.0 M $CaCl_2$ (final 10 mM) and 200 μL of 5% BSA (final 0.1%) and mix well. Filter the enzyme solution using a 0.45 μm syringe filter and aliquot into two 60 × 15 mm Petri plates evenly (*see* **Note 5**). Prepare the solution freshly before each experiment.
2. W5 solution: For 300 mL, mix 3 mL of 0.2 M MES buffer (final 2 mM), 92.4 mL of 0.5 M NaCl (final 154 mM), 37.5 mL of 1 M of $CaCl_2$ (final 125 mM), and 3 mL of 0.5 M of KCl (final 5 mM). Then add additional dH_2O to bring to the final volume (*see* **Note 4**).
3. WI solution: For 300 mL, add 6 mL of 0.2 M MES buffer (final 4 mM), 187.5 mL of 0.8 M mannitol (final 0.5 M), and 12 mL of 0.5 M KCl (final 20 mM). Then add additional dH_2O to bring to the final volume (*see* **Note 4**).
4. MMG solution: For 300 mL, mix 6 mL of 0.2 M MES buffer (final 4 mM), 150 mL of 0.8 M mannitol (final 0.4 M), and 4.5 mL of 1 M $MgCl_2$ (final 15 mM). Then add additional dH_2O to bring to the final volume (*see* **Note 4**).
5. Transfection solution: To prepare 2 mL, add 0.8 g PEG-4000 (final 40%), 500 μL of 0.8 M mannitol (final 0.2 M), and 200 μL of 1.0 M $CaCl_2$ (final 0.1 M) in a 14 mL round bottom tube (*see* **Note 6**). Adjust the final volume to 2 mL by adding sterile dH_2O. Vortex to dissolve the PEG-4000 flakes completely. Prepare the solution freshly before each experiment (*see* **Note 7**).
6. MS medium: In 900 mL of water, dissolve 4.43 g Murashige and Skoog (MS) basal salts with vitamin, and 30 g sucrose. Adjust the pH to 5.7 with 1 N KOH. Add additional water to bring to 1 L. Then add 2.4 g Gelrite. Autoclave at 121 °C for 20 min. After the medium has cooled down to 55 °C, pour into 12 oz. sundae cup approximately 83 mL per each cup (*see* **Note 8**).

7. Seed sterilization solution: For 10 mL, add 2 mL of commercial bleach (8.25% sodium hypochlorite) to 8 mL of sterile dH_2O, then add 1 μL of TWEEN® 20 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and mix well. Prepare the solution freshly before each experiment.
8. FDA staining solution: To make 1 mL, add 20 μL of FDA stock in 980 μL MMG solution. Make it fresh just before use.

2.4 Other Supplies

1. Sundae cup, 12 oz. (~355 mL) sundae cups and lids, SD12 and SDL12 from Solo Cup Company (Lake Forest, Illinois, USA).
2. Soilless substrate LC1 from Sun Gro Horticulture (Agawam, Massachusetts, USA).
3. 0.22 μm micro syringe filters and Stericup filtration system.
4. Petri dish, 60 mm × 15 mm.
5. Forceps.
6. Double edge razor blade.
7. Benchtop orbital shaker.
8. 40 μm cell strainer.
9. 50 mL conical tube.
10. Wide-bore pipette tips, 1000 μL and 200 μL.
11. 14 mL round bottom tube with cap.
12. Swing bucket rotor centrifuge with 15 mL conical tube adapters.
13. Hemocytometer and cover glass.
14. Microcentrifuge.
15. 6-well cell culture plate.
16. Fluorescent microscope, Zeiss Axiostar Plus with mercury lamp illuminator, Carl Zeiss AG (Oberkochen, Germany).
17. Grayscale microscope camera, DFC3000 G, Leica (Wetzlar, Germany).

2.5 Plasmid DNA

1. pKL2188 [44] (Fig. 1a): This is a 7.3 kb plasmid vector carrying dual fluorescent marker genes ZsGreen1 and tdTomato. The green fluorescent protein (GFP) ZsGreen1 is driven by a double cauliflower mosaic virus (CaMV) 35S promoter (2xP35S) and terminated by the potato proteinase inhibitor II terminator (TpinII). The red fluorescent protein (RFP) tdTomato is driven by 2xP35S and terminated by nopaline synthase terminator (Tnos). The plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, Düsseldorf, Germany) and eluted in nuclease-free water. Final plasmid DNA concentration was adjusted to 1 μg/μL (± 50 ng/μL is acceptable).

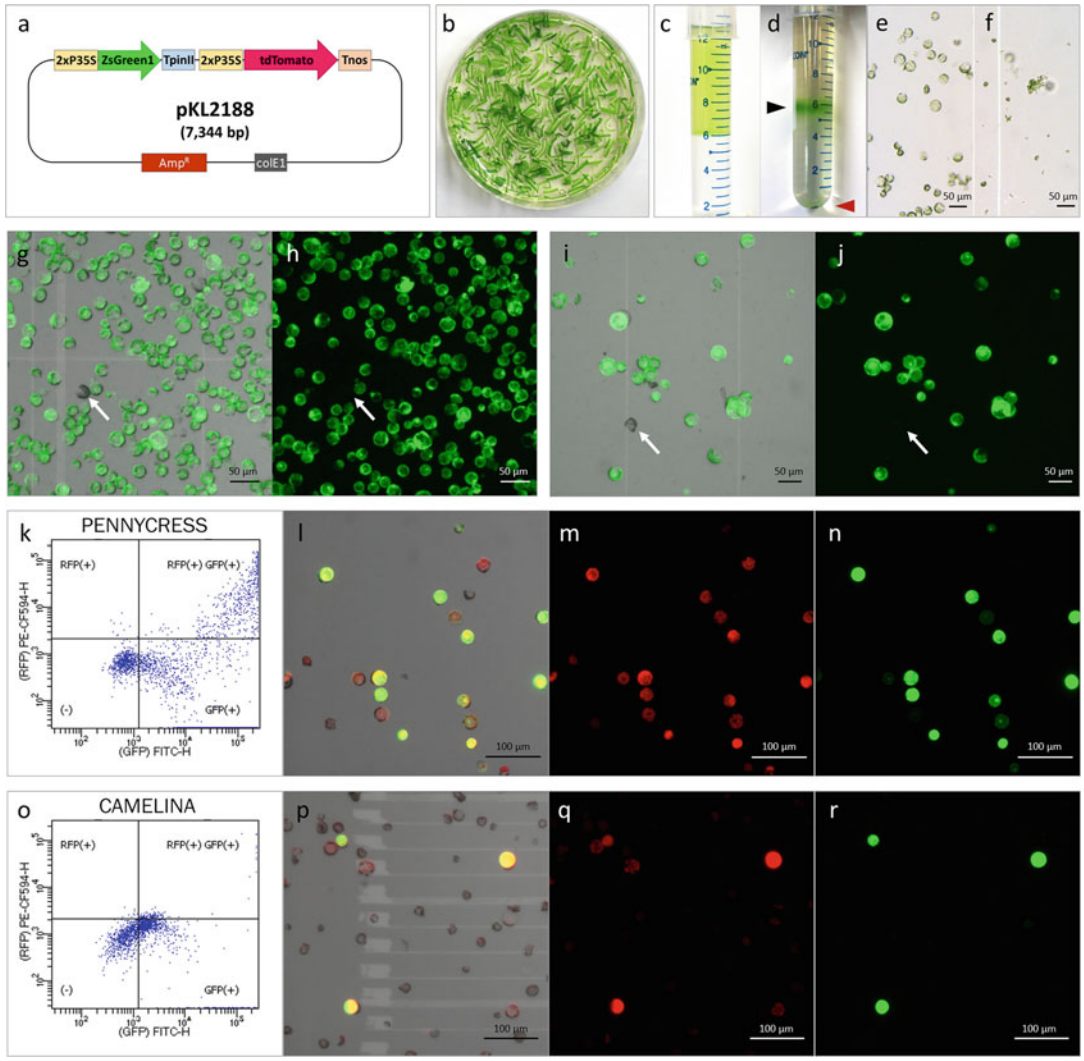

Fig. 1 Stages in protoplast isolation, cell viability test, and transient assay for PEG-mediated DNA transfection. (**a**) Plasmid DNA pKL2188 (Miller et al. 2021) used for transfection and transient assay carries ZsGreen1 and tdTomato as fluorescence markers. 2xP35S, double cauliflower mosaic virus 35S RNA gene promoter; TpinII, potato proteinase inhibitor II terminator; Tnos, nopaline synthase terminator. (**b**) 60 × 15 mm Petri plate containing enzyme solution and leaf strips. (**c**) Protoplast suspension (light green) over 0.6 M sucrose solution before centrifugation. (**d**) Dark green protoplast fraction (black arrowhead) and cell debris (red arrowhead) after centrifugation. (**e**) Protoplasts collected from the dark green fraction after sucrose gradient centrifugation. (**f**) Cell debris collected from the bottom of the tube after centrifugation. Pennycress protoplast after FDA staining, bright field—green channel overlayed (**g**) and green channel (**h**). Camelina protoplast after FDA staining, bright field—green channel overlayed (**i**) and green channel (**j**). White arrows indicate dead cells. (**k**) Flow cytometry of transfected pennycress protoplasts, 43 h after transfection. Pennycress protoplasts 40 h after transfection. Overlayed (**l**), RFP channel (**m**), and GFP channel (**n**). (**o**) Flow cytometry of transfected camelina protoplasts, 40 h after transfection. Camelina protoplasts 42 h after transfection. Overlayed (**p**), RFP channel (**q**), and GFP channel (**r**). Due to high expression of GFP, some of double fluorescent cells were classified to GFP-expressing cells in (**k**) and (**o**)

3 Methods

The protocol described below is modified from a previously published method for *Arabidopsis thaliana* [40].

3.1 Plant Material Preparation

3.1.1 Growing Pennycress In Vitro

1. Place seeds (~1.5 g) in a 50 mL tube and add 10 mL of 70% ethanol. Manually swirl the tube gently about ten times; allow the tube to sit on the bench for 3 min.
2. Remove the ethanol by pipetting. Rinse the seeds with 10 mL of sterile water once.
3. Remove the excess water and add 10 mL of seed sterilization solution. Close the tube and place it straight on a benchtop orbital shaker and shake the seeds at 60 rpm for 15 min.
4. Remove the seed sterilization solution by pipetting. Wash the seeds thoroughly five times (30 s per wash) with 15 mL of sterile dH_2O each wash.
5. Using a pair of sterile forceps, place four seeds onto plant growth medium in a sundae cup and close the lid. No need to seal the cup.
6. Grow plants in a biological incubator at 23 °C with 16 h/8 h (day/night; ~50 μmol/m^2/s) for 3 weeks (*see* **Note 9**).

3.1.2 Growing Camelina in Soil

1. Place two to three seeds in a 3.5 inch (8.8 cm) square pot filled with wet potting mix (soilless substrate).
2. Grow plants in a growth facility at 20–22 °C with 16 h/8 h (day/night; ~75 μmol/m^2/s light intensity) for 3–4 weeks (*see* **Note 10**).
3. After germination, remove extra plants and keep one plant per pot.

3.2 Protoplast Isolation

3.2.1 Leaf Explant Preparation (See **Note 11**)

1. For the in vitro-grown plants, bring the plants in the sundae cups to a horizonal laminar flow hood. Using a sterile sharp scalpel blade and a pair of sterile forceps, collect fully expended two to three top young leaves with 1 cm of petiole (size may vary, choose the leaves larger than 1 cm^2).
2. Gently stack the leaves on a sterile surface such as autoclaved paper towels or paper sheet. Hold the petioles with a pair of forceps to avoid damage on leaf surfaces (*see* **Note 12**).
3. For the soil-grown plants, collect one to two fully expended leaves from the top (size may vary, choose the leaves larger than 2–4 cm^2) (*see* **Note 13**).
4. Follow **step 2** in this section for soil-grown plants. Use sterile materials and wear gloves to reduce further bacterial and fungal contamination.

*3.2.2 Leaf Cutting and Enzyme Digestion (See **Note 14**)*

1. Prepare a 60 × 15 mm Petri plate containing 4.7 mL of freshly prepared enzyme solution as described in **step 1** of Subheading 2.3.
2. On the sterile paper towel, carefully cut the stacked leaf explants into thin leaf strips of 1 mm or less in size using a sterile sharp razor blade without trampling the tissue (*see* **Note 15**). Thinner strips (<1 mm) are preferred for better yield (Fig. 1b).
3. After cutting one stack of leaves, gently transfer and immerse the leaf strips immediately to the Petri plate containing enzyme solution using a pair of sterile forceps (*see* **Note 12**).
4. Cover the Petri plates with aluminum foil to prevent light exposure.
5. Place the Petri plates on a benchtop orbital shaker and gently shake the plates at 60 rpm for 3 h.

*3.2.3 Protoplast Separation (See **Note 16**)*

1. In a clean hood, gently swirl the Petri plates by hand 20–30 times to release protoplasts from the leaf strips (*see* **Note 17**).
2. Slowly add 5 mL of W5 solution to each Petri plate. Gently swirl the dish to mix.
3. Place a 40 μm sterile cell strainer (*see* **Note 18**) onto a sterile 50 mL cell culture tube with conical bottom.
4. Carefully and slowly transfer the protoplast suspension to the cell strainer with wide-bore pipette tips (*see* **Note 19**).
5. Add another 5 mL of W5 solution to the Petri plate containing the leaf strips and gently swirl the plate to release more protoplasts.
6. Transfer the protoplast suspension to the cell strainer as described in **step 4** of this section to combine with the first batch of the flow-through suspension in the tube.
7. For each Petri plate of protoplasts, prepare two sterile 14 mL tubes containing 6 mL of 0.6 M sucrose (*see* **Note 20**).
8. Transfer no more than 7 mL of strained protoplast suspension to each of the 14 mL tube by releasing the suspension slowly along the side of the tube onto the top of the sucrose solution. It will form a green protoplast suspension layer over the clear sucrose solution (Fig. 1c).
9. Centrifuge the tubes at 1500 × *g* for 10 min in a swing bucket rotor at room temperature (22–25 °C) with minimum or no deceleration setting (*see* **Note 21**). A green fraction containing the protoplasts should be clearly visible in the middle of the tube after the centrifugation (Fig. 1d).

3.2.4 Washing

1. To collect protoplasts, carefully insert a wide-bore pipette tip into the tube to reach to the green fraction and slowly withdraw the protoplasts and transfer them to a new 14 mL round bottom tube that contains 6 mL of W5 solution (*see* **Note 22**).
2. Gently mix the tube content by slightly tapping the tube wall.
3. Centrifuge the tube at 100 × *g* for 5 min at room temperature (22–25 °C) in a swing bucket rotor to pellet the protoplasts.
4. Carefully remove the supernatant as much as possible by pipetting.
5. Slowly add 1 mL of W5 solution to the pellet and swirl the tube gently to resuspend the protoplasts.
6. Slowly add 6 mL of W5 solution and carefully mix the tube content.
7. Centrifuge the tube at 100 × *g* for 2 min in a swing bucket rotor.
8. Repeat **steps 4–6** in this section.

3.2.5 Resting

1. Wrap the tube containing 7 mL of protoplast suspension in W5 solution with aluminum foil and place it on ice for 30 min.
2. Centrifuge at 100 × *g* for 2 min as described in **step 7** of Subheading 3.2.4.
3. Carefully remove the supernatant as described in **step 4** of Subheading 3.2.4.
4. Slowly add 1 mL of MMG solution. Resuspend the protoplasts by gentle swirling or tapping (*see* **Note 23**). Cover the tube with aluminum foil and keep it on benchtop.

3.2.6 Cell Counting and Density Calculation

It is important to determine the cell density and make appropriate adjustment for further transfection experiments. We typically adjust the protoplast density to 2×10^5 cells per mL in MMG solution.

1. Gently swirl the tube to mix the protoplasts (*see* **Note 24**); take 10 μL protoplast suspension and load it in between a hemocytometer and a cover glass by capillary action.
2. Place the hemocytometer under microscope using 100× magnification (10× ocular and 10× objective lens).
3. Count the number of cells in all four corner squares and the central squares as shown in Fig. 2 (*see* **Note 25**).
4. The cell density is (number of cells/5) × 10^4 per mL.
5. If the density is below 2×10^5 cells/mL, concentrate the suspension by centrifugation as described in **step 7** of Subheading 3.2.4. Then gently resuspend the pellet using an appropriate amount of MMG to reach 2×10^5 cells/mL (*see* **Note 26**).

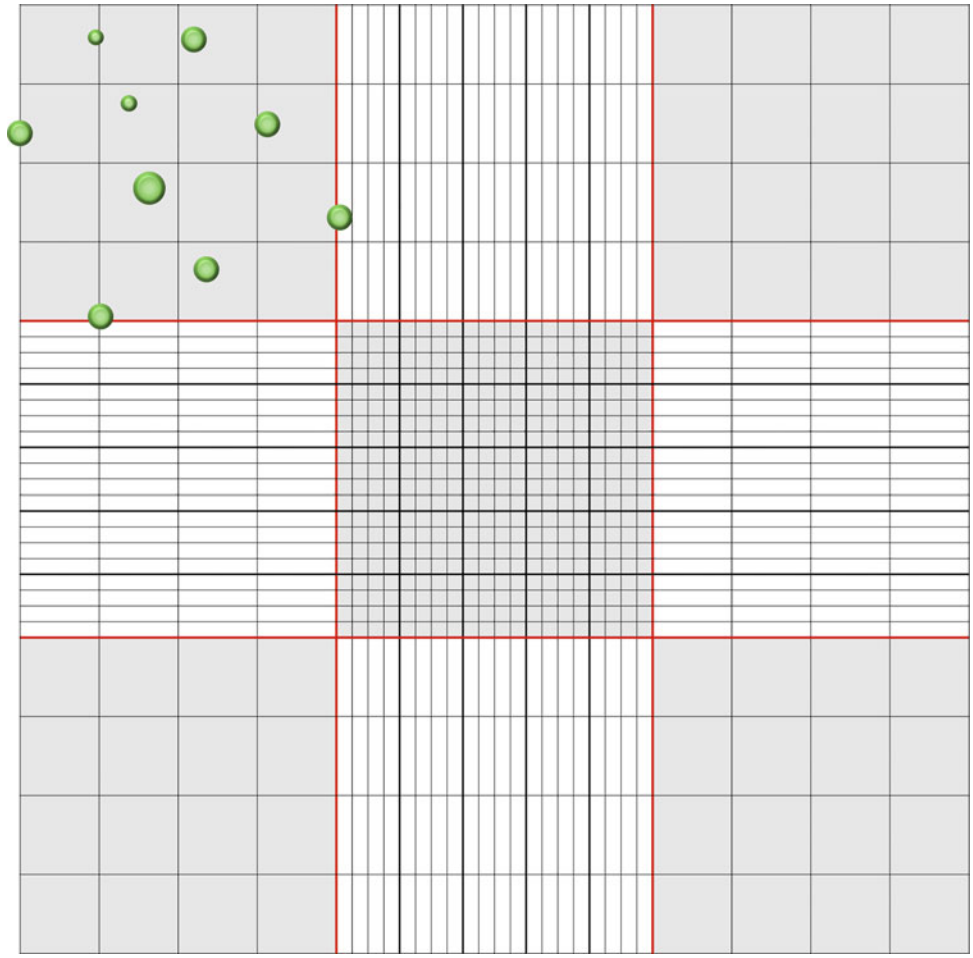

Fig. 2 Standard hemocytometer grid. Count the number of cells in the five gray squares. Do not count the cells touching red lines. For example, the number of cells in the top left square is 7

6. If the density is over 2×10^5 cells/mL, dilute the suspension by adding additional MMG solutions (*see* **Note 27**).

3.2.7 Cell Viability Test

1. Gently swirl the tube to mix the protoplast; transfer 50 μL the suspension to a microcentrifuge tube and add 50 μL of freshly made FDA staining solution.
2. Gently mix it by tapping and incubate at least 10 min in the dark at room temperature (*see* **Note 28**).
3. Collect 10 μL of stained protoplast suspension from the bottom of the tube and transfer onto a hemocytometer (*see* **Notes 29** and **30**). Check the cell viability using fluorescent microscope with 100× magnification (10× ocular and 10× objective lens).

4. Viable cells would express bright green under GFP channel (excitation 455–485 nm, emission 505–545 nm) (Fig. 1g–j) and DAPI channel (excitation 310–390 nm, emission >420 nm). Dead cells are not detectable under the GFP channel. Under the DAPI channel, dead cells are visible, but they do not show bright green color.

3.3 PEG-Calcium Transfection (See Note 31)

1. Place 10 μL of 1 μg/μL of DNA in nuclease-free water into a 2 mL round bottom tube (*see* **Note 32**). Slowly add 200 μL of protoplast suspension (at a density of 2×10^5 cells/mL) using a wide-bore tip and mix by gentle tapping.
2. Slowly add 210 μL of 40% PEG-calcium transfection solution and mix by gentle tapping until the refraction pattern disappears. Incubate the tube at room temperature for at least 10 min, but do not exceed 15 min.
3. Prepare a 6-well cell culture plate by adding 2 mL of WI solution into each well (*see* **Note 33**).
4. Slowly add 800 μL of W5 solution to the protoplast/DNA/PEG suspension and mix gently by inverting the tube until the refraction pattern disappears.
5. Place the tube in a tabletop microcentrifuge and centrifuge at 500 rpm (approximately $23 \times g$) for 2 min at room temperature (22–25 °C). Rotate the tube 180° and centrifuge again with the same setting (*see* **Note 34**).
6. After centrifugation, remove approximately 1 mL of the supernatant. Gently tap the tube two to three times to resuspend the protoplasts in the leftover solution (*see* **Note 35**).
7. Transfer all resuspended protoplasts into the 6-well plate described in **step 3** of this section, one transfection tube per well. Swirl the plate a few times to mix.
8. Incubate the protoplasts at 25 °C in a dark biological incubator for a desired period, up to 3 days (*see* **Note 36**).
9. To observe the cells under a microscope, collect the protoplast suspension into a 2 mL tube and centrifuge at $100 \times g$ for 2 min with minimum or no deceleration setting.
10. Remove the supernatant but leave about 100–200 μL in the tube. Gently resuspend by tapping.
11. Load 10 μL of the concentrated protoplast suspension on a hemocytometer to check for marker gene expression.
12. Typically, the transient gene expression can be detected 16–36 h after transfection (*see* **Note 37**). Our transfection rates validated by flow cytometry were 49.3–74.9% for pennycress (Fig. 1k–n) and 7.6–56.2% for camelina (Fig. 1o–r) using this protocol (*see* **Note 38**).

4 Notes

1. The spring-type pennycress does not require a vernalization process. For winter-type pennycress, vernalization is required for propagation. Place the seedlings in the cotyledon stage in cold chamber (4 °C) with 10/14 (day/night) photo period for 16 days before transferring to the greenhouse [45, 46].
2. Individual plants were planted in 5.5 cubic inch (15 cm^3) square pots and grown in the greenhouse with the condition of 16/8 h (day/night; ~40 μmol/m^2/s for the additional light at night) photo period and 28/20 °C (day/night) temperature.
3. The enzymes can be purchased from the vendors listed here: Research Products International (Mount Prospect, Illinois, USA), Sigma-Aldrich (St. Louis, Missouri, USA), and Yakult (Tokyo, Japan). The Yakult product is distributed by Kanematsu USA (Arlington Heights, Illinois, USA) in the USA. The enzyme activity from different manufacturers may vary. We have achieved our result using the product from RPI.
4. The chemicals for stock solutions are dissolved in sterile deionized water. Filter sterilize the stock solutions using a 0.22 μm Stericup filtration system to remove unwanted microbes and dust in the solution. We recommend making small quantity solution and using small size Stericup (300 mL). To prevent cross contamination, each solution in Stericup can be aliquoted into 50 mL conical tubes. The solutions do not need to be kept in the dark but avoid direct sunlight. The stock solutions can be stored for up to 1 year in room temperature (22–25 °C). However, the mannitol solution tends to form precipitates after 6 months.
5. The final volume would be approximately 4.7 mL for each plate due to loss of solution by syringe filter.
6. The transfection solution is very viscous. Use round bottom tubes to prevent clogged PEG flakes at the tip. We recommend using 14 mL graduated round bottom tubes.
7. Prepare the PEG solution at least 1 h before transfection to allow to dissolve PEG completely and remove bubbles in the solution.
8. Seeds produced from the field often face contamination issues. In general, adding benomyl (50 mg/mL stock in acetone, 1 mL in 1 L of medium) is an effective way to reduce fungal contamination in plant medium [47, 48]. However, we observed growth defects from the pennycress 1371 genotype in benomyl supplemented medium.

9. The pennycress plants with two to three fully expended true leaves are ideal for protoplast isolation. In vitro tissue culture material allows incubating protoplast over 3 days in antibiotic-free solution without bacterial or fungal contamination.
10. The camelina plants for protoplast isolation were grown indoor with artificial lighting source. Individual plants were planted in 3.5 inch (8.8 cm) square pots. Two fluorescent lamps were installed above the plants with outlet timer for photoperiod control. The space between the pots and the light source was 40 cm.
11. Top young leaves from the plants in the vegetative stage are preferred for protoplast isolation. We often observed poor mesophyll protoplast yield from plants close to the flowering stage.
12. Leaves from the in vitro-grown plants are fragile and can be dried out in a couple of minutes. We recommend handling two to three leaves collected from one sundae cup at a time. Slice the leaves immediately after collecting from the plant. Hold the petiole with forceps to avoid damages to the mesophyll tissue.
13. Leaves from the soil-grown plants are larger than those from the in vitro culture-grown plants. To keep the clean lab environments, bringing a plant in the pot is not recommended. The leaves can be collected from the plant and kept in the Petri dish for 5 min before cutting to prevent dry out.
14. The amount of enzyme solution and leaf material can be determined by experimental purpose. Using this protocol, preparing protoplasts for up to 20 transfections can be achieved with 10 mL of enzyme solution. The yield of protoplast depends on several factors: age of plant, amount of leaf material, thickness of leaf strips, incubation time, and the washing step. With this protocol, we obtained 3–8 $\times$ 10^6 cells/g of pennycress leaf tissue (20–22 days old, in vitro culture grown) and 3–3.5 $\times$ 10^6 cells/g of camelina leaf tissue (30–37 days old, soil grown). The approximate leaf size was 1 cm^2 for pennycress 2–4 cm^2 for camelina.
15. A new razor blade is recommended to cut each stack of leaf explants. Dull blades smush the leaves leaving significant amount of green leaf juice on the paper towels or paper. Use 70% ethanol to sterilize a new razor blade and air dry it before each cutting. We do not cut leaves in Petri plates, because the hard surface can dull the blade very quickly.
16. From this stage on, always use wide-bore pipette tips to transfer protoplast suspension to minimize cell damage. Pasteur pipette or cut tips (at least 2 mm wide tip) can be used as alternatives.

17. The enzyme solution will turn green when the protoplasts are released. At this stage, you can identify the green round cells in the suspension. To observe the cells, bring the Petri plates under a dissecting microscope after swirling.
18. Different sizes of cell strainers larger than 40 μm (e.g., 75 μm) can be used depending on the experimental purpose. In this protocol, a 40 μm cell strainer was used for further flow cytometry analysis.
19. Tilt the tube slightly so the flow-through will run down the side rather than dripping. The use of a cell strainer is to remove undigested leaf tissues.
20. Sucrose gradient step is required to remove damaged cells and debris after enzyme digestion (Fig. 1e and f).
21. For the rest of the isolation step, always use minimum or no deceleration setting for centrifugation.
22. To collect the green fraction, move the pipette tip along with the tube wall to withdraw as much protoplasts as possible. It is acceptable if some sucrose solution is also withdrawn.
23. Protoplast suspension with a high cell density ($>10^6$ cells per mL) appears deep green, and this is undesirable for cell counting. To ease the cell counting, add 1 mL of MMG again to adjust the cell density.
24. It is important to mix the protoplast suspension before density estimation. If the protoplasts are not adequately mixed, the volume taken for cell counting may not represent the true cell density.
25. Count the healthy round cells located in each gray square, do not count damaged cells and cells touching the red middle grid (Fig. 2).
26. Low protoplast density calculation example:
    (a) Number of cells from each square: 11, 15, 23, 17, 21. The total is 87.
    (b) The protoplast density is $(87/5) \times 10^4$ per mL $= 17.4 \times 10^4$ per mL.
    (c) You have 2 mL of protoplast suspension. Therefore, you have a total of $2 \times 17.4 \times 10^4 = 3.48 \times 10^5$ protoplasts.
    (d) To reach desired 2×10^5 cells/mL, you need to concentrate the suspension by centrifugation (as described in **step 7** of Subheading 3.2.4). Then remove the supernatant and add appropriate amount of MMG solution.
    (e) In this case, bring the final volume to 1.74 mL after centrifugation: $(3.48 \times 10^5)/(2 \times 10^5) = 1.74$ mL.

(f) Gently resuspend it well and check the cell density again. The density in the range of 1.8–2.2 $\times$ 10^5 cells/mL is acceptable.

27. High protoplast density calculation example:
    (a) Number of cells from each square: 35, 37, 42, 40, 35. The total is 189.
    (b) The protoplast density is (189/5) $\times$ 10^4 per mL = 37.8 $\times$ 10^4 per mL.
    (c) You have 2 mL of protoplast suspension. Therefore, you have a total of 2 $\times$ 37.8 $\times$ 10^4 = 7.56 $\times$ 10^5 protoplasts.
    (d) To reach the desired 2 $\times$ 10^5 cells/mL, you need to dilute the protoplast suspension by adding additional MMG solution.
    (e) In this case, add 1.78 mL of MMG solution to 2 mL of protoplast suspension to bring the final volume to 3.78 mL: (7.56 $\times$ 10^5)/(2 $\times$ 10^5) = 3.78 mL.
    (f) Gently resuspend it well and check the cell density again. The density in the range of 1.8–2.2 $\times$ 10^5 cells/mL is acceptable.
28. The FDA staining solution would be used immediately, and the incubation time would be less than 30 min. The old FDA staining solution can make bright background level of fluorescence. To avoid false positive, dead protoplasts after 10 min incubation at 70 °C can be used as negative control.
29. For microscopy, load the protoplast suspension on a hemocytometer. Regular slide glasses and cover slips are not recommended due to the narrow space between the glass pieces.
30. Centrifuge the suspension at 100 $\times$ *g* for 2 min to collect more cells if necessary.
31. PEG concentration can affect transfection efficiency. We observed that higher PEG concentration (40%) yielded higher transfection rate in *Nicotiana benthamiana* mesophyll protoplast transfection (data is not shown). For further transfection optimization for pennycress and camelina, PEG-4000 concentrations between 10% and 40% can be tested.
32. Handle up to six samples for each transfection in one batch to reduce PEG incubation time variation among the samples.
33. Even if the number of samples is fewer than six, fill all wells on the plate with the WI solution to reduce evaporation during the incubation period.
34. For the swing bucket rotor centrifuge, rotation is not required. Centrifuge for 2 min at 100 $\times$ *g* to collect cells.

35. Sometimes, protoplast cells may not be visible after transfection. It depends on the number of cells remaining and the chlorophyll level of the cells.
36. Aeration is necessary during the incubation. However, to reduce evaporation of the WI solution, place the 6-well plate in a plastic bag and leave the bag open.
37. Autofluorescence from the green chloroplast under RFP channel is common. The autofluorescence showed dot pattern within the cells and overlap with chloroplasts. However, tdTomato expression from pKL2188 does not overlap with the chloroplasts and showed a center-gradient pattern.
38. Flow cytometry analysis by FACSCanto™ system was conducted in the Flow Cytometry Facility at the Iowa State University (Ames, Iowa, USA). Transfected protoplasts were resuspended in 300 μL of WI solution for flow cytometry analysis.

Acknowledgments

The authors thank Oluwatoyosi Akintayo of Dr. Madan Bhattacharyya lab for providing camelina plantlets and Shawn Rigby of the Flow Cytometry Facility for technical assistance. This project was partially supported by National Science Foundation Plant Genome Research Program Grants 1917138, to K.W., by the USDA NIFA Hatch project #IOW04714, by State of Iowa funds, and by the Crop Bioengineering Center of Iowa State University.

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Chapter 13

Protoplast Isolation, Transfection, and Gene Editing for Soybean (*Glycine max*)

Gunvant B. Patil, Robert M. Stupar, and Feng Zhang

Abstract

Protoplast is a versatile system for conducting cell-based assays, analyzing diverse signaling pathways, studying functions of cellular machineries, and functional genomics screening. Protoplast engineering has become an important tool for basic plant molecular biology research and developing genome-edited crops. This system allows the direct delivery of DNA, RNA, or proteins into plant cells and provides a high-throughput system to validate gene-editing reagents. It also facilitates the delivery of homology-directed repair templates (donor molecules) into plant cells, enabling precise DNA edits in the genome. There is a great deal of interest in the plant community to develop these precise edits, as they may expand the potential for developing value-added traits which may be difficult to achieve by other gene-editing applications and/or traditional breeding alone. This chapter provides improved working protocols for isolating and transforming protoplast from immature soybean seeds with 44% of transfection efficiency validated by the green fluorescent protein reporter. We also describe a method for gene editing in soybean protoplasts using single guide RNA molecules.

Key words CRISPR/Cas9, Immature seeds, Green fluorescent protein (GFP), Polyethylene glycol (PEG), Transformation

1 Introduction

Genetic engineering has become a routine technology for crop improvement. The recent development of gene-editing technologies, particularly those involving CRISPR/Cas9 reagents, has further revolutionized the field of crop biotechnology [1, 2]. However, not all crop species are equally accessible to these technologies. For example, crop species from the Solanaceae family are more readily transformed and manipulated in tissue culture compared to other plant families [3]. Historically, the Fabaceae (or Leguminosae) family has been more recalcitrant to biotechnology applications. Therefore, crop species within this

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_13,

family (soybean, common bean, pea, etc.) may require the development of specialized methods to achieve favorable outcomes [4].

Soybean (*Glycine max* (L) Merr.) is an important crop because of its unique blend of protein, oil, and carbohydrates [5, 6]. In soybean, substantial public research investments have been made to sequence large diverse germplasm [7–9], and several quantitative trait loci and genes controlling specific traits [10, 11] are publicly available. These resources are important to functionally characterize and engineer specific traits in soybean. However, compared to some plant species such as *Arabidopsis*, tobacco, and rice, delivering CRISPR/Cas reagents into soybean using *Agrobacterium* and biolistic transformation is relatively difficult and often suffers from lower transformation efficiencies and genotype dependency [12]. Protoplast transformation could offer several advantages, such as a higher efficiency of delivery and editing in cells, non-transgenic delivery of editing reagents (e.g., RNA and/or protein delivery), and efficient editing of multiple genes simultaneously.

Protoplast isolation from tomato root tips dates back to the 1960s, and since then, the protocol for protoplast isolation and transformation has been improved in several plant species [13]. There are limited reports on protoplast isolation and genetic transformation in soybean, and the majority of protocols focused on protoplast isolation from soybean leaf tissue with lower transformation efficiency [14–18]. In this chapter, we describe an efficient method for isolating protoplasts from immature soybean seeds and its application in transient gene expression using green fluorescent protein (GFP). The average transfection efficiency using 3.86 kb construct (pMOD_A3001) was 44%. Furthermore, we describe a platform for cloning single CRISPR guide RNAs and report a simplified procedure for gene editing using CRISPR/Cas9 technology. We also describe a protocol for detecting mutations using cleaved amplified polymorphic sequence (CAPS) PCR and deep-amplicon sequencing. Altogether, this chapter provides an updated working protocol for employing gene-editing technology to generate targeted DNA edits in soybean.

2 Materials

2.1 Plant Materials

1. Soybean cultivars 'Williams 82', 'Bert', and 'Maverick' are used for protoplast isolation. Seeds are propagated in the University of Minnesota's greenhouse (Saint Paul, MN, USA). Plants are grown at 24 °C at 18:6 (light: dark) photoperiod. Immature pods at reproductive (R) stages R2–R3 are collected for protoplast isolation.

2.2 Chemicals and Stock Solutions

1. Cellulase (Onozuka R-10): Yakult Pharmaceutical Ind. Co., Ltd., Kanematsu USA Inc., NJ, USA. Dissolve 2 g of Cellulase in 90 mL SoyPIS solution (Table 1) and add additional SoyPIS to the final volume (*see* **Notes 1–3**).
2. Macerozyme R-10: Yakult Pharmaceutical Ind. Co., Ltd., Kanematsu USA Inc., NJ, USA. Dissolve 500 mg of Cellulase in 90 mL and add additional dH_2O to the final volume (*see* **Notes 1–3**).
3. MES buffer (0.2 M, pH 5.7): For 500 mL, dissolve 11.71 g 2-(*N*-morpholino)ethanesulfonic acid in 400 mL dH_2O. Adjust the pH to 5.7 using 10N NaOH and add additional dH_2O to the final volume (*see* **Note 2**).
4. 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 (*see* **Note 2**).
5. 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 (*see* **Note 2**).
6. Magnesium chloride (2.0 M): For 500 mL, dissolve 95.21 g $MgCl_2$ in 400 mL dH_2O, then add additional dH_2O to the final volume (*see* **Note 2**).
7. 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 (*see* **Note 2**).
8. 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 (*see* **Note 2**).
9. Sucrose (0.55 M): For 200 mL, dissolve 37.65 g sucrose in 100 mL dH_2O, then add additional dH_2O to the final volume (*see* **Note 2**).
10. PEG solution: For 50 mL, dissolve 20 g of PEG 4000 in 20 mL solution containing 0.2 M mannitol and 100 mM $CaCl_2$ (*see* Table 1, buffer D; PEG-calcium transfection buffer). Add additional solution D to the final volume (*see* **Note 4**).

2.3 Solutions and Media

1. Prepare working solutions and media as shown in Table 1 (*see* **Notes 1–4**).
2. Bleach solution (5%): For 100 mL, add 5 mL of commercial bleach (Clorox® 8.25% sodium hypochlorite) to 95 mL of sterile dH_2O, then add 50 μL of TWEEN® 20.

2.4 Other Supplies

1. Clean bench/laminar flow hood.
2. Forceps and razor blade (single-edged carbon steel blades; Fisherbrand #12-640).

Table 1
Reagents and solutions for protoplast isolation and transformation. Formulations are derived and modified from Hammatt et al. [15]; Dhir et al. [17]; and Schwenk et al. [19]

#	Chemical	Stock concentration	Final concentration
A	SoyPIS#* (soybean protoplast isolation solution)—pH 5.7		
1	Mannitol D (-)	0.8 M	0.45 M
2	MES (pH 5.7)	0.2 M	20 mM
3	Cellulase R-10	–	2.0%
4	Macerozyme R-10	–	0.5%
B	WB-N# (washing buffer)—pH 5.7		
1	Mannitol D (-)	0.8 M	0.45 M
2	$CaCl_2$	1.0 M	10 mM
C	MMG# solution—pH 5.7		
1	Mannitol D (-)	0.8 M	0.4 M
2	MES (pH 5.7)	0.2 M	4 mM
3	$MgCl_2$	2.0 M	15 mM
D	PEG (PEG-calcium transfection buffer)—pH 5.7		
1	PEG 4000	–	40%
2	D (-) Mannitol	0.8 M	0.2 M
3	$CaCl_2$	2.0 M	100 mM
E	PM (plating media)—pH 5.7		
1	KCl	0.1 M	5 mM
2	$CaCl_2$	1.0 M	120 mM
3	NaCl	2.0 M	150 mM
4	MES (pH 5.7)	0.2 M	2 mM

#Sterilize solution using 0.2 μm filter
*Prepare Fresh each time

3. 20 μm filter units.
4. Glassware (100, 250, and 500 mL beakers and flasks).
5. Benchtop orbital shaker.
6. Petri dish, 60 mm × 15 mm.
7. 100 μm cell strainer.
8. 15 and 50 mL conical tube.
9. Centrifuge with 15 and 50 mL conical tube adapters.
10. Microcentrifuge for 1.5 and 2.0 mL tubes.

11. Regular and wide-bore pipette tips, 1000 and 200 μL.
12. Hemocytometer and cover glass.
13. 6-well cell culture plate.
14. Compound microscope with 10×, 20×, and 40× lenses.
15. Fluorescent microscope, EVOS M5000 Imaging System, Thermo Fischer Scientific, with color fluorescence, transmitted light, and color images (Product #AMF5000).

2.5 Plasmid DNA

1. Reporter gene construct: Gene construct (pMOD_A3001) carrying green fluorescent protein (GFP) under 35S promoter was obtained from Dr. Daniel Voytas, University of Minnesota, St. Paul, MN, USA. This construct is available at Addgene #91042 [20].
2. Gene-editing construct: Guide RNAs (gRNAs) were cloned in pMOD_B2515 (Addgene #91072) using modular cloning method [20].

3 Methods

3.1 Cloning gRNA to Target Soybean Genes

A two-step procedure is conducted in this example for cloning a single gRNA into the modular vector (pMOD_B2515). The final vector carrying the gRNA is referred to as pGP009. After cloning the gRNA, gene-editing experiments can be conducted by transforming the pGP009 and pMOD_A0521 (carrying GmUBI:AtCas9) constructs together. Gene-editing efficiency can be assessed as described below. Alternatively, researcher can choose to clone both the gRNA and Cas9 cassettes in a single construct.

3.1.1 Design gRNA Oligonucleotide

1. In this example, the gRNA sequence is identified for gene (Glyma.13g068800) using the method described by Liu et al. [12] (*see* **Note 5**). Synthesize two oligonucleotides containing the gRNA spacer sequence (20 bp) and 4 bp overhangs specific for a Pol III promoter (AtU6) as described previously [20]. The vectors used in this study can be obtained from Addgene (plasmids 90997 and 91225) (*see* **Note 5**).

3.1.2 Cloning gRNA in Modular Vector

This protocol is adapted from Čermák et al. [20].

1. Phosphorylate the oligonucleotide with 3 μL each of 100 μM sense and antisense gRNA oligonucleotide, 3 μL T4 DNA ligase buffer (contains ATP), 2 μL T4 polynucleotide kinase, and 19 μL deionized water. Incubate for 1 h at 37 °C.
2. After 1 h, place the reaction in boiling water for 2 min and allow it to cool down gradually at room temperature (22–24 °C).

3. Dilute the reaction 25-fold with PCR grade water.
4. Set up Golden Gate reaction as follows: 50 ng pMOD_B2515, 1 μL diluted oligos, 0.5 μL Esp3I, 2 μL 10× T4 DNA ligase buffer, 1 μL DNA ligase and deionized water up to 20 μL.
5. Place the Golden Gate reaction in a thermocycler and run as follows: 37 °C/5 min + 16 °C/10 min + 37 °C/15 min + 80 °C/5 min.
6. Transform 5 μL of the Golden Gate reaction into *E. coli* (DH5α) and plate on LB + 50 mg/L ampicillin or carbenicillin.
7. Correct clones can be identified using primer (5′- GGAA TAAGGGCGACACGGAAATG -3′) via colony PCR or from plasmid DNA PCR (for background information and details, refer to [20]).

3.2 Protoplast Isolation and Transfection

3.2.1 Tissue Preparation and Digestion

1. Prepare a fresh soybean protoplast isolation solution (SoyPIS) as described in Table 1 (*see* **Notes 1–4**).
2. Collect 10–15 soybean pods (R2–R3 stages; 12–18 days after flowering) from greenhouse-grown soybean plants (Fig. 1a). Keep the pods in sterile water until the immature seeds are harvested from the pods. For best protoplast recovery, select pods with seed size between 2 and 5 mm (Fig. 1) (*see* **Note 6**).
3. Surface sterilize soybean pods with sterile distilled water two to three times, followed by soaking in 70% ethanol for 30 s. Then sterilize the pods by soaking with 5% bleach solution (approx. 0.4% of sodium hypochlorite) for 7–10 min (Fig. 1b). Manually swirl the flask every 1–2 min. Rinse the pods three to five times with sterile water to wash out the bleach.
4. In the tissue culture hood, cut to open the pods (Fig. 1c) with a sterile razor blade and transfer the seeds to a new Petri dish (*see* **Notes 7** and **8**).
5. After collecting 30–35 immature seeds, remove the seed coat with a sharp forceps and transfer the seeds into a low retention tissue culture plate (100 × 25 mm). Pour 15–20 mL of SoyPIS into the plate (*see* **Note 9**).
6. With a sharp sterile razor blade, slice the immature seeds into 0.5 mm pieces (Fig. 1d).
7. Seal the Petri plate with Parafilm and cover them with aluminum foil to maintain dark conditions.
8. Place the plates on a shaker set to 25 °C. Shake at 35 rpm for 12–16 h (*see* **Note 10**).

3.2.2 Protoplast Isolation

1. Add approximately 5 mL SoyPIS to the plates and incubate for an additional 30 min on the shaker at 45 rpm.

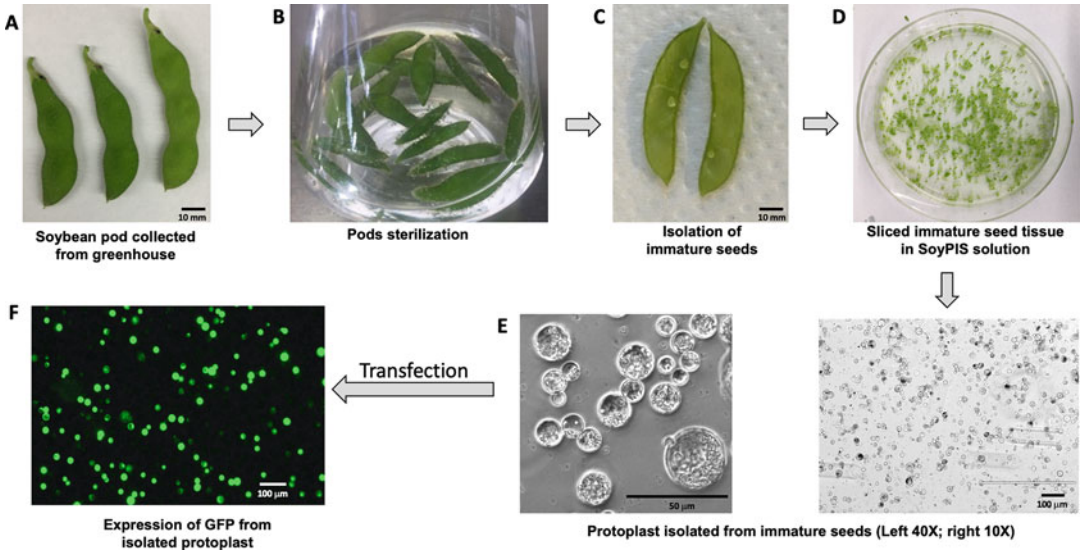

Fig. 1 Steps involved in protoplast isolation and transformation. (**a**) Soybean pods selected at R2–3 stages. (**b**) Surface sterilization of pods. (**c**) Pods showing immature seeds. (**d**) Enzymatic digestion of sliced immature seeds. (**e**) Freshly isolated protoplast under 10× and 40× magnification. (**f**) Soybean protoplast expressing GFP after 48 h of transformation

2. While the plates are shaking, prepare the laminar flow hood by sterilizing with 70% ethanol.
3. Remove the PEG 4000 and plating media from the refrigeration and allow them to warm to room temperature.
4. After the laminar flow hood is sterilized, place all reagents and required plastic wear in the hood and turn ON a UV light to sterilize for 15 min.
5. Pour approximately 40 mL of the WB-N washing buffer into a new sterile Petri plate.
6. Aseptically place a 100 μm cell filter onto a 50 mL Falcon tube.
7. Use an angled plate holder and tube rack to hold the Falcon tube at a 45-degree angle.
8. Pipette 5 mL of WB-N buffer through the filter to moisten the filter and tube. This will help to reduce protoplast loss.
9. Bring the digested seed tissue plate to the laminar flow hood. Remove the aluminum foil and Parafilm.
10. Gently pipette the digested tissue suspension into a 25 mL serological pipette. Slowly filter the solution through the 100 μm cell filter and into the 50 mL Falcon tube.
11. Using centrifuge settings Accel = 5 and Decel = 3, centrifuge the 50 mL tube for 5 min, at 200 rcf at 22 °C.

12. During this time, pipette 10 mL of 0.55 M sucrose into a 15 mL Falcon tube.
13. Carefully retrieve tubes from the centrifuge, remove most of the supernatant, and gently resuspend protoplasts to 5 mL with WB-N.
14. Pipette the resuspended protoplasts using a wide-bore 5 mL serological pipette *very* gently on to the top of the 0.55 M sucrose solution in the 15 mL Falcon tube.
15. Centrifuge the tube for 5 min, at 150 rcf at 22 °C (Accel = 5, Decel = 1).
16. Prepare a 50 mL tube containing 20 mL of WB-N solution.
17. Carefully remove the protoplast layer found at the interphase using a 1 mL pipette using wide-bore pipette tips (*see* **Note 11**) and slowly eject it into the 50 mL Falcon tube containing 20 mL of WB-N solution as mentioned in **step 16**.
18. Centrifuge 5 min at 200 rcf at 22 °C (centrifuge settings Accel = 5, Decel = 4).
19. Remove the supernatant using a pipette and quickly replace with 2 mL of MMG solution.
20. Using a wide-bore pipette tip, draw 20 μL of resuspended protoplasts and place into a hemocytometer.
21. Count five sets of large squares (each consisting of 16 small squares). Count healthy round cells; do not count damaged cells (Fig. 1e).
22. Determine the protoplast yield via the equation found on https://www.hemocytometer.org. For example, cell density = average number of cells $\times 10^4$ per mL.
23. Centrifuge protoplasts for 5 min at 200 rcf at room temperature (Accel = 5; Decel = 4).
24. Remove the supernatant using a pipette.
25. Add the appropriate volume of MMG to create a concentration of 2×10^6 protoplasts per mL. For example, if the protoplast density is 8×10^5 per mL, centrifuge the protoplast for 5 min at 200 rcf, remove the supernatant, and resuspend in 400 μL MMG buffer (*see* **Note 12**).

3.2.3 PEG-Mediated Protoplast Transfection

1. Prepare a DNA sample for transfection by mixing 20–25 μg of DNA constructs and MMG to a total volume of 100 μL in a 2 mL tube (*see* **Notes 13–15**).
2. Add 100 μL of protoplast (from **step 25** of Subheading 3.2.2) using a wide-bore tip to each transfection tube and flick gently. For this experiment, we used approximately 2×10^5 cells per transfection (*see* **Note 12**).

3. Incubate this DNA/protoplast solution at 22 °C temperature in the dark for 5 min.
4. Add an equal volume of PEG 4000 (approx. 200 μL) and mix *very* gently by flicking with finger until it becomes homogeneous.
5. Incubate the tubes at 22 °C for 30 min in the dark.
6. Stop the transfection by adding 1000 μL of WB-N. Invert tubes several times until the PEG 4000 is completely mixed.
7. Centrifuge at 200 rcf for 5 min.
8. Remove the supernatant using a 200 μL pipette tip, being careful not to disturb the pellet.
9. Wash the pellet with 1000 μL of WB-N solution and centrifuge at 200 rcf for 5 min.
10. Carefully remove the supernatant using a 1 mL pipette (do not disturb the pellet).
11. Add 1000 μL of plating media to resuspend the pellet, and invert gently four to five times.
12. Centrifuge at 200 rcf for 5 min. Remove the supernatant using a 200 μL pipette tip.
13. Add 1 mL of plating media, and invert gently until protoplasts are resuspended.
14. Transfer the protoplast suspension to a low retention 6-well sterile culture plate.
15. Seal the Petri plates with Parafilm. Incubate at 22 °C in the dark for 24–48 h.

3.3 Analysis of Protoplast Transformation Efficiency

In our practice, we use the reporter gene expression construct (e.g., GFP, YFP) to check the cell viability and transfection efficiency after 16, 24, and 48 h (see the GFP image in Fig. 1f) using the fluorescence microscope (EVOS M5000, Invitrogen) and/or counting transformed vs. non-transformed cells using a hemocytometer. In this example, we assessed the transformation efficiency after 48 h.

3.4 Analysis of Gene-Editing Efficiency

3.4.1 Cleaved-Amplified Polymorphic Site (CAPS) PCR

1. Design a primer pair flanking gRNA (Fig. 2a; in our case, the forward primer 5′- CACCATTGCTATTGCATCAATCCT -3′ and the reverse primer 5′- AGACACGAATGTGGTTCCGT -3′). We used the HotStarTaq Master mix PCR kit (Qiagen, CA, USA) (*see* **Note 16**).
2. PCR reaction setup: DNA 2 μL (10–50 ng) + 15 μL 2× Hot Start Primer mix + 2 μL forward primer + 2 μL reverse primer + 9 μL deionized water.
3. Thermocycler conditions: 96 °C for 5 min, 30 cycles of (96 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min), and a final hold of 72 °C for 10 min.

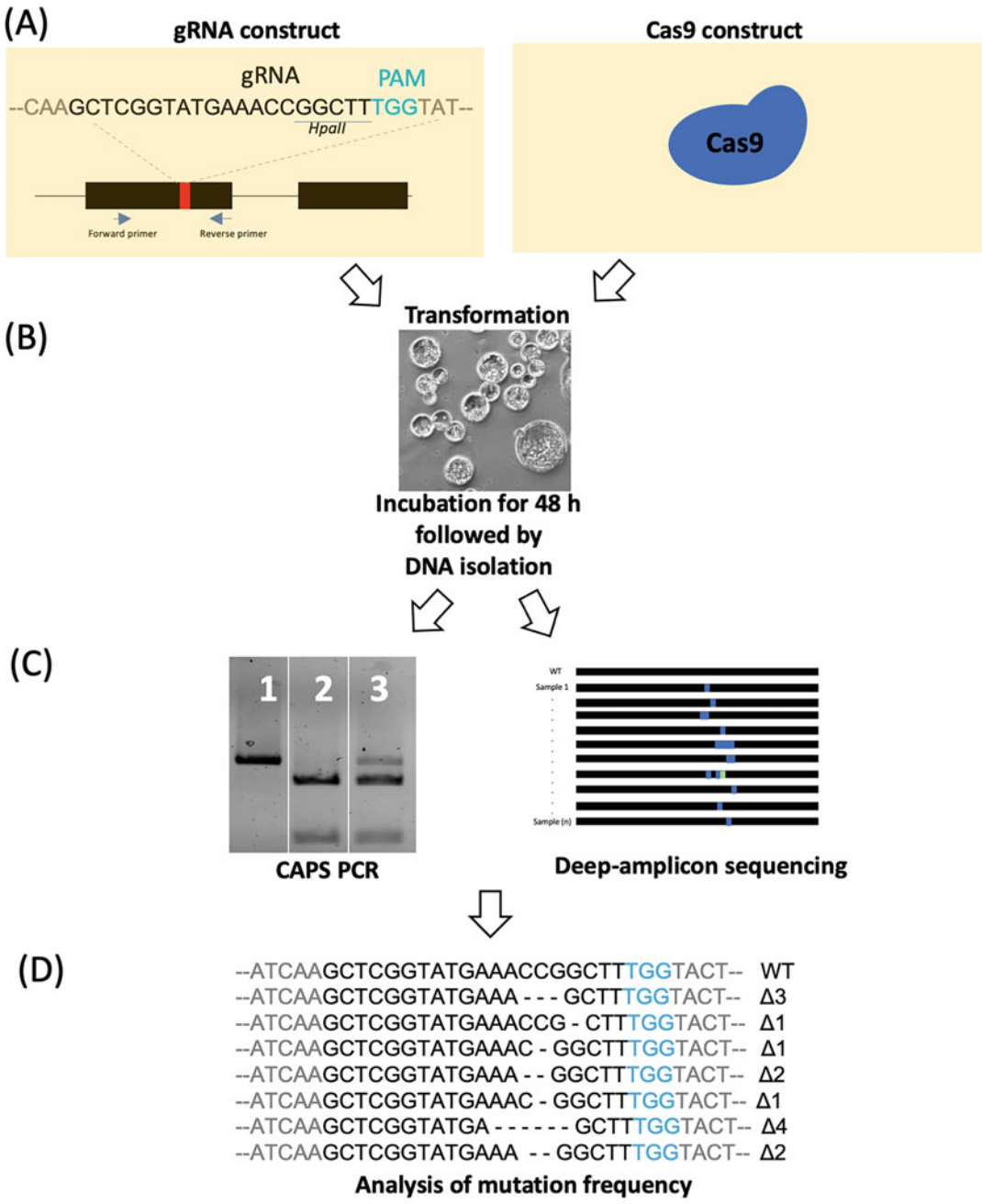

Fig. 2 Schematic showing transformation of CRISPR/Cas9 reagents and analysis of mutation frequency using protoplast transfection system. (**a**) Position of gRNA target sequence (marked with red line) from Glyma.13G068800 and Cas9 constructs. Arrow showing location of PCR primers used in this example. (**b**) Both gRNA and Cas9 constructs were transformed into freshly isolated protoplast. (**c**) Detection of mutations. (Left) The gel image depicts the PCR products. (Lane 1) Undigested PCR product as control. (Lane 2) Wild-type DNA is completely digested. (Lane 3) Mutant DNA is resistant to digestion, resulting in a band of similar size to the control. (Right) Schematic showing the position of mutations using deep-amplicon sequencing. (**d**) Multiple sequence alignment showing the presence of mutation in the target site

4. Following the reaction, the PCR products were digested with the *HpaII* restriction enzyme (site present in the gRNA) (Fig. 2c). When the cells have experienced the intended site-directed mutations, the restriction site in the mutated DNA will be disturbed and will no longer be recognized by the restriction enzyme. Thus, the wild-type alleles will migrate as lower bands on the gel, while the mutated bands will migrate to a higher position on the gel (see lanes 1, 2, and 3 on the agarose gel in Fig. 2c).

3.4.2 Next Generation Amplicon Sequencing

1. For comprehensive mutation analysis, deep-amplicon sequencing can be used (Fig. 2d). Perform the PCR amplification with a primer pair flanking a target region of less than 500 bp. In this example, the primers similar to CAPS PCR were used.
2. Check for the presence of the PCR product by agarose gel electrophoresis.
3. Purify the PCR product with Qiagen PCR purification kit.
4. Dilute the purified sample to 30 ng in 20 μL water or low TE buffer (<0.1 mM EDTA).
5. Send the samples for Illumina next generation sequencing as per manufacturer's instructions to obtain >50,000 reads per sample. In this example, the mutation frequency from each sequenced sample was analyzed using CRISPResso software [21].

4 Notes

1. For the current experiment, the cell wall-digesting enzymes were purchased from Yakult (Tokyo, Japan; distributed by Kanematsu USA). The activity of enzyme from different vendors may vary.
2. Filter sterilize using 0.22 μm 500 mL filter units. We have used Cole-Parmer filter units (Product #UX-06730-31).
3. SoyPIS solutions containing Cellulase and Macerozyme should be prepared fresh. PEG takes up volume when dissolved in water; therefore, start by adding only half the desired volume of buffer.
4. Prepare the PEG solution at least few hours before transfection to allow to dissolve PEG completely and remove bubbles in the solution. Filter sterilization of PEG solutions takes longer time due to viscosity. Use syringe filters for the sterilization process.
5. Example: Guide RNA sequence, GCTCGGTAT GAAACCGGCTT**TGG**; forward oligo, GATTGCTCGGTAT GAAACCGGCTT ; reverse oligo, AAAC

AAGCCGGTTTCATACCGAGC. The PAM site is shown in bold letters. Overhangs specific to the vector backbone are underlined. The method to select a CRISPR targets is not included in this book chapter. This process is greatly simplified by using web tools. Resources for designing and cloning multiple CRISPR targets can be found at http://crispr-multiplex.cbs.umn.edu//allmaps.php. There are also several other web tools that are publicly available for target design. Vector information is also available at http://www.addgene.org.

6. We have observed that immature seeds at R2–R4 stages are ideal for protoplast isolation. Select immature seeds with uniform size.
7. Make sure the seeds are not drying out during seed isolation. Whenever necessary, cover the Petri dish and add few drops of SoyPIS solution to prevent from drying out.
8. Use a new sterile razor blade to cut open and slice the immature seeds. Razor blade can be sterilized by 85% ethanol.
9. Use culture plates with 25 mm or more depth.
10. Start orbital shaker on lowest speed and then increase speed slowly.
11. It is recommended to use precut wide-bore pipette tips or alternately cut the end of the tip using a sharp sterile razor to increase the diameter of tips.
12. Researchers use protoplast density between 5×10^5 and 2×10^6 protoplasts per mL, and the final concentration of protoplast transfection per sample varies from 4×10^4 to 6×10^5 cells per reaction. However, it is recommended to test the ratio of DNA/protoplast. In this example, we used approximately 5×10^5 cells per reaction.
13. In the following example, two transformation reactions were set up. First transformation with GFP construct (pMOD_A3001 carrying 35S::GFP) was carried out to access the transformation efficiency and the second transformation was carried out with two constructs carrying gene-editing reagents (AtU6::gRNA and GmUBI::Cas9; 15 μg each; *see* Subheading 3.1.2) to assess the gene-editing efficiencies.
14. It is recommended to perform six to eight transformations in one batch to avoid PEG transfection time variations between multiple reactions. PEG solution was prepared freshly.
15. Prepare high-quality and high-quantity plasmid DNA using Midi or mega plasmid isolation kit. Qiagen midiprep kit was used for this experiment.
16. PCR conditions are optimized according to the desired PCR products prior to CAPS PCR.

Acknowledgments

This work was partially supported by a grant from United Soybean Board (1920-152-0131-B) to F.Z. and R.S.

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Chapter 14

Protoplast Isolation and Transformation in Oil Palm

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Abstract

The protocol outlined in this chapter describes a detailed procedure for protoplast isolation and transformation using polyethylene glycol (PEG)-mediated transfection and DNA microinjection, highlighting also the critical steps associated with the method. Briefly, we will describe the efficient isolation of protoplasts from 3-month-old suspension calli collected at 14 days after cultured. Digestion of the calli with an optimal composition of enzyme solution yielded over 2×10^6 protoplasts/mL with the viability of more than 80%. The concentrations of DNA, PEG, and magnesium chloride and application of heat shock treatment are the crucial determinants for efficient PEG-mediated transfection. Using the optimal PEG transfection conditions, a transfection efficiency of more than 20% could be obtained. At the same time, protoplasts embedded in alginate layer cultured for 3 days and injected with 100 ng/μL of total DNA solution are the optimal factors for microinjection. We successfully regenerated the injected protoplasts to calli expressing green fluorescent protein (GFP) signals when cultured in optimal medium and cultivation procedures.

Key words DNA transfection, *Elaeis guineensis*, Microinjection, Polyethylene glycol (PEG), Suspension culture

1 Introduction

Elaeis guineensis, commonly known as oil palm, is a perennial crop of major economic importance. In 2019, the production of oil palm generated a total of USD 15.71 billion for Malaysia [1]. The cultivation area of oil palm in Malaysia is about 5.8 million hectares with no large expansion since 2000 [2]. With limited cultivation area and issue on labor shortage, oil palm industry needs to be competitive and sustainable to fulfill the increasing demands every year [2]. Applications of breeding and genetic engineering are among the strategies to assist the oil palm industry [3]. Now, 14 planting series (PS) of valuable traits such as high bunch index palms (PS7) and high oleic acid palms (PS12) are available from oil

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_14,

palm breeding program [4]. However, introgression and selection of these PS series to produce commercial planting materials is a long-term process. Practically, the oil palm breeding cycle is between 10 and 12 years and requires a large amount of planting materials for field evaluation [5]. The open-pollinated behavior of oil palm also contributed significantly to the slowness of the breeding process. Taken together, breeding technology for oil palm improvements requires nearly 30 years before planting materials with the introgressed traits could be released to the oil palm industry [5].

Genetic engineering of oil palm was pursued in early 2000 to assist the oil palm breeding [6]. Introduction of particular valuable traits into oil palm through genetic engineering was postulated to be faster than breeding technology [3]. Transgenic oil palm could be produced in 10–12 years, and traits that are impossible to be introgressed through a breeding approach also could be achieved [3]. Currently, two DNA delivery methods, biolistic [7] and *Agrobacterium*-mediated transformation [8], are extensively used for gene function analyses, characterization of isolated promoters, and production of transgenic oil palms [9]. Both methods are used primarily to deliver gene constructs for the modification of fatty acid biosynthesis to increase oleic acid content, modify oil quality such as higher content of stearic acid, and produce value-added oils such as palmitoleic and ricinoleic acid, as well as novel products such as biodegradable plastic [10, 11].

Genetically engineered oil palms that are generated through biolistic and *Agrobacterium*-mediated transformation solely used callus as target tissues [8, 9]. It is a clump of thousands of cells with an intact cell wall which poses as a major barrier that influences the DNA delivery efficiency mediated by biolistic and *Agrobacterium*-mediated methods [8]. Thus, a plant cell without a cell wall or protoplast is the most suitable transformation material as the DNA could easily pass through the cell membrane. The cell wall can be removed by either mechanical approach or enzymatic hydrolysis, which the latter method is easier to perform and widely being used for high protoplast yield [12]. Protoplasts are suitable for DNA delivery by either polyethylene glycol (PEG), electroporation, or DNA microinjection [13]. Among these methods, PEG transfection is a widely used protocol as it is cheap and straightforward and does not require special equipment. With the surge of genome editing technology especially clustered regularly interspaced short palindromic repeats-associated endonuclease Cas9 (CRISPR-Cas9), protoplast transformation system becomes an essential tool for the evaluation of the efficiency of CRISPR-Cas9 system for almost all plant species such as rice, watermelon, soybean, grape, potato, wheat, maize, and apple [14].

Even though protoplasts are valuable and attractive cells for plant transformation, the use of protoplasts for oil palm

transformation is still at the early stage of development as oil palm is a long regeneration species and recalcitrant to DNA transformation [9–11]. Before 2013, only a few reports are available within the scientific literature describing protoplast isolation but no description for transformation [15–17]. A few years ago, we successfully developed the first successful protocol for plant regeneration from protoplast isolated from oil palm suspension calli [18]. We also demonstrated that the oil palm protoplasts could be transformed using PEG transfection and DNA microinjection [19]. Recently, several reports describing PEG transformation of oil palm protoplasts for gene function studies have been published [20, 21], but no detailed procedure has been described. In this chapter, we describe a step-by-step protocol for protoplast isolation and transformation using PEG transfection and DNA microinjection. The protocols presented here are the improved version that resulted from modification of our published protocols [18, 19].

2 Materials

2.1 Plant Material and DNA Constructs

1. Friable calli derived from the primary embryoid initiation are used as starting calli for oil palm liquid culture (*see* **Note 1**).
2. DNA constructs, namely, pAMGFP (*see* Fig. 1a), pAMDsRED (*see* Fig. 1b) [22], and pAMYFP (*see* Fig. 1c), containing green fluorescent protein (GFP), red fluorescent protein (RFP), and yellow fluorescent protein (YFP) genes driven by CaMV35S promoter are used for PEG transfection experiment. Meanwhile, CFDVhrGFP (*see* Fig. 1d) [19] containing humanized recombinant GFP (hrGFP) gene driven by coconut foliar decay virus (CFDV) promoter is used for DNA microinjection experiment (*see* **Note 2**).

2.2 Stocks

1. Y3 Macro (20× [18]): In 900 mL ddH_2O, dissolve 10.7 g ammonium chloride (NH_4Cl), 40.4 g potassium nitrate (KNO_3), 4.9 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 5.8 g calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), 29.8 g potassium chloride (KCl), and 5.4 g sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$). Add ddH_2O to 1 L final volume. Filter sterilize and store at 4 °C for up to 3 years (*see* **Note 3**).
2. Y3 Micro (100× [18]): In 900 mL ddH_2O, dissolve 0.85 g manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$), 0.72 g zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 0.31 g boric acid (H_3BO_3), 0.83 g potassium iodide (KI), 0.016 g cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 0.024 g cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 0.024 g sodium molybdate dehydrate ($Na_2MoO_4 \cdot 2H_2O$), and 0.001 g nickel chloride

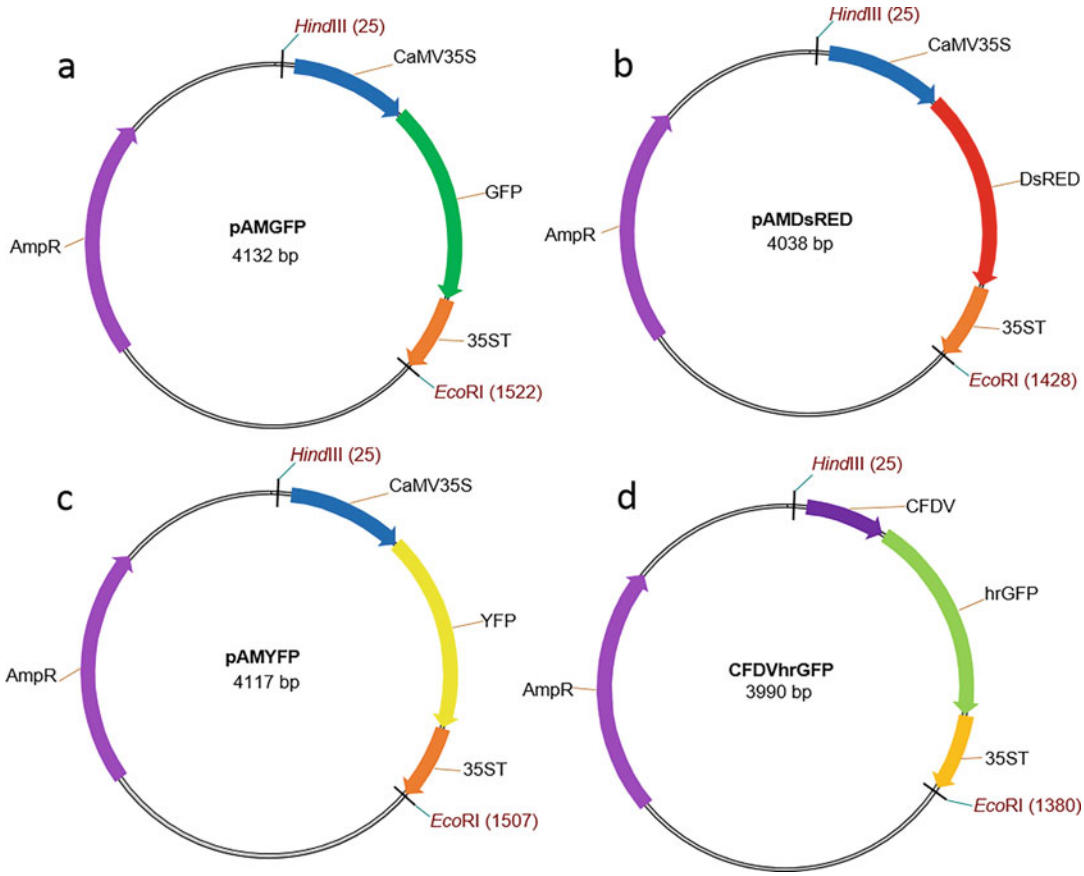

Fig. 1 Schematic diagram of DNA constructs pAMGFP (**a**), pAMDsRED (**b**), pAMYFP (**c**), and CFDV-hrGFP (**d**). Arrow indicates the orientation of each DNA fragment assembled. *CaMV35S* cauliflower mosaic virus 35S promoter, *GFP* gene for green fluorescent protein, *DsRED* gene for red fluorescent protein, *YFP* gene for yellow fluorescent protein, *35ST* CaMV35S terminator, *AmpR* gene confers resistance to ampicillin

hexahydrate ($NiCl_2 \cdot 6H_2O$). Add ddH_2O to 1 L final volume. Filter sterilize and store at 4 °C for up to 3 years (*see* **Note 3**).

3. Y3 vitamins (1000× [18]): In 150 mL, dissolve 0.2 g thiamine hydrochloride, 0.2 g pyridoxine hydrochloride, and 0.2 g nicotinic acid. Add ddH_2O to 200 mL final volume. Filter sterilize with a 0.22 μm syringe filter, and aliquot 50 mL into 50 mL conical centrifuge tubes. Store at 4 °C for up to 3 years.
4. Naphthalene acetic acid (NAA, 1 mg/mL) (*see* **Note 4**).
5. 2,4-Dichlorophenoxyacetic acid (2,4-D, 1 mg/mL) (*see* **Note 4**).
6. 2-γ-Dimethylallylaminopurine (2iP, 1 mg/mL) (*see* **Note 4**).
7. Gibberellic acid (GA3, 1 mg/mL) (*see* **Note 4**).
8. Indole-3-butyric acid (IBA, 1 mg/mL) (*see* **Note 4**).
9. 6-Benzylaminopurine (BAP, 1 mg/mL) (*see* **Note 4**).

2.3 Solutions and Media

1. Y35N5D2iP liquid medium: In 900 mL ddH_2O, while stirring, add 50 mL of 20× Y3 Macro (final 1×), 10 mL of 100× Y3 Micro (final 1×), 1 mL Y3 vitamins (final 1×), 0.2 g glutamine (final 1.36 mM), 0.1 g myo-inositol (final 0.55 mM), 0.1 g asparagine (final 0.76 mM), 0.1 g arginine (final 0.57 mM), 0.0375 g ethylenediaminetetraacetic acid ferric sodium salt (NaFeEDTA) (final 0.1 mM), 0.25 g 2-(*N*-morpholino)ethanesulfonic acid (MES) (final 1.28 mM), 0.25 g potassium phosphate monobasic (final 1.83 mM), 0.004 g glycine (final 0.05 mM), 0.2 g ascorbic acid (final 1.14 mM), 40 g sucrose (final 0.13 mM), 5 mL of 1 mg/mL NAA (final 27 μM), 5 mL of 1 mg/mL 2,4-D (final 22.6 μM), and 2 mL of 1 mg/mL 2iP (final 6.14 μM). Adjust the pH to 5.8 with 1 M KOH and add ddH_2O to 1 L final volume. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).
2. Enzyme solution: Dissolve 0.05 g pectolyase Y-23 (final 0.1% (w/v)), 0.25 g Cellulase Onozuka R-10 (final 0.5% (w/v)), 1.5 g KCl (final 0.4 M), 0.25 g $CaCl_2 \cdot 2H_2O$ (final 0.034 M), 1.82 g mannitol (final 0.2 M), and 0.25 g MES (final 0.026 M) in 25 mL ddH_2O. While stirring, add 1 mL of cellulase from *Trichoderma reesei* (final 2% (v/v)) and 0.5 mL of pectinase from *Aspergillus aculeatus* (final 1% (v/v)). Adjust the pH to 5.6 with 1 M KOH and add ddH_2O to 50 mL. Filter sterilize with a 0.45 μm syringe filter, and aliquot 10 mL enzyme solution into 50 mL sterile centrifuge tubes. Wrap the tubes with aluminum foil. Store at −20 °C for up to 6 months (*see* **Note 5**).
3. Washing solution: In 950 mL ddH_2O, dissolve 30 g KCl (final 0.4 M), 5 g $CaCl_2 \cdot 2H_2O$ (final 0.034 M), 36.4 g mannitol (final 0.2 M), and 1 g MES (final 5.12 mM). Adjust the pH to 5.6 with 1 M KOH and add ddH_2O to 1 L final volume. Filter sterilize and store at room temperature (22–25 °C) for up to 3 years (*see* **Notes 3** and **6**).
4. PEG solution (40% (w/v)): Weigh 8 g PEG 4000 and 0.5 g $MgCl_2 \cdot 6H_2O$ (final 50 μM), and then place in a 50 mL glass beaker. Add washing solution to 20 mL and heat using a microwave for 10 s to dissolve the powder. While stirring, adjust the pH to 6.0 with 1 M KOH. Filter sterilize with a 0.45 μm syringe filter into a 50 mL sterile centrifuge tube and store at room temperature (22–25 °C) (*see* **Note 7**).
5. Rinse solution: Dissolve 30 g KCl (final 0.4 M), 72.8 g mannitol (final 0.4 M), and 1 g MES (final 5.12 mM) in 900 mL ddH_2O. Adjust the pH to 5.6 with 1 M KOH and add ddH_2O to 1 L. Filter sterilize and store at room temperature (22–25 °C) for up to 3 years (*see* **Notes 3** and **6**).

6. Y3A liquid medium: Mix 50 mL of 20× Y3 Macro (final 1×), 10 mL of 100× Y3 Micro (final 1×), 1 mL of 1000× Y3 vitamins (final 1×), 0.2 g glutamine (final 1.36 mM), 0.1 g myo-inositol (final 0.55 mM), 0.1 g asparagine (final 0.76 mM), 0.1 g arginine (final 0.57 mM), 0.0375 g NaFeEDTA (final 0.1 mM), 0.25 g MES (final 1.28 mM), 0.25 g KH_2PO_4 (final 1.83 mM), 0.004 g glycine (final 0.05 mM), 0.2 g ascorbic acid (final 1.14 mM), 55 g sucrose (final 0.16 M), 115 g glucose (final 0.66 M), 1.8 mL of 1 mg/mL NAA (final 10 μM), 1.0 mL of 1 mg/mL IBA (final 2 μM), 300 μL of 1 mg/mL 2iP (final 2 μM), 300 μL of 1 mg/mL GA3 (final 2 μM), and 485 μL of 1 mg/mL 2,4-D (final 2 μM) to 900 mL ddH_2O. Adjust the pH to 5.8 with 1 M KOH and add ddH_2O to 1 L. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).
7. Alginate solution (1% (w/v)): Weigh 0.5 g alginate and place into a 250 mL Erlenmeyer flask, and add 50 mL calcium-free Y3A liquid medium (see below **item 9**). Seal the flask with aluminum foil and then incubate at 37 °C, shake at 100 rpm for overnight (16 h). Transfer the alginate solution into a 50 mL centrifuge tube and spin down at 850 × *g* for 10 min. Filter sterilize the supernatant with a 0.22 μm syringe filter into a 50 mL sterile centrifuge tube and store at room temperature (*see* **Note 8**).
8. Supporting medium: Add 0.5 g SeaPlaque agarose and 0.05 g $CaCl_2 \cdot 2H_2O$ into a 250 mL Erlenmeyer flask, and then add Y3A liquid medium (see below **item 9**) to 50 mL. Heat at 70 °C in water bath to dissolve the powder. Filter sterilize with a 0.45 μm syringe filter into a 50 mL sterile centrifuge tube and store at 50 °C (*see* **Note 9**).
9. Supporting culture dish: Fill the 35 mm Petri dish with 3 mL warm supporting medium as described in **item 7** above. Seal the Petri dish with Parafilm. Place the Petri dish in a 60 mm Petri dish and store at 4 °C.
10. Y3A-1 liquid medium: Same composition of Y3A liquid medium but reduce the glucose to 82 g/L. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).
11. Y3A-2 liquid medium: Same composition of Y3A liquid medium but reduce sucrose to 40 g/L and glucose to 72 g/L. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).
12. Y3A-3 liquid medium: Same as the composition of Y3A-2 liquid medium with only 40 g/L sucrose. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).
13. Y3A-4 liquid medium: In 900 mL ddH_2O, add 50 mL of 20× Y3 Macro (final 1×), 10 mL of 100× Y3 Micro (final 1×),

1 mL Y3 vitamins (final 1×), 0.2 g glutamine (final 1.36 mM), 0.1 g myo-inositol (final 0.55 mM), 0.1 g asparagine (final 0.76 mM), 0.1 g arginine (final 0.57 mM), 0.0375 g NaFeEDTA (final 0.1 mM), 0.25 g MES (final 1.28 mM), 0.25 g potassium phosphate monobasic (final 1.83 mM), 0.004 g glycine (final 0.05 mM), 0.2 g ascorbic acid (final 1.14 mM), 30 g sucrose (final 0.088 M), 185 μL of 1 mg/mL NAA (final 1 μM), and 200 μL of 1 mg/mL BAP (final 0.88 μM). Adjust the pH to 5.8 with 1 M KOH and add ddH_2O to 1 L final volume. Filter sterilize and store at 4 °C for up to 6 months (*see* **Note 3**).

14. Fluorescent dye stock solution (10 mg/mL): Dissolve 25 mg Lucifer Yellow CH dilithium salt (Cat. No. L0259, Sigma) with 2.5 mL sterile ddH_2O. Aliquot 500 μL into a 1.5 mL sterile microcentrifuge. Cover the tube with aluminum foil. Store at −20 °C for up to 3 years.

2.4 Equipment and Related Supplies

1. Vacuum filtration system 1 L.
2. Cell culture dishes, 35 mm × 10 mm, 60 mm × 15 mm.
3. Falcon 50 mL conical centrifuge tubes.
4. Miracloth.
5. PCR cleanup gel extraction kit.
6. Leica DM LFS upright microscope with a joystick-controlled motorized objective (10×, 20×, 40×, and 63×).
7. Luigs and Neumann manipulator set with a control system SM-5 and SM-6 (Luigs and Neumann, Germany).
8. Microinjector CellTram Vario (Cat. No. 5176000033, Eppendorf).
9. Clean bench laminar flow.
10. Ultrafree-MC centrifugal filter.
11. Microloader (Cat. No. 5242 956.003, Eppendorf).
12. Pre-pulled microinjection needle, Femtotip II (Cat. No. 5242 957.000, Eppendorf).
13. Pure mineral oil.

3 Methods

3.1 Preparation of Calli for Protoplast Isolation

1. Add 0.1 g friable callus into a sterile 50 mL Erlenmeyer flask containing 20 mL Y35N5D2iP liquid medium.
2. Incubate at 28 °C in the dark on a rotary shaker at 120 rpm.
3. After 14 days, leave the flask on a benchtop for a few minutes to sediment the calli, and discard half of the supernatant.

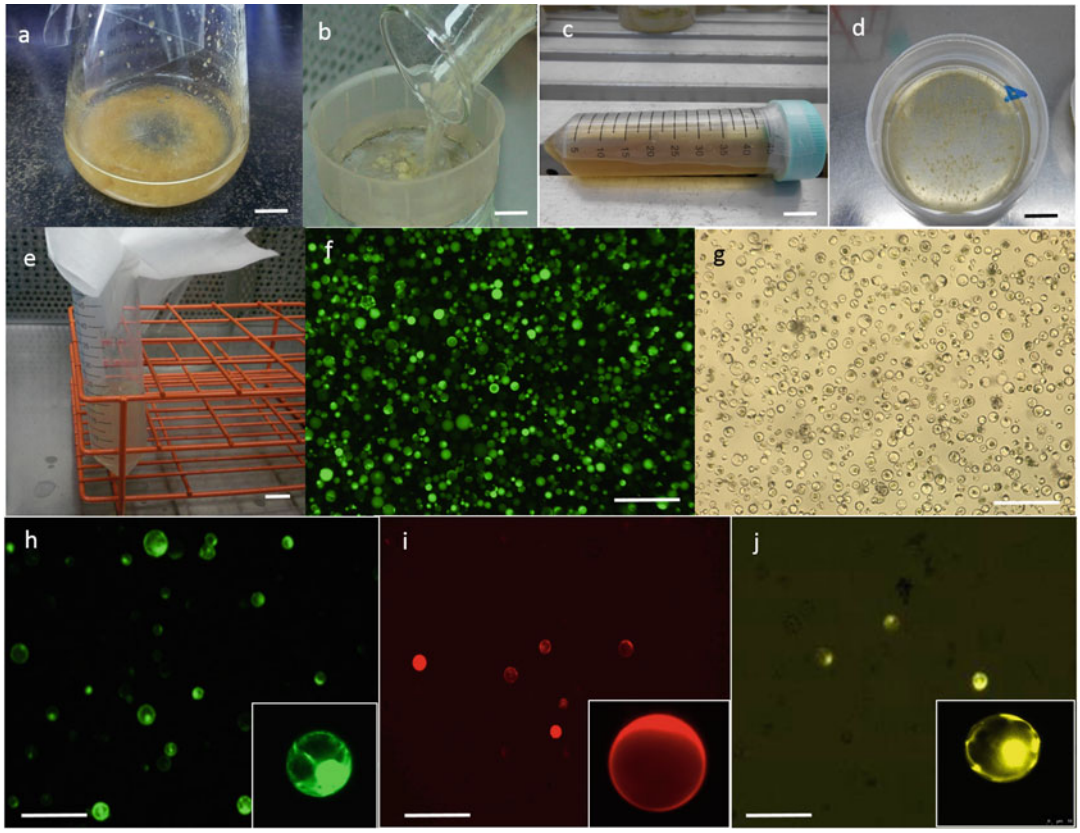

Fig. 2 Protoplast isolation and PEG transfection of oil palm protoplasts. Three-month-old oil palm liquid culture (**a**). Calli are collected by filtration through a 300 μm nylon mesh (**b**). Calli in enzyme solution are incubated in the dark at 26 °C (**c**). Digestion mixture in a 30 mm Petri dish for observation under a microscope for examination of the digestion status (**d**). Digestion mixture is filtered through a double layer of miracloth (**e**). CFDA stained protoplasts observed under a fluorescent microscope (**f**). Clean protoplasts are ready for DNA transformation (**g**). Oil palm protoplasts showing GFP (**h**), RFP (**i**), and YFP (**j**) fluorescence observed after 3 days of PEG transfection (protoplast in the box shows the gene fluorescence expressions that are distributed throughout the cytoplasm and nucleus and extended to the plasma membrane). Scale bar: 1 cm in (**a**)–(**e**); 100 μm in (**f**) and (**g**), 50 μm in (**h**)–(**j**)

4. Add 10 mL Y35N5D2iP liquid medium and then incubate at 28 °C in the dark on a rotary shaker at 120 rpm.
5. Refresh the medium every 14 days as **step 4** for 3 months (*see* Fig. 2a) (*see* **Note 10**).

3.2 Protoplast Isolation

1. Collect the calli from liquid culture by filtration with a sterile 300 μm mesh (*see* Fig. 2b).
2. Transfer the calli (0.5–1.0 g) into a 50 mL centrifuge tube containing 10 mL enzyme solution and then invert the tube four to five times.

3. Place the tube horizontally in an incubator (*see* Fig. 2c), at 26 °C in the dark for overnight (14–16 h). Using a cut tip, take 1 mL into a 35 mm Petri dish to check the digestion status under a microscope (*see* Fig. 2d) (*see* **Note 11**).
4. Add washing solution into a digestion mixture to 20 mL. Invert gently four to five times. Depending on the volume of digestion mixture, add an equal volume of washing solution (*see* **Note 12**).
5. Using a serological pipette, transfer the digestion mixture onto a sterile double-layer miracloth placed on a 50 mL centrifuge tube (*see* Fig. 2e) (*see* **Note 13**).
6. Centrifuge at 100 × *g* for 5 min at 16 °C, and then discard the supernatant (*see* **Note 14**).
7. Add 20 mL washing solution into the tube and invert gently four to five times to resuspend the protoplast pellet (*see* **Note 12**).
8. Centrifuge at 100 × *g* for 5 min at 16 °C. Discard most of the supernatant but leave 2–3 mL to resuspend the pellet.
9. Invert gently four to five times to resuspend the protoplast pellet.
10. Check the viability of the isolated protoplasts by cell staining procedure and determine the yield of protoplasts using a hemocytometer (*see* **Note 15**; also *see* Fig. 2f, g).
11. Store the protoplast suspension at room temperature (22–25 °C) for PEG transfection or DNA microinjection experiments.

3.3 PEG Transfection

1. Add 50 μL (50 μg) of DNA into a 50 mL centrifuge tube.
2. Place the tube at 45 °C for 5 min, then immediately place on ice for 1 min. Then incubate at room temperature (22–25 °C) for 10 min (*see* **Note 16**).
3. Add 1 mL protoplast suspension (1×10^6 protoplasts/mL) to the DNA solution, then gently mix by tapping the tube.
4. Carefully add 1 mL 40% (w/v) PEG solution into the protoplast-DNA mixture. Gently mix by tapping the tube (*see* **Note 17**).
5. Incubate the protoplast-PEG mixture at room temperature in the dark for no more than 10 min (*see* **Note 18**).
6. Slowly add 20 mL washing solution into the protoplast-PEG mixture. Gently invert four to five times to mix.
7. Centrifuge at 100 × *g* for 5 min. Remove the supernatant as much as possible.

8. Carefully add 3 mL Y3A liquid medium into the protoplast pellet. Gently invert for a few times to resuspend the protoplast pellet.
9. Transfer the protoplast suspension into a 60 mm Petri dish, then seal the lid with Parafilm.
10. Incubate the protoplast culture at 26 °C for at least 3–5 days in dark condition.
11. Observe the protoplast culture under a fluorescent microscope. Depending on the vitality of the transfected protoplasts, the fluorescing protoplasts could also be observed at 24 h after PEG transfection (*see* **Note 19**; also *see* Fig. 2h–j).

3.4 DNA Microinjection

3.4.1 Preparation of Alginate Layer

1. Carefully transfer the protoplast suspension (~3 mL) from the Petri dish into a 50 mL centrifuge tube. Add rinse solution to 20 mL. Invert gently four to five times to mix (*see* **Note 20**).
2. Centrifuge at 100 × *g* for 5 min. Remove the supernatant as much as possible.
3. Add 1 mL of 1% alginate solution. Gently tap the tube several times to resuspend the protoplast pellet.
4. Using cut 200 μL yellow tip, pipette out 100 μL protoplast-alginate suspension and slowly place it at the edge of supporting medium. Immediately tilt the dish until the drop runs down to the lower edge leaving a thin layer on the medium. Adjust the volume to form the alginate layer as thin as possible (*see* Fig. 3a).
5. Immediately remove excess of protoplast-alginate suspension. Leave the dish at room temperature for 10–15 min to solidify the alginate layer.
6. Put the lid on and seal with Parafilm. Place the sealed dish in a 60 mm Petri dish and seal with Parafilm. Incubate at 26 °C in the dark for 3 days (*see* Fig. 3b).

3.4.2 Preparation of DNA Injection Solution

1. Extract the plasmid DNA CFDVhrGFP using plasmid DNA purification kit according to the manufacturer's instructions (*see* **Note 2**).
2. Digest the plasmid DNA with *Hind*III and *Eco*RI (*see* Fig. 1d). Run DNA on 1% agarose gel and cut the 1.3 kb band corresponding to CFDV-hrGFP-Nos DNA fragment using a clean blade. Purify the 1.3 kb CFDV-hrGFP-Nos DNA fragment using the PCR cleanup gel extraction kit according to the manufacturer's instructions.
3. Dilute the purified DNA fragment in sterile ddH_2O to a concentration of 100 ng/μL (*see* **Note 21**).

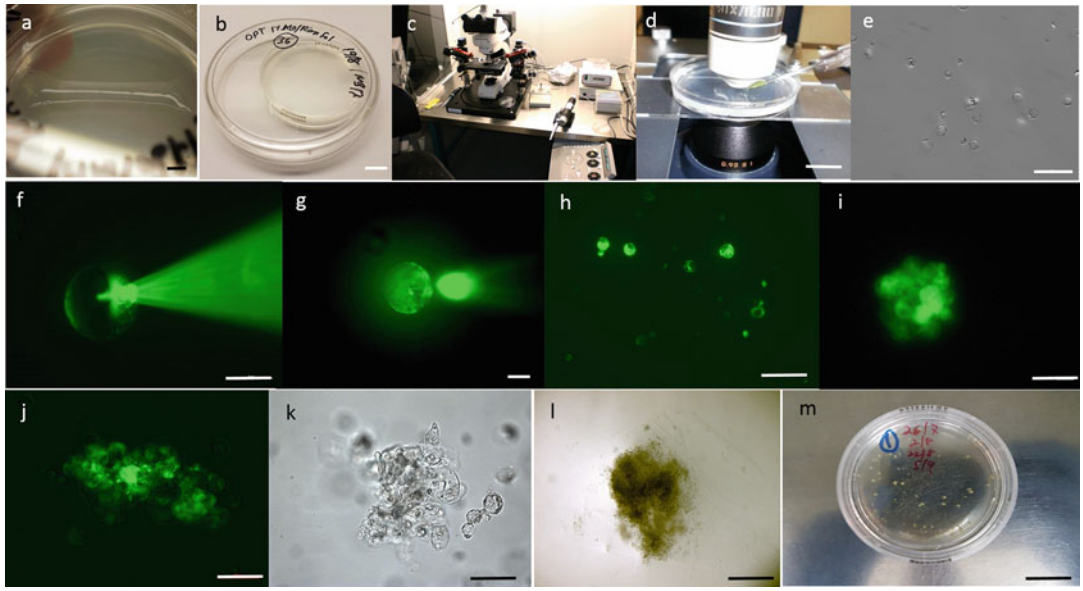

Fig. 3 Microinjection of DNA into oil palm protoplasts and alginate layer culture. The mixture of protoplasts with 1% alginate solution is distributed as a thin layer onto supporting medium (**a**). The alginate layer on supporting medium in a 35 mm dish is cultured for 3 days (**b**). Overview of the microinjection workstation in a sterile environment (**c**). The needle is fixed at an angle of 35° immediately above the alginate layer on the microscope stage during DNA microinjection (**d**). The viable protoplasts embedded in alginate layer observed using the 20× objective (**e**). The needle tip is carefully inserted into the cytoplasm compartment of protoplast (**f**). The DNA solution is injected into the protoplast confirmed by fluorescence illumination (**g**). GFP fluorescing injected protoplasts are observed under a microscope after 3 days of injection (**h**). Development of micro-colony (**i**), macrocallus (**j**, **k**), and calli (**l**). The calli are cultured in a 60 mm Petri dish containing Y3A-4 liquid medium (**m**). Scale bar: 1 cm in (**b**), (**d**), and (**m**); 1 mm in (**a**) and (**l**); 100 μm in (**i**), (**j**), and (**k**); 50 μm in (**e**) and (**h**); 10 μm in (**f**) and (**g**)

4. Mix 20 μL of 100 ng/μL DNA with 2 μL fluorescent dye and then transfer the mixture into Ultrafree-MC centrifugal filter (*see* **Note 22**).
5. Centrifuge at 12,100 × *g* for 5 min at 16 °C. Collect the supernatant and aliquot 5 μL DNA injection solution into sterile microcentrifuge tubes. Cover the tubes with aluminum foil. Store at −20 °C for up to 6 months.

3.4.3 Loading the DNA Solution into Microinjection Needle

1. Centrifuge the DNA solution at maximum speed for 20 min at 4 °C.
2. Slowly fill the Microloader with 5 μL DNA solution and then insert the tip of the Microloader into microinjection needle as close as possible to the needle tip.
3. Slowly pipette 3 μL DNA solution into the needle and slowly withdraw the Microloader tip (*see* **Note 23**).

4. Carefully tap the needle to remove any air bubbles. Leave the needle on an appropriate stand for at least 30 min. Be very gentle when tapping the needle as the needle tip is very fragile. Remove any trap air bubbles to avoid needle clogging.
5. Using a 200 μL yellow tip, fill Microloader with 60 μL sterile mineral oil and slowly pipette the oil into the needle, and then leave the needle on an appropriate stand for at least 10 min.

*3.4.4 Microinjection of Protoplasts (*See ***Note 24****; Also* See *Fig. 3c)*

1. Tightly screw the needle in the capillary holder of a microinjector. Then, tightly fix the capillary holder onto a micromanipulator.
2. Adjust the position of the capillary holder at 30–45° and observe the needle tip through the microscope. Then, move the needle tip with a $Z/X/Y$-axis manipulator controller to the focal plane and the center of the field view (*see* **Note 25**).
3. Raise the needle with a Z-axis controller as high as possible from the microscope stage. Put the dish lid containing alginate layer at the center of microscope stage (*see* **Note 26**; also *see* Fig. 3d).
4. Focus on the protoplast cells using the 20× objective and lower the needle tip as close as possible to the alginate layer with a Z-axis controller (*see* Fig. 3e).
5. Move the needle tip immediately above the target protoplast cell with an X/Y-axis controller and then lower the needle tip immediately adjacent to the target protoplast using a Z-axis controller.
6. Slowly move the needle tip with an X-axis controller to penetrate into the protoplast.
7. Switch on the fluorescence illumination (*see* Fig. 3f).
8. Using fluorescent dye as a guide, slowly inject the DNA solution into the protoplast using a microinjector (*see* Fig. 3g).
9. Switch off the fluorescence illumination.
10. Carefully withdraw the needle tip from the protoplast. Move the needle tip to the next target protoplast cell.
11. Repeat the injection procedure (**steps 7–10** in this Subheading) for at least 50–100 protoplasts for each alginate layer.
12. Raise the needle with a Z-axis controller as high as possible from the microscope stage and put back the lid dish containing alginate layer in a 60 mm Petri dish.
13. Seal the lid of Petri dish with Parafilm and incubate at 26 °C in the dark for 5–7 days.
14. After 3 days, observe the injected protoplast under a fluorescent microscope (*see* Fig. 3h).

3.4.5 Alginate Layer Culture

1. After 5–7 days, separate the alginate layer from supporting medium using a sterile scalpel. Transfer it into a 60 mm Petri dish containing 3 mL Y3A liquid medium.
2. Seal the Petri dish with Parafilm and incubate in the dark at 26 °C on a rotary shaker at 60 rpm.
3. After 2 weeks, discard the Y3A liquid medium and add 3 mL Y3A-1 liquid medium. Refresh the Y3A-1 liquid medium at 14-days intervals and culture the alginate layer in this medium for at least 2 months. At this stage, the protoplasts are actively divided to form microcolonies (*see* Fig. 3i). Extend incubation for another 2 months if no colony developed.
4. Replace the Y3A-1 liquid medium with 3 mL Y3A-2 liquid medium. Refresh with this medium at 14-days intervals for at least 2 months. At this stage, most of the colonies developed into macrocalli (*see* Fig. 3j, k).
5. Discard the Y3A-2 liquid medium and add 3 mL Y3A-3 liquid medium. Refresh with this medium at 14-days intervals until the calli are observed on the alginate layer (*see* Fig. 3l, m). The calli with a size of 1 mm can be observed after 6–7 months. Extend for another 2 months in this medium if no calli appeared.
6. Transfer the calli onto Y3A-4 solid medium for plant development. Embryoid could be regenerated after 5–8 months cultured on Y3A-4 medium [18].

4 Notes

1. Friable calli are initiated from selected ortet oil palm identified as very responsive to tissue culture and with efficient generation rate. The transformation of oil palm is genotype dependent. Thus, it is essential first to assess the amenability of the calli for DNA transformation using conventional gene transient assays such as biolistic and *Agrobacterium*-mediated method.
2. We routinely use the Qiagen Maxiprep kit for yielding high concentration and high-quality DNA. Any plasmid DNA purification kit can be successfully used. Use any fluorescent gene, but the efficiency depends on the size and concentration of plasmid DNA, and also the use of a strong constitutive promoter. For oil palm, we recommend using CaMV35S and maize ubiquitin promoters.
3. We routinely use vacuum filtration system 1 L (Cat. No. 431098, Corning) to sterilize 1 L of stocks, solution, and media.

4. All plant growth regulators (PGR) are prepared by dissolving 0.03 g PGR in 1 mL 1 M KOH in a 50 mL conical centrifuge tube. Add ddH_2O to 30 mL final volume. Filter sterilize with a 0.22 μm syringe filter into a 50 mL sterile conical centrifuge tube. Store at 4 °C for up to 6 months.
5. We recommend using chemicals of a similar brand for the preparation of enzyme solution as the digestion efficiency is higher compared to using other brands. Use pectolyase Y-23 (Cat. No. Y-011, Kyowa Chemical Products Co. Ltd., Japan), Cellulase Onozuka R-10 (Cat. No. 190517-01, Yakult, Pharmaceutical Ind. Co. Ltd., Japan), cellulase from *Trichoderma reesei* (Cat. No. C2730, Sigma), and pectinase from *Aspergillus aculeatus* (Cat. No. P2611, Sigma). The enzymes are sensitive to light, cover with aluminum foil during the preparation of enzyme solution as well as storage in −20 °C.
6. Do not autoclave as the solution contains a high concentration of KCl and mannitol, which will crystalize and subsequently damage the protoplasts and decrease the protoplast yield.
7. Always prepare fresh the PEG solution as the pH will increase with time. We recommend using PEG 4000 from Sigma (Cat. No. 81240).
8. Do not autoclave or heat with a microwave. The alginate will only dissolve after shaking overnight at 37 °C and exhibit a cloudy color. We routinely use alginic acid sodium salt from Sigma (Cat. No. A2158). Always prepare fresh the alginate solution.
9. Do not autoclave as SeaPlaque agarose does not withstand a high temperature. We recommend using SeaPlaque agarose from Duchefa (Cat. No. S1202).
10. We recommend using 3-month-old liquid culture calli collected at Day 14 after subculture as at this stage the calli are homogeneous and at exponential growth phase.
11. Do not incubate the enzyme solution more than 16 h to avoid over-digestion, thus decrease the protoplast yield.
12. Do not invert the tube more than five times to avoid protoplast aggregates or clumps.
13. We routinely use a miracloth to remove the debris from the digestion mixture. Commercial cell strainers with a size of 100 μm can also be successfully used.
14. We recommend using a vertical swing bucket to precipitate the protoplasts at the bottom of the centrifuge tube. Depending on the type of centrifuge, the speed should be experimentally determined to find the optimal speed.
15. We routinely use 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, Cat. No. C1356, Invitrogen). Dissolve the CFDA to 5 μg/mL with acetone as a stock solution. By

using our protoplast isolation protocol, the yield of more than 2×10^6 protoplasts per gram of callus suspension culture with the viability of more than 80% could be successfully obtained.

16. We recommend applying heat shock treatment as the transfection efficiency is significantly high compared to the untreated sample.
17. Be very gentle when adding the PEG solution. We recommend immediately adding the PEG solution once the DNA is mixed with protoplast suspension, which can sometimes increase the transfection efficiency.
18. Prolonging the incubation period for more than 10 min will decrease the transfection efficiency.
19. The transfection efficiency could be achieved at >20% following our optimized protoplast isolation and PEG transfection protocols. The protocol could also be used for protoplast isolation and PEG transfection for other oil palm tissues such as embryoid, mature leaf, and mesocarp by altering the enzyme composition and the osmotic strength of the solution used.
20. Rinse solution is used to clean up the protoplasts from $CaCl \cdot 2H_2O$ as the alginate will polymerize when mixing with a calcium solution.
21. The optimal concentration of DNA injection solution for microinjection of oil palm protoplast is 100 ng/μL [19].
22. Use any fluorescent dye if the dye is not toxic to the protoplast.
23. Do not pipette all the DNA solution (5 μL) to avoid accidental insertion of air bubbles inside the needle tip.
24. The procedure described here is based on microinjection workstation (*see* Subheading 2.4). Any microinjection workstation can be successfully used.
25. Depending on the thickness of the alginate layer, the injection angle should be experimentally determined to find the optimal angle.
26. The protoplast should be injected at Day 3 after being embedded in the alginate layer, as at this stage, the cell wall is partially developed [19].

Acknowledgments

The authors wish to thank the management of MPOB for permission to publish this chapter. The authors would also like to thank Clonal Propagation group for the supply of oil palm friable callus. Thanks are also due to all members of Transgenic Technology Group (MPOB) and AG Molecular Biotechnology (IME, Muenster, Germany) for their assistance and Dr. Omar Rasid for editing the manuscript. This research is fully funded by the Malaysian Palm Oil Board (MPOB).

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Part II

Advanced Systems for High Throughput Analysis and Automation

Chapter 15

Protoplast Preparation and Fluorescence-Activated Cell Sorting for the Evaluation of Targeted Mutagenesis in Plant Cells

Ward Decaestecker, Norbert Bollier, Rafael Andrade Buono, Moritz K. Nowack, and Thomas B. Jacobs

Abstract

Fluorescence-activated cell sorting (FACS) allows for the enrichment of specific plant cell populations after protoplasting. In this book chapter, we describe the transformation and protoplasting of an *Arabidopsis thaliana* cell suspension culture (PSB-D, derived from MM2d) that can be used for the evaluation of CRISPR vectors in a subpopulation of cells. We also describe the protoplasting of *Arabidopsis thaliana* cells from the roots and stomatal lineage for the evaluation of tissue-specific gene editing. These protocols allow us to rapidly and accurately quantify various CRISPR systems in plant cells.

Key words Cell suspension cultures, CRISPR, FACS, Lateral root cap, Protoplasts, Stomata

1 Introduction

The development of CRISPR systems as biotechnological tools has allowed researchers to perform targeted mutagenesis with unprecedented ease and flexibility. Applications of the system rely on the expression and/or delivery of a guide RNA (gRNA) and CRISPR-associated (Cas) nuclease to target cells. The gRNA-Cas complex identifies DNA target sites and can be used to induce a variety of DNA mutations. In most experimental contexts, such as the transformation of plant cell cultures, tissue-specific gene knockout (TSKO), and protoplast transfections, the expression of the gene editing reagents (and, therefore, the associated DNA mutations) is limited to subpopulations of cells (Fig. 1 [1–3]).

In plant transformation experiments, technical and biological factors determine the transformation efficiency and CRISPR expression [4] and the proportion of transformed cells can be enriched over time through antibiotic or herbicide selection.

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_15,

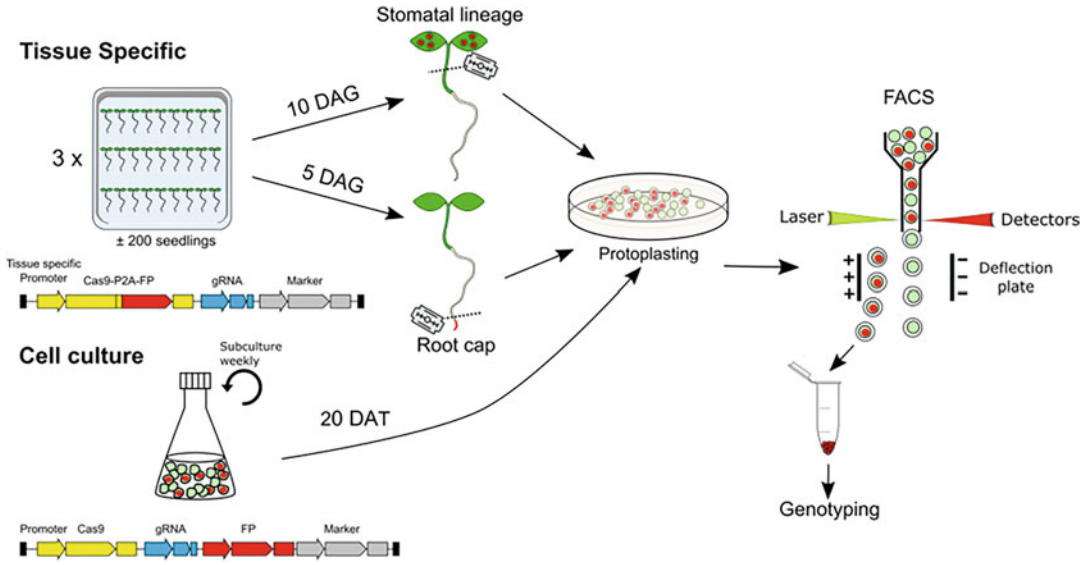

Fig. 1 Schematic diagram of the protoplast preparation and FACS for the evaluation of targeted mutagenesis in plant cells. In one workflow (tissue specific), ~600 *Arabidopsis thaliana* T2 seedlings transformed with a vector for tissue-specific gene knockout (CRISPR-TSKO) are grown for 5 days on 1/2 MS medium. Root tips or cotyledons are collected for protoplasting, typically yielding 50–100 K protoplasts. In a second workflow (Cell culture), PSB-D cell suspension cultures are transformed with a CRISPR vector and protoplasted 20 days after transformation, typically yielding >750 K protoplasts. Protoplasting is followed by FACS to enrich for the population of cells expressing the fluorescent marker that is expressed as a polycistronic 2A expression system (tissue specific) or a separate cassette (cell culture). The enriched cells are then used in genotyping assays for the accurate quantification of CRISPR experiments

Depending on the transformation platform, this process can take weeks to months and is not compatible with the evaluation of CRISPR vectors in a rapid and high-throughput manner. The variable and residual levels of non-transformed cells within the cultivated cell population also prevent the accurate quantification of DNA mutations. In CRISPR-TSKO approaches *in planta*, the expression of Cas9 is restricted to certain cell types, tissues, or organs to evaluate the function of one or several genes in specific developmental or physiological processes [1–3]. The expression profiles can be restricted to such a small percentage of cells that it is not possible to confirm targeted mutagenesis using standard bulk tissue sampling methods. In both types of applications, the accurate quantification of targeted mutagenesis requires the enrichment of Cas9-expressing cells for genotyping.

One of the most common methods used for cell type-specific enrichment is fluorescence-activated cell sorting (FACS) [5]. FACS is a method combining a flow cytometer and a cell separator allowing for the isolation of individual cells based on their physical characteristics (size and morphology, as well as expression of fluorescent proteins or labelling with fluorescent dyes or antibodies).

Because flow cytometry and cell sorting require single cell suspensions, plant protoplast preparations by cell wall removal are necessary to use FACS on most plant tissues. To isolate transformed or Cas9-expressing cells, these cells need to express a fluorescent marker either from a separate cassette or as a transcriptional fusion to Cas9 using a 2A peptide (Fig. 1). It is also possible to translationally fuse a fluorescent marker to Cas9 [2]; however, our experience is that such vectors give low and inconsistent fluorescence levels despite high levels of Cas9 activity [6]. Therefore, we prefer polycistronic 2A expression systems to report Cas9 expression. The sorted protoplasts can then be used for determining DNA repair outcomes only in the cell types of interest. Additionally, multiple fluorescent markers can be used to sort for transformed/Cas9-expressing cells and a reporter for targeted mutagenesis (Fig. 3), thus allowing one to sort multiple cell populations from a single sample.

In this chapter, we describe the transformation (*see* Subheading 3.2) and protoplast preparation (*see* Subheading 3.3) of the *Arabidopsis thaliana* cell suspension culture ecotype Landsberg *erecta* (PSB-D, derived from MM2d). The protocol for the transformation of PSB-D cells is based on Van Leene et al. [7]. Using this protocol, one can obtain *Agrobacterium*-transformed material within 3 weeks of transformation (Fig. 1), which is much faster than standard transformation experiments. We can also normalize for transformation efficiency by enriching for cells expressing the CRISPR vector. Using this method, we typically obtain >750 K protoplasts per sample. We also describe the protoplast preparation from *Arabidopsis thaliana* roots and cotyledons (*see* Subheading 3.5), which was reported earlier by Bargmann and Birnbaum [5]. We use these protocols to enrich for transformed or Cas9-expressing subpopulations of cells (*see* Subheading 3.6) to quantify the mutagenesis efficiency of CRISPR-TSKO vectors (*see* Subheading 3.7). This allows us to precisely quantify the level of DNA mutagenesis in specific cells, tissues, and organs that would not be possible with typical bulk sampling approaches [1]. This protocol generally yields 50–100 K protoplasts from ~600 *Arabidopsis thaliana* root tips. A slight modification to the protocol can be used to enrich for cells from *Arabidopsis thaliana* cotyledons.

2 Materials

All solutions and media are prepared using Type I water (Arium® pro DI Ultrapure Water System) and stored at standard laboratory room temperature, 22–25 °C, unless indicated otherwise. Filter sterilization is performed with 0.22 μm filter units into sterile containers, unless indicated otherwise.

2.1 PSB-D Cell Suspension Culture Maintenance

1. Naphthalene acetic acid (NAA) stock (1 mg/mL): Dissolve 10 mg NAA in ~8 mL of 0.1 M NaOH, and adjust to 10 mL with water (*see* **Note 1**). Aliquot into microcentrifuge tubes and store at −20 °C.
2. Kinetin stock (1 mg/mL): Dissolve 50 mg in 50 mL dimethyl sulfoxide (DMSO) (*see* **Note 1**). Aliquot in microcentrifuge tubes and store at −20 °C.
3. MSMO medium: Add ~800 mL water to a 1 L beaker. Weigh 4.43 g Murashige and Skoog (MS) basal salts with minimal organics and 30 g sucrose and add to the beaker. Adjust to pH 5.7 using 1 M KOH. Bring the volume to 1 L with water. Add 500 μL of 1 mg/mL NAA stock and 50 μL of 1 mg/mL kinetin stock. Autoclave. Store at room temperature. Media is stable for at least 3 months.

2.2 PSB-D Transformation

1. $MgSO_4$ stock (1 M): Weigh 24.65 g $MgSO_4 \cdot 7H_2O$ and dissolve in 100 mL water. The stock does not need to be sterilized and can be stored for at least 3 months.
2. Rifampicin stock (100 mg/mL): Dissolve in DMSO (*see* **Note 1**), aliquot in microcentrifuge tubes, and store in the freezer at −20 °C.
3. Gentamicin sulfate stock (10 mg/mL): Dissolve in water (*see* **Note 1**), filter sterilize, aliquot in microcentrifuge tubes, and store at −20 °C.
4. Spectinomycin stock (100 mg/mL): Dissolve in water (*see* **Note 1**), filter sterilize, aliquot in microcentrifuge tubes, and store at −20 °C.
5. Kanamycin stock (50 mg/mL): Dissolve in water (*see* **Note 1**), filter sterilize, aliquot in microcentrifuge tubes, and store at −20 °C.
6. Vancomycin hydrochloride stock (50 mg/mL): Dissolve in water (*see* **Note 1**), filter sterilize, aliquot in microcentrifuge tubes, and store at −20 °C.
7. Carbenicillin disodium stock (100 mg/mL): Dissolve in water (*see* **Note 1**), filter sterilize, aliquot in microcentrifuge tubes, and store at −20 °C.
8. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) stock (100 mM): Weigh 196.19 mg and dissolve in 10 mL DMSO (*see* **Note 1**), aliquot in microcentrifuge tubes, and store at −20 °C for up to 6 months.
9. YEB medium: Add ~800 mL water to a 1 L beaker. Weigh 5 g beef extract powder, 1 g yeast extract, 5 g peptone, 5 g sucrose and add to the beaker. Add 2 mL of 1 M $MgSO_4$ stock. Bring the volume to 1 L with water and autoclave. Store at room temperature for up to 6 months.

10. YEB + RGS medium: Add 1 mL of 100 mg/mL rifampicin stock, 1 mL of 40 mg/mL gentamicin sulfate stock, and 1 mL of 100 mg/mL spectinomycin stock to 1 L of YEB medium. Store at 4 °C for up to 1 month.
11. MSMO + KVC medium: Add 1 mL of 50 mg/mL kanamycin stock, 10 mL of 50 mg/mL vancomycin hydrochloride stock, and 5 mL of 100 mg/mL carbenicillin disodium stock to 1 L of MSMO medium. Store at 4 °C for up to 1 month.

2.3 PSB-D Protoplasting

1. Digestion buffer: 0.4 M D-mannitol, 20 mM KCl, 20 mM MES monohydrate. Add ~800 mL water to a 1 L beaker. Weigh 72.87 g D-mannitol, 1.49 g KCl, and 4.27 g MES monohydrate and add to the beaker. Adjust to pH 5.7 using 1 M KOH. Bring the volume to 1 L with water and filter sterilize.
2. Wash buffer: 0.4 M D-mannitol, 2.5 mM $CaCl_2$, 1 mM MES monohydrate, pH 5.7. Add ~800 mL water to a 1 L beaker. Weigh 72.87 g D-mannitol, 0.37 g $CaCl_2 \cdot 2H_2O$, and 0.21 g MES monohydrate and add to the beaker. Adjust the buffer to pH 5.7 using 1 M KOH. Bring the volume to 1 L with water. Filter sterilize.
3. Resuspension buffer: 0.4 M D-mannitol, 15 mM $MgCl_2$, 5 mM MES monohydrate, pH 5.6. Add ~800 mL water to a 1 L beaker. Weigh 72.87 g D-mannitol, 3.05 g $MgCl_2 \cdot 6H_2O$, and 1.07 g MES monohydrate and add to the beaker. Adjust the buffer to pH 5.6 using 1 M KOH. Bring the volume to 1 L with water. Filter sterilize.
4. Cellulase Y-C (*see* **Note 2**).
5. Macerozyme R-10 (*see* **Note 2**).
6. 100 μm cell strainer.

2.4 Arabidopsis Growth and Sterilization

1. *Arabidopsis* lines: Wild-type *Arabidopsis thaliana* Col-0 or a transgenic line expressing a fluorescent reporter, such as GFP (*pHTR5:NLS-GFP-GUS* [8]; *see* **Note 3**), is transformed with a CRISPR-TSKO vector for tissue-specific gene knockout. The construct expresses Cas9-P2A tagged with a fluorescent protein of choice such as mCherry [1], mTagBFP2 [6], or GFP and one or several gRNAs. In these vectors, the expression of Cas9 is restricted to the tissue of choice by the promoter used and marked by the P2A-fluorescent protein.
2. Growth medium: Add ~800 mL to a 1 L beaker. Weigh 2.15 g MS basal medium, 0.1 g MES monohydrate. Adjust to pH 5.7 using 1 M KOH. Bring the volume to 1 L with water. Add 10 g plant tissue culture grade agar and autoclave. Cool the bottles to ~60 °C and dispense ~50 mL of growth medium per 12 × 12 cm square Petri dish. Allow the agar to solidify in a

laminar flow hood for ~30 min. We use freshly prepared plates due to the scale of the experiment.

3. Sterilizing solution: 1.3% (v/v) sodium hypochlorite, 0.05% Tween® 20 (v/v).
4. 70% ethanol.
5. Autoclaved water.

2.5 Arabidopsis Protoplasting

1. Digestion buffer: Same as for PSB-D cells (*see* Subheading 2.3, **item 1**).
2. Wash buffer: 0.4 M mannitol, 20 mM MES, 20 mM KCl, 0.1% bovine serum albumin (BSA) (w/v), 10 mM $CaCl_2$, pH 5.7. Add ~800 mL water to a 1 L beaker. Weigh 72.87 g D-mannitol, 1.49 g KCl, 4.27 g MES monohydrate, 1 g BSA, 1.48 g $CaCl_2 \cdot 2H_2O$ and add to the beaker. Adjust the pH to 5.7 using 1 M Tris–HCl pH 5.7. Bring the volume to 1 L with water. Filter sterilize.
3. Cellulase Onozuka R-10 (*see* **Note 2**).
4. Macerozyme R-10 (*see* **Note 2**).
5. 40 μM cell strainer.

2.6 Plasmids and Bacterial Strains

1. Our general cloning strategy for generating CRISPR vectors is based on the Golden Gate technology and modified GreenGate vectors [9, 10]. Briefly, six different Golden Gate entry modules are combined via Golden Gate assembly in a binary destination vector. Generally, the A-B module contains the promoter, C-D contains the nuclease which can be combined with a N-terminal (B-C) or C-terminal tag (D-E), and the E-F module contains the terminator. The F-G module contains the gRNA or cloning sites for iterative cloning (e.g., when cloning >2 gRNAs) [1]. Depending on the purpose, a fluorescent marker can be cloned as a C-terminal tag of Cas9 using a P2A ribosomal-skipping peptide or can be a separate expression cassette on the destination vector [1, 6]. To select PSB-D cells, we generally use the kanamycin resistance marker *nptII*, though hygromycin and BASTA selection are also possible. Vectors, associated sequences, and maps are available and distributed through the following website: https://gatewayvectors.vib.be/.
2. Expression vectors are transformed in *Agrobacterium tumefaciens* strain C58C1 pMP90 by electroporation. This strain contains rifampicin resistance in the genomic DNA. The pMP90 helper plasmid contains a gentamycin resistance cassette. As our expression vectors generally contain a spectinomycin resistance cassette, transformed *Agrobacterium* cells are grown in YEB medium supplemented with rifampicin,

gentamicin sulfate, and spectinomycin (YEB + RGS; *see* Subheading 2.2, **item 10**).

2.7 Genotyping

1. Modified Edwards DNA extraction buffer: 100 mM Tris–HCl pH 8.0, 500 mM NaCl, 50 mM EDTA, 0.7% [w/v] SDS.
2. Thermo Scientific™ Phire™ Plant Direct PCR Master Mix.
3. ALLin™ HS Red Taq Mastermix (highQu).
4. Genotyping oligos Primer 1, 5′- CTTA TACGTGTGTGTGCGCG ; Primer 2, 5′- GCAGCTGAGT CAGGGCTAAA.
5. DNA Clean and Concentrator-5 (Zymo Research).
6. HighPrep™ PCR Clean-up System (MagBio).

3 Methods

3.1 PSB-D Cell Suspension Culture Maintenance

1. Grow 100 mL of PSB-D cell suspension culture in 250 mL Erlenmeyer flasks with screw caps. Close with surgical tape (*see* **Note 4**). Incubate at 25 °C with shaking at 130 rpm in the dark.
2. Subculture the cell suspension culture weekly by diluting 20 mL of the 1-week-old culture into 80 mL MSMO medium in a fresh Erlenmeyer flask. Swirl the Erlenmeyer flask before the transfer to homogenize the culture, since cells settle quickly (*see* **Note 5**).

3.2 PSB-D Transformation

1. Day 1. Repeat **step 2** in Subheading 3.1 (*see* **Note 5**).
2. Day 2. Establish an *Agrobacterium* liquid culture by picking three colonies from a solid plate (*see* **Note 6**) and inoculate them into 2 mL YEB + RGS in a 15 mL cell culture tube. Incubate at 28 °C in the dark with shaking at 200 rpm.
3. Day 3. Transfer 1 mL of the *Agrobacterium* culture to 20 mL YEB + RGS medium in a 100 mL Erlenmeyer flask. Incubate at 28 °C in the dark with shaking at 200 rpm.
4. Day 4. Transfer 10 mL of the *Agrobacterium* culture to a 50 mL centrifuge tube.
5. Centrifuge at 3095 × *g* for 15 min. Decant the supernatant.
6. Add 20 mL MSMO medium to the tube. Vortex until the pellet completely resuspends.
7. Repeat **steps 5** and **6**.
8. Measure the optical density (O.D.) at 600 nm (*see* **Note 7**).
9. Repeat **step 5**.

10. Resuspend *Agrobacterium* with adequate volume of MSMO to achieve a final $O.D._{600}$ of 1.0 based on the O.D. measurement in **step 8** (*see* **Note 8**).
11. Take a 6-well plate and add per well: 3 mL of the 2-day-old cell suspension culture (*see* **Note 5**), 200 μL of *Agrobacterium* culture ($O.D._{600} = 1$), and 6 μL of 100 mM acetosyringone (*see* **Note 9**).
12. Close the 6-well plate with surgical tape. Incubate for 3 days at 25 °C with shaking at 130 rpm in the dark.
13. Day 7. Transfer the contents of one well into a 25 mL Erlenmeyer flask containing 7 mL MSMO + KVC medium using a wide-bore pipette (*see* **Note 10**). Tape the screw cap with surgical tape (*see* **Note 4**).
14. Incubate for 8 days at 25 °C with shaking at 130 rpm in the dark.
15. Day 15. Transfer the entire 10 mL to a 100 mL Erlenmeyer flask containing 25 mL MSMO + KVC medium using a wide-bore pipette (*see* **Note 5**). Tape the screw cap with surgical tape (*see* **Note 4**).
16. Incubate for 8 days at 25 °C with shaking at 130 rpm in the dark.
17. Day 22. Transfer 10 mL of sunk cells to 35 mL MSMO + KVC medium using a wide-bore pipette (*see* **Note 11**). Tape the screw cap with surgical tape (*see* **Note 4**).

3.3 PSB-D Protoplasting

1. Optional: In the days before the protoplasting experiment, use a fluorescence microscope to quickly check cultures for expression of the fluorescent marker gene. Approximately 20–30% of cells should express the marker. If a culture shows very low numbers of transformed cells (e.g., <5%), we generally omit these from the experiment as these require too much time to sort within the working day. If none of the cultures are >5%, consider abandoning the experiment and retransforming or scaling back the size of the experiment to allow sufficient time to sort.
2. Day 24. Prepare the digestion solution fresh by adding 1.5% cellulase and 0.4% macerozyme to the digestion buffer (*see* **Note 12**).
3. Transfer 25 mL of the transformed PSB-D culture to a 50 mL tube.
4. Centrifuge at 124 × *g* for 5 min. Remove the supernatant with a wide-bore pipette.

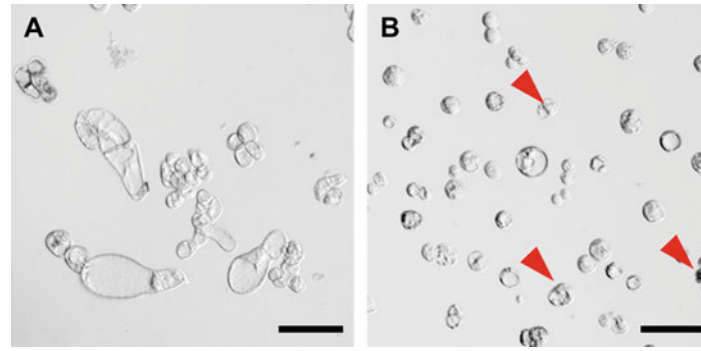

Fig. 2 PSB-D protoplasting. (**a**) Picture of a PSB-D cell culture at Day 24 (20 days after transformation) showing typical clustering of cells during propagation. (**b**) Protoplasting of the transformed PSB-D cell culture generates separate and spherical protoplasts 20–100 μm in diameter. Red arrows indicate damaged protoplasts. Picture taken after 6 h of treatment with digestion solution (*see* Subheading 2.3). Microscopic images are shown under bright field using an Olympus BX51 microscope. Scale bar represents 100 μm

5. Add 25 mL of the prepared digestion solution (*see* **step 2**). Tilt the tube multiple times until the soft pellet is completely resuspended.
6. Pour the tube content into a 145 mm Petri dish. Incubate under gentle shaking (*see* **Note 13**) in the dark for at least 4 h.
7. Check the solution under the microscope to confirm that the cells are separated into individual and spherical protoplasts (Fig. 2) (*see* **Note 14**). The size of the protoplasts ranges from 20 to 100 μm.
8. Transfer the solution from the Petri dish to a 50 mL tube using a wide-bore pipette (*see* **Note 10**).
9. Centrifuge at 124 × *g* for 5 min and discard the supernatant by pouring.
10. Add 20 mL wash buffer (*see* **Note 15**). Tilt the tube multiple times until the protoplast pellet is completely resuspended.
11. Repeat **steps 9** and **10**, for a total of two washing steps.
12. Add 3 mL resuspension buffer (*see* **Note 15**). Tilt the tube multiple times until the pellet is completely resuspended.
13. Filter through a 100 μm cell strainer.
14. Maintain the protoplasts on ice or at 4 °C. Protoplasts can be stored at 4 °C overnight if needed.
15. The PSB-D protoplasting process typically yields >750 K protoplasts (as determined by FACS).

3.4 Arabidopsis Seedling Growth

1. Put 600–800 seeds in a 1.5 mL microcentrifuge tube (*see* **Note 16**), add 1 mL 70% ethanol, and shake it using a tube rotator at 30 rpm for 1 min.
2. Discard the ethanol. Add 1 mL of sterilizing solution and shake it using a tube rotator at 30 rpm for 10 min.
3. Discard the sterilizing solution. Wash the seeds five times with 1 mL of sterile water.
4. Place ~200 *Arabidopsis* seeds across three dense rows per 12 × 12 cm square plate containing growth medium using a P1000 micropipette, with three plates per genotype for 600 seeds in total (*see* **Note 16**).
5. Seal the plate with surgical tape.
6. Stratify *Arabidopsis* seeds by placing the plates at 4 °C in the dark for 2 days for an even germination.
7. Grow the plates vertically inside 21 °C growth chambers under continuous light. We use SpectraluxPlus NL 36 W/840 Plus (Radium Lampenwerk) fluorescent bulbs at 100 μmol m^{-2} s^{-1}.

3.5 Arabidopsis Root/Cotyledon Protoplasting

1. Prepare the digestion solution fresh by adding 1.25% (w/v) cellulase and 0.3% (w/v) macerozyme to the digestion buffer (*see* **Notes 12** and **17**).
2. Add 0.1% BSA (w/v) and 10 mM $CaCl_2$.
3. Filter the solution using a syringe and a 0.45 μm membrane filter.
4. Add 10 mL of digestion solution into a 60 × 15 mm Petri dish.
5. Harvest the 600 root tips or cotyledons using a surgical blade and a pair of forceps. Slice root tips or the cotyledons to ~0.5 cm in size. Transfer them into the protoplasting solution using a pair of forceps. Make sure to not allow harvested material to dry before transferring to protoplasting solution.
6. Put the Petri dish containing the protoplasting solution on a horizontal orbital shaker at 75 rpm for 2–3 h for root tips, or 12 h for cotyledons, at room temperature. In our experiments, we have not used dark incubation for this step.
7. Collect the protoplasts with a wide-bore pipette and filter through a 40 μm cell strainer into a 50 mL tube (*see* **Note 18**).
8. Centrifuge at 150 × *g* for 10 min using a swing-bucket rotor; a pellet should be visible.
9. Discard the supernatant with a wide-bore pipette. Add 1.5 mL of ice-cold wash buffer. Gently tilt the tube multiple times until the protoplast pellet is completely resuspended (*see* **Note 19**).
10. A typical yield is ~50–100 K protoplasts.

3.6 FACS

1. Set up the fluorescence-activated cell sorter according to the manufacturer's procedure. Here, a BD FACSMelody™ cell sorter (*see* **Note 20**) is used with a 100 μm nozzle and sheath pressure set at 20 psi.
2. Select the needed parameters in the BD FACSChorus™ acquisition software: forward scatter (FSC), side scatter (SSC), and the appropriate fluorescence detectors. The detectors have a bandpass filter of 448/45 for mTagBFP2 (excitation by 405 nm laser), a bandpass filter of 527/32 nm for GFP (excitation by 488 nm laser), a bandpass of 582/15 (excitation by 561 nm laser; *see* **Note 22**), and a bandpass filter of 613/18 nm for mCherry (excitation by 561 nm laser).
3. Start by running a control sample with the plant protoplasts from the background line used (e.g., non-transformed or GFP reporter lines) on the cell sorter (*see* **Note 21**).
4. Generate a dot plot of FSC versus SSC (use linear scaling). Adapt the scatter voltage settings to centralize the measured events on the plot. Adjust the FSC threshold if needed.
5. Then create an appropriate dot plot based on the particular experiment (use biexponential scaling) (*see* **Note 22** and Fig. 3). Make sure events do not go off-scale on the fluorescence channels and lower detector voltage slightly if needed.

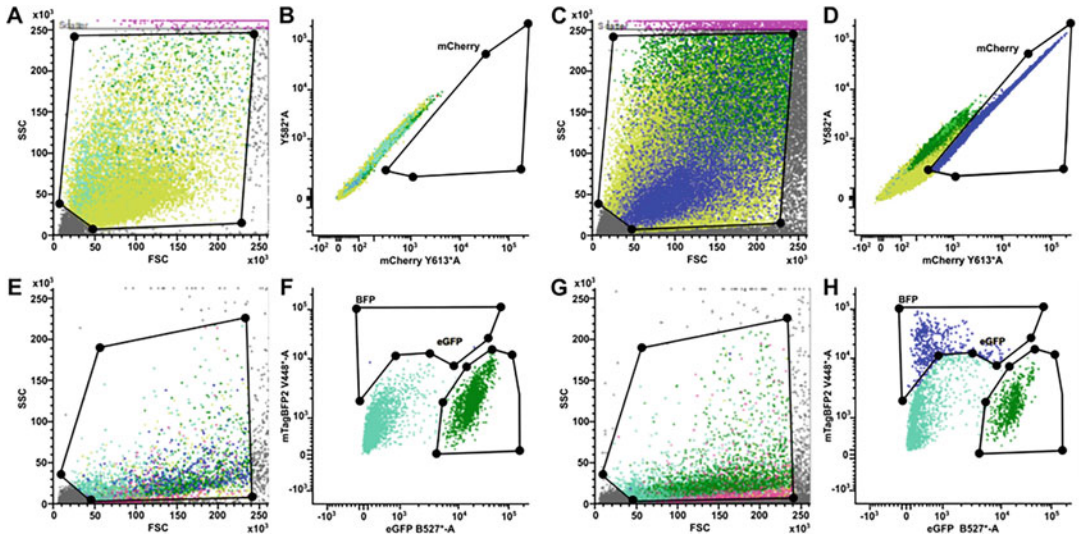

Fig. 3 Dot plots generated during FACS. (**a** and **b**) PSB-D background line. (**c** and **d**) PSB-D transformed line. (**a** and **c**) Dot plot of FSC versus SSC for PSB-D protoplasts and the gating strategy to enrich for protoplasts and reduce the amount of cell debris. (**b** and **d**) Dot plot of Y582 versus mCherry for the gated protoplasts in A and C. The represented gating is used to enrich for protoplasts expressing mCherry. (**e** and **f**) *pHTR5:NLS-GFP-GUS* background line. (**g** and **h**) *pHTR5:NLS-GFP-GUS* transformed line. (**e** and **g**) Dot plot of FSC versus SSC for *Arabidopsis* root protoplasts and the gating strategy to enrich for protoplasts and reduce the amount of cell debris. (**f** and **h**) Dot plot of mTagBFP2 versus eGFP for the gated protoplasts in **e** and **g**

6. Load a fluorescent marker-expressing sample on the FACS cell sorter. A shifted population of events should be visible on the dot plot, which should not be seen in the dot plot of the background sample (Fig. 3).
7. Set up a gate to select for cells expressing the fluorescent marker indicating the presence of Cas9.
8. Select the "Purity" sort mode in the acquisition software.
9. Sort the selected protoplasts into 1.5 mL Eppendorf tubes, which are held in a water-cooled sort collection device (*see* **Notes 23** and **24**).
10. Maintain the Eppendorf tubes on ice after sorting.
11. Centrifuge the tube at 6010 × *g* for 2 min.
12. Remove the supernatant and store at −20 °C until DNA extraction.

Figure 3 shows typical sorting results for a wild-type or background line and transformed PSB-D and *Arabidopsis* root protoplasts. The first graphs of each set (Fig. 3a, c, e, g) plots FSC vs. SSC and is used to sort protoplasts from debris. The second set of graphs (Fig. 3b, d, f, h) plots the intensity of one or two different fluorescent markers for each event mCherry (Fig. 3b, d) or eGFP and mTagBFP2 (Fig. 3f, h). By comparing the control vs. transformed lines, the gating strategy can be determined. For example, Fig. 3b is wild-type PSB-D protoplasts so the cells line up on the diagonal of mCherry vs. Y582 (*see* **Note 22**). In Fig. 3d, the transformed PSB-D cells express the mCherry marker and can be visualized as a shift in fluorescence intensity. The gates are then drawn around this population, ensuring maximum enrichment for transformed cells and that a minimum of, if any, wild-type cells are captured, as in Fig. 3b. In Fig. 3e, f, the background line already expresses the GFP marker gene. In Fig. 3g, h, the mTagBFP2 marker is used to select Cas9-expressing protoplasts. In this case, two gates are used. A population of cells only expressing GFP, but not mTagBFP2, is sorted as a Cas9 negative control. Another gate is drawn around the population of cells expressing mTagBFP2 and are thus Cas9 positive. In the example in Fig. 3e–h, the Cas9 construct also targets the GFP. Thus, the presence of Cas9 often results in the absence of GFP signal. A smaller population displays both GFP and mTagBFP2 signals and is also captured by this gating strategy. The ability to draw different gating strategies allows for researchers to select and enrich for specific cell populations. For example, the aforementioned populations could be broken into smaller ones. The Cas9-positive population (mTagBFP2 positive in this example) could be sorted as two distinct subpopulations: one GFP positive and another GFP negative, but both Cas9 positive. This would allow for investigations on

the possible different editing efficiencies and outcomes in these two populations. This versatility in establishing gating strategies is one of the benefits of using FACS.

3.7 Genotyping

The enriched population of protoplasts can be used to evaluate targeted mutagenesis in CRISPR experiments. Generally, DNA is extracted from the protoplasts followed by PCR amplification of the target sequence(s) and quantification of the mutations. A variety of methods are available for each step. Our standard procedure is to extract genomic DNA using a modified DNA extraction buffer from Edwards et al. [11] (*see* Subheading 2.7) and a 70% (v/v) ethanol washing step before the final pellet is dissolved. The extracted DNA is used as a template for PCR amplification of fragments containing the target site(s). Alternatively, a direct PCR (we have used Thermo Scientific™ Phire™ Plant Direct PCR Master Mix) can be performed, thus omitting the DNA extraction step. PCR products are purified (we use magnetic beads from MagBio or silica columns from Zymo Research) and sent for Sanger sequencing. As the chromatograms usually contain a mixture of mutant and wild-type sequences, the Sanger sequences are evaluated by sequence alignment tools or Tracking of Indels by Decomposition (TIDE) [12] or Inference of CRISPR Edits (ICE) [13]. Alternatively, amplicon sequencing using massively parallel sequencing can be performed with technologies such as Illumina sequencing [6].

3.8 Example: CRISPR-TSKO in the Lateral Root Cap

The NAC transcription factor SOMBRERO/ANAC033 (SMB) is expressed specifically in the lateral root cap (LRC) and promotes programmed cell death. Loss-of-function smb mutants have aberrant LRC cells that fail to undergo programmed cell death and are unable to perform corpse clearance, resulting in a larger LRC. We used the SMB promoter to drive Cas9-P2A-mCherry expression to induce knockout of a GFP reporter specifically in the LRC [1]. We used the same SMB promoter to drive Cas9-P2A-mCherry expression and target SMB itself and GFP, resulting in smb and gfp mutant phenotypes in the T1 and T2 generations. Importantly, the phenotypes were only observed in T2s carrying the T-DNA, indicating that the mutations were strictly somatic in nature. To confirm that DNA mutations were causing these phenotypes, the LRC cells were genotyped by protoplasting root tips of T2 seedlings followed by enrichment of mCherry-expressing cells via FACS. A population of protoplasts expressing GFP but not mCherry was also sorted to provide a Cas9 negative control.

1. Germinate 600 5-day-old T2 seedlings on 1/2 MS and isolate root tips (*see* Subheading 3.4).
2. Prepare protoplasts (*see* Subheading 3.5) and sort (*see* Subheading 3.6) using a gating strategy to enrich for mCherry-

expressing cells (*see* Supplemental Figure 6 in [1] for the gating strategy).

3. Extract DNA from protoplasts using a modified protocol from Edwards et al. [11] (*see* Subheading 3.7).
4. Perform PCR with primers 1 and 2 with the ALLin Red Taq Master Mix 2× (highQu) with the thermal cycler conditions of 95 °C for 3 min, 35 cycles (30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C), and 72 °C for 5 min in a 20 μL reaction. Include wild-type and water controls.
5. Check ~5 μL of PCR products by gel electrophoresis.
6. If the water control is free of contaminating bands, purify the remaining PCR products with magnetic beads (HighPrep) or columns (DNA Clean and Concentrator-5) and send for Sanger sequencing via Mix2Seq (Eurofins Scientific).
7. Analyze chromatograms with the online software Tracking of Indels by Decomposition (TIDE) [12] by uploading the sample and wild-type chromatograms and ensure an R2 of 0.9 or higher.

By using this FACS and genotyping approach, we are able to demonstrate that ~98% of the SMB sequences are mutated in mCherry-expressing cells [1]. This indicates that CRISPR-TSKO is highly efficient at inducing mutagenesis in the LRC and is responsible for the smb phenotypes observed in those lines. Such confirmation would not be possible using manual dissection methods as non-LRC cells would contaminate the samples and it would be impossible to determine the precise mutagenesis rate in the LRC. Thus, plant cell protoplasting followed by FACS allows us to make precise estimations on DNA mutagenesis which is useful for characterizing novel CRISPR vectors and to provide evidence for mutagenesis in specific cells, tissues, and organs targeted by CRISPR-TSKO.

4 Notes

1. Dissolve salt in indicated solvent in a glass beaker with a magnetic stirrer using a smaller solvent volume (~80%) than required. Remove the magnet and transfer the solution into a volumetric flask. Rinse the beaker and collect in the same volumetric flask. Adjust the volume with solvent or diluent. Cover the flask with a sealing film, such as Parafilm®, and tilt the flask multiple times to mix.
2. The quality of enzymes might differ between manufacturers. For the PSB-D protoplasting, we use Cellulase Y-C from Kyowa Chemical Industry Co., Ltd. and Macerozyme R-10

from Duchefa Biochemie. For the *Arabidopsis* root and cotyledon protoplasting, we use Cellulase Onozuka R-10 and Macerozyme R-10 both from Yakult.

3. Seeds for the line *pHTR5:NLS-GFP-GUS* are available from the Nottingham Arabidopsis Stock Centre (NASC), ID N2109788 [8].
4. Leave the screw cap somewhat loose on the Erlenmeyer flask before taping with surgical tape to ensure adequate air exchange.
5. Our weekly subculture is performed on Tuesdays. As such, the transformation transfers take place on the following weekdays: Day 1 (Tuesday), Day 2 (Wednesday), Day 3 (Thursday), Day 4 (Friday), Day 7 (Monday), Day 15 (Tuesday), Day 22 (Tuesday), Day 24 (Thursday).
6. We have found that some individual *Agrobacterium* clones from the same electroporation consistently have a lower transformation efficiency than others. Therefore, for each transformation, we inoculate three different clones of *Agrobacterium* containing the same plasmid in a single culture tube.
7. The calculated $O.D._{600}$ of the starting solution generally ranges between 0.8 and 3.0. If the measured $O.D._{600}$ is greater than 1.0, additional dilutions are required to obtain an accurate measurement.
8. Resuspension volume to add to achieve a final $O.D._{600}$ of 1

   Measured O. D. value × (20 mL − mL taken for O. D. measurement)

   For example, if a sample has a measured $O.D._{600}$ of 1.4 and 1 mL was used to measure the $O.D._{600}$, add 1.4 × 19 mL = 26.6 mL of MSMO.
9. Acetosyringone is added directly to each individual well to limit the waste of the acetosyringone stock.
10. Before transferring, tilt the plate and pipette some of the solution to the top of the plate, to collect the cells at the bottom.
11. Let the cells settle and pipette them up while moving around on the bottom of the Erlenmeyer much like a vacuum cleaner. We refer to this as "vacuuming" (*stofzuigen*).
12. Dissolve the digestion solution using a magnetic stirrer.
13. The gentle shaking should cause a tiny wave that goes around in the plate. Our shaker is set at ~30 rpm.
14. We check our protoplasts with the inverted Diavert microscope (Leitz) using a 125 × magnification.

15. Avoid adding the buffer directly to the top of the pellet, pour against the side wall of the falcon. The volume of wash buffer added might be adjusted depending on the yield of protoplasts.
16. A seed volume of 100 μL usually corresponds to ~1000 seeds.
17. Depending on the enzyme quality, it is sometimes recommended to heat the solution at 55 °C for 10 min to help dissolve the enzyme and inactivate proteases from the cellulase and macerozyme products.
18. At this step, the protoplasts can be transferred into a round-bottomed tube for centrifugation to avoid breaking them during the resuspension; we also recommend using slow acceleration and breaking in swing-bucket rotors for the centrifugation.
19. At this stage, it is possible to inspect the protoplasts microscopically and to use a hemacytometer to estimate the quality and number of protoplasts. Living protoplasts are round shaped and very little debris should be visible. Ideally, a density of 10^6 mL^{-1} protoplasts can be obtained.
20. Our BD FACSMelody™ cell sorter is equipped with three lasers (405 nm, 488 nm, and 561 nm).
21. Make use of the agitation option to keep the protoplasts in suspension.
22. For the PSB-D cells transformed with mCherry, we plot mCherry versus Y582 (Fig. 3b, d). By applying the "neighboring" detector Y582, we create a centered diagonal pattern when evaluating the PSB-D background line (Fig. 3b [5]). A transformed PSB-D line will generate a population of mCherry-expressing cells that was not seen in the PSB-D background line (Fig. 3c). This strategy works well to avoid capturing autofluorescent cells. For the pHTR5:NLS-GFP-GUS line transformed with Cas9-P2A-mTagBFP2 vectors, we plot eGFP versus mTagBFP2 (Fig. 3f, h).
23. We have successfully sorted a wide number of protoplasts for genotyping. In our initial protocols, we sorted ~75–100 K protoplasts [1]. However in later experiments, we have sorted as few as 8 K protoplasts per sample and were still able to obtain PCR products from the samples. This is particularly important when transformation efficiencies are low (e.g., >5%), as sorting tens of thousands of cells can require a significant amount of time at the sorter. The number of sorted protoplasts needed for an assay depends on a number of variables, namely, the DNA extraction method and efficiency of the PCR. Therefore, we recommend researchers empirically determine the number of protoplasts they need to collect for reproducible PCR before

conducting a large experiment, for example, a series of samples with 5, 10, 20, 50, and 100 K.

24. The final volume of the sample depends on the number of protoplasts sorted. For ~75–100 K protoplasts, it is usually ~400–500 μL.

Acknowledgments

We thank Julie Van Duyse and Gert Van Isterdael from the VIB Flow Core (VIB Center for Inflammation Research, Ghent, Belgium) for their input on the FACS settings.

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Chapter 16

Characterization of Programmable Transcription Activators in the Model Monocot *Setaria viridis* Via Protoplast Transfection

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Abstract

Recent advances in DNA synthesis and assembly allow for genetic constructs to be designed and constructed in high throughput. Characterizing large numbers of variant genetic designs is not feasible with low-throughput and time-consuming plant transformation workflows. Protoplast transformation offers a rapid, high-throughput compatible alternative for testing genetic constructs in plant-relevant molecular environments. Here, we describe a protocol for protoplast transformation using a recent experiment in genetic optimization of dCas9-based programmable transcription activators as an example.

Key words *Setaria viridis*, Protoplasts, Transfection, Monocot, dCas9, Transcription, High throughput

1 Introduction

Just as other engineering disciplines, plant genetic engineering and synthetic biology benefit from an iterative Design, Build, Test, Learn (DBTL) cycle. The completion of each cycle generates improved performance and increased understanding of the system. Progress toward engineering objectives is largely determined by the rate at which the DBTL cycle can be iterated.

For plant engineering, the Test step is the rate-limiting bottleneck within each DBTL cycle. Designing and building a large, combinatorial set of genetic constructs is fast and inexpensive. Decreasing costs of DNA sequencing and increased computational power have generated large databases of DNA and protein sequences that can be mined to identify genes of interest [1–4]. These can function as parts libraries for genetic engineering efforts. Large genetic parts libraries, coupled with algorithmic

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_16,

genetic design tools, allow for the design of hundreds to thousands of unique constructs [5, 6]. The modernization of DNA synthesis and assembly technologies, especially through isothermal and Golden Gate assembly, allows relatively rapid and economical fabrication of large combinatorial libraries of constructs [7, 8]. Combinatorial libraries of genetic constructs can be built in vitro, with the total number of unique and testable designs limited only by transfection efficiency. In good conditions, these processes bring the Build step to the order of hundreds to thousands of constructs within a week even for relatively small labs. Lastly, machine learning and artificial intelligence workflows [9] are widely available to facilitate and accelerate the Learn step of each DBTL cycle.

In comparison, plant engineering greatly lags in the Test step of the engineering cycle. We illustrate this using *Setaria viridis*, a model monocot plant, as a model system. *S. viridis* produces seeds 6–8 weeks post-germination and the seeds remain dormant for 6 months, a timeline that can be shortened to 1 week post-harvest through a variety of treatments [10]. The time to generate a homozygous transgenic plant is approximately 1 year and requires intensive resources to generate and screen putative transgenics. The process of transformation requires plant tissue culture, a selection stage, and transplantation, in addition to phenotypic screening. For *S. viridis*, 100 transformations would require approximately 1 m^2 in a growth chamber and up to 100 m^2 of growth chamber or greenhouse space for homozygosing (Fig. 1).

For transgenic phenotypes that can be screened at the tissue culture stage, less time and space are required compared to generating homozygous transgenics. The turnaround time for construct characterization is on the order of weeks. However, a balanced treatment with hormones is necessary to maintain the callus state [11]. In practice, a single individual would be expected to be able to screen 15 constructs per cycle for either of these methodologies. Thus, even tissue culture would be a substantial bottleneck for plant DBTL cycles.

Agrobacterium rhizogenes naturally co-opts the metabolism of dicots to produce its preferred food source, opines, and transforms the tissue into immortalized "hairy root" tissue. The tissue may be subsequently transformed with *Agrobacterium tumefaciens* allowing for multiple rounds of genetic delivery. The generation of a hairy root culture is advantageous over callus culture as hormone treatment is not required. Additionally, cultures can be grown in liquid media. In the case of either culture technique, some species of plants may be regenerated from the culture. However, the generation of a hairy root requires approximately 3 weeks, and space requirements are comparable to that of tissue culture [12–14]. A spike dip infiltration allows for *in planta* transformation by dipping the spike of *S. viridis* in a suspension of *A. tumefaciens*. This methodology has the advantage of resembling the eventual full

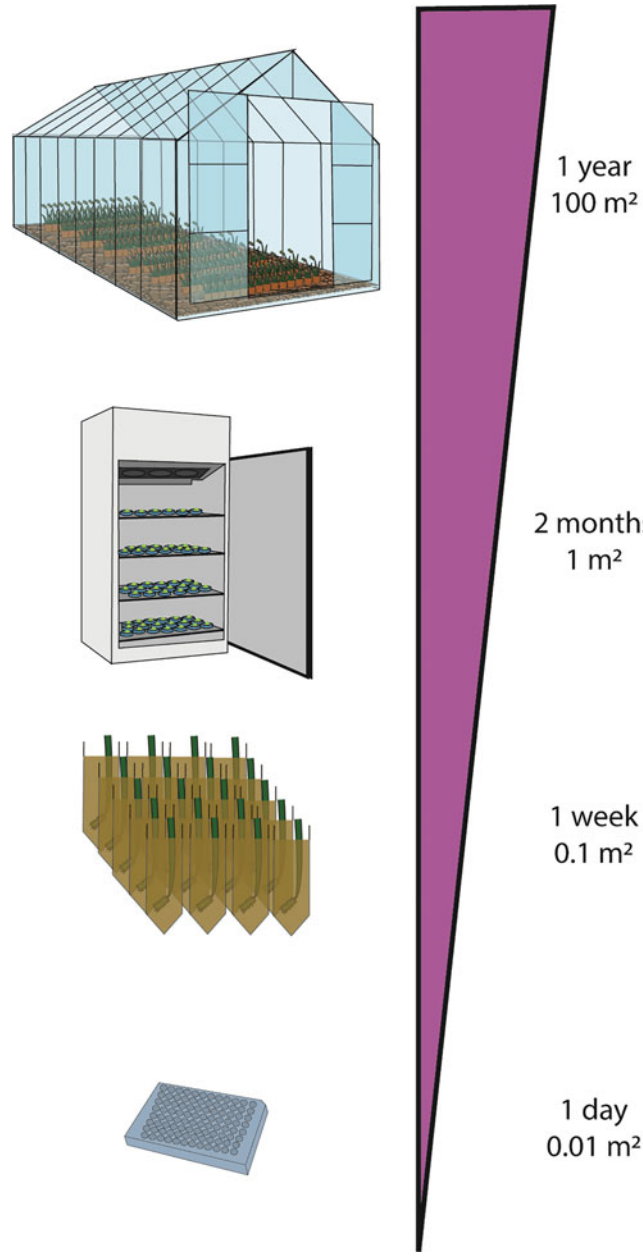

Fig. 1 A comparison of plant genetic construct characterization paradigms. On the right are the approximate time and space requirements to test 96 unique constructs. Top to bottom: Generating transgenic plants, hairy root or plant tissue culture, spike dip transformation, and high-throughput protoplast transformation

plant target. Spike dips require 5–7 days before they can be screened. Space requirements are lower by another factor of ten compared to culture [15, 16]. In addition to the space and time

requirements, several agriculturally relevant plant species are recalcitrant to *A. tumefaciens* [17].

In all the above-described methods, screening can be done by qRT-PCR, quantification of protein, or detection of a reporter such as luciferase. In addition to each methodology having limitations in terms of time and space necessary to perform a transformation, downstream analysis suffers from several points. First, reporter readouts typically must be done in a low-throughput manner to accommodate whole plants or culture plates. Protein quantification or qRT-PCR needs the respective material to be extracted from heterogeneous tissue, lowering the signal-to-noise ratio.

The use of plant protoplasts overcomes many of the bottlenecks associated with testing alternative genetic constructs in plants. Protoplasts can be generated from a variety of plant species by enzymatic removal of the cell wall [18]. The resulting protoplasts are more amiable to DNA transfection than their walled counterparts [19–21]. This property, along with the ability for high-throughput experimentation, makes them an ideal system for screening and selecting variant genetic constructs.

Here, we describe a protocol for the generation of *S. viridis* protoplasts with typical yields of 1.0–1.4 $\times$ 10^6 protoplasts/gram of leaf tissue. We then provide two transfection protocols for high- and low-throughput methods. With the high-throughput pipeline, we can reliably transfect 96 constructs in a single day, using a single 96-well cell culture dish. This methodology is reasonably scalable to multiple 96-well plates per day. Furthermore, we provide methods for downstream analysis by either luciferase or qRT-PCR in the 96-well format. From start to finish, isolation, transfecting, and screening nearly 100 unique constructs can be done in less than 36 h.

As an example experimental system, we describe the characterization of CRISPR-Cas activators (also known as programmable transcription activators, or PTAs). PTAs embody the combinatorial screening problem described above. Several PTAs that efficiently induce transcription have been developed using different strategies to recruit activation domains to the promoter of its target genes [22]. The strength of the activation provided by these systems is strongly influenced by their binding (provided by the sgRNA) proximity to the transcription start site (TSS). For instance, in plants, targeting of a PTA to the region −300 to +1 bp relative to the TSS provides the most efficient gene overexpression [23]. In addition, the recruitment of multiple copies of the PTA by different sgRNAs provides a synergistic effect on transcriptional activation [24]. Thus, selection of the most efficient PTA system and the use of several sgRNAs targeting the region upstream of the TSS should be considered to achieve optimal gene activation. Efficiency of a sgRNA, even when targeting the region upstream of the TSS, is difficult to predict and must be determined empirically. For this

reason, multiple sgRNAs are commonly designed for target genes and tested individually for activation efficiency. Once the most efficient sgRNAs are identified, they could be combined to yield even stronger gene activation. Either single or multiple sgRNAs could be used to compare different PTA systems in the target organism [24]. Selection of a PTA and sgRNAs driving the most effective gene activation will therefore require at least three iterations of activation assays. Protoplast transient assays are uniquely suited for PTA optimizing tasks.

2 Materials

Prepare all solutions using ultrapure water (*see* **Note 1**) and analytical grade reagents. All reagents can be stored at room temperature (22–26 °C) unless indicated. All stock solutions are filter sterilized using a 0.2 μm filter.

2.1 Stock Solutions

1. NaCl (1 M): Weigh out 58.44 g of NaCl. Suspend in a total volume of 1 L of H_2O stirring until dissolved.
2. $CaCl_2$ (1 M): Weigh out 55.49 g of $CaCl_2$. Suspend in a total volume of 500 mL of H_2O stirring until dissolved.
3. KCl (1 M): Weigh out 18.64 g of KCl. Suspend in a total volume of 250 mL of H_2O stirring until dissolved.
4. Mannitol (0.8 M): Weigh out 145.74 g of mannitol. Suspend in a total volume of 1 L of H_2O stirring until dissolved.
5. Glucose (1 M): Weigh out 45.04 g of glucose. Suspend in a total volume of 250 mL of H_2O stirring until dissolved.
6. $MgCl_2$ (1 M): Weigh out 50.83 g of $MgCl_2$ hexahydrate. Suspend in a total volume of 250 mL of H_2O stirring until dissolved.
7. MES buffer (2 M, pH 5.7): In a 250 mL container, add 200 mL H_2O. Weigh out 97.6 g of 2-(*N*-morpholino)ethanesulfonic acid and add to the H_2O. Mix and adjust to pH 5.7 using 1 N HCl. Bring up to 250 mL. Store at 4 °C.
8. Passive Lysis Buffer (PLB, 5×): Promega Cat. Number E1941.

2.2 Working Solutions and Media

1. LB medium: Weigh out 12.5 g of LB media powder. Add 500 mL H_2O. Autoclave at 121 °C and 15 psi for 40 min.
2. Enzymatic solution: Make fresh for every protoplast isolation. Weigh out 0.225 g Cellulase R-10 (Yakult Pharmaceutical Industry Co.) and 0.1125 g Macerozyme R-10 (Yakult Pharmaceutical Industry Co.). Combine these in a 50 mL conical tube, adding 11.25 mL 0.8 M mannitol. Place the tube in a 50–55 °C water bath for 10 min. Remove and allow to cool to

room temperature. Add 1.5 mL 1% BSA, 150 µL of 1 M $CaCl_2$, and H_2O to bring up to 15 mL (*see* **Note 2**).

3. W5: In a laminar flow hood, prepare a sterile 1 L bottle. Add 154 mL of 1 M NaCl, 125 mL of 1 M $CaCl_2$, 5 mL of 1 M KCl, 1 mL of 2 M MES (pH 5.7) buffer, 5 mL of 1 M glucose. Add 710 mL of sterile H_2O for a final volume of 1 L.
4. MMG buffer: In a laminar flow hood, prepare a sterile 500 mL bottle. Add 250 mL of 0.8 M mannitol, 15 mL of 1 M $MgCl_2$, 2 mL of 2 M MES (pH 5.7) buffer, and 233 mL of sterile H_2O for a final volume of 1 L. Store at room temperature wrapped in foil.
5. PEG 4000 solution: Make fresh once protoplasts are isolated. To prepare 10 mL 40% PEG 4000 with MMG buffer, add 4 g PEG 4000 in a 50 mL Falcon tube, then add MMG buffer to 10 mL. Place the tube on a shaker set to 225 rpm until dissolved, briefly vortexing every 2 min. You will need a total of 750 µL/low-throughput transfection reaction or 100 µL/high-throughput transfection reaction of PEG 4000 solution rounding up to the nearest mL.
6. Passive Lysis Buffer (PLB, 1×): Add 1 volume of 5× PLB to 4 volumes of H_2O. Mix well. Store at 4 °C for no more than 1 month.

2.3 Supplies and Equipment

1. Wide orifice micropipette tips (*see* **Note 3**).
2. Potting soil: Sunshine Grower's Professional Potting mix SS#8-F2.
3. Cellulase R-10: Yakult Pharmaceutical Industry Co.
4. Macerozyme R-10: Yakult Pharmaceutical Industry Co.
5. PEG 4000: polyethylene glycol 4000.
6. Round-bottom 96-well tissue culture plate.
7. 15 mL round-bottom culture tube.
8. 50 mL conical tubes.
9. Centrifuges (swing-rotor, tabletop centrifuge with refrigeration, PCR plate compatible centrifuge).
10. Multichannel pipette.
11. Hemocytometer.
12. Luminometer (Promega: GM3000).
13. DNA midi-prep kit (Macherey-Nagel: 740410).
14. Kits for quantitation of firefly and *Renilla* luciferase activities (Promega: E1910, E1960, and E1980).
15. LB media powder (Fisher Scientific: DF0446-17-3).
16. TRIzol reagent (Thermo-Fisher: 15596018).

17. RNase-free glycogen (5 μg/μL) (Invitrogen: AM9510).
18. Isopropanol.
19. 75% ethanol (in nuclease-free H_2O).
20. TURBO DNase (Invitrogen: AM2238).
21. RNase removing spray.
22. RT-qPCR Kit (New England Biolabs: E3005).

2.4 Plant Materials

1. *Setaria viridis* cv ME034V: Seeds were kindly provided by Dr. Daniel Voytas of University of Minnesota.

3 Methods

Carry out all methods at room temperature (22–26 °C) unless otherwise indicated.

3.1 DNA Preparation

1. For every construct, you will be transfecting (*see* **Note 4**), aliquot 50 mL of LB media with the appropriate selection agent into a 15 mL label shake flask (*see* **Note 5**).
2. Retrieve glycerol stock containing the desired construct and keep on ice. Using a 20 μL micropipette with tip, stab the glycerol stock, ejecting the tip into the appropriate shake flask.
3. Incubate the shake flasks at 37 °C for 16 h. Ensure that they are shaking at 200 rpm.
4. Transfer cultures by directly pouring into labeled 50 mL conical flasks. In a centrifuge with appropriate balances, spin down at 6500 rcf for 5 min.
5. Discard the supernatant (*see* **Note 6**).
6. If the pellet was frozen, thaw at room temperature. Utilizing a midi-prep kit, isolate the plasmid DNA (*see* **Notes 7** and **8**).
7. Quantify the concentration of each plasmid using a UV spectrophotometer. Ensure that the 260 nm/280 nm ratio is above 1.75. The DNA can be stored at −20 °C without affecting transfection efficiency for at least 6 months (*see* **Note 9**).

3.2 Growing S. viridis

1. We routinely grow *Setaria viridis* cv ME034V in a growth chamber set at 31 °C and 21 °C of diurnal and nocturnal temperatures, respectively. The photoperiod is set at 12 h and 400–450 PAR (photosynthetically active radiation) of light intensity.
2. For planting, pots (sized 9 × 9 × 9 cm) are filled with potting soil leaving 1 cm of space from the top. Pots are placed in a tray and then the soil allowed to moisten by capillarity.

3. Enough seeds to produce 10–15 seedlings (*see* **Note 10**) are sprinkled on the top of the moistened soil and then covered with an additional 1 cm of soil.
4. Pots are then placed into the growth chamber. Seedlings usually appear after 5–6 days in the growth chamber.
5. Pots are watered by capillarity every 2 days using a diluted fertilizer solution (*see* **Note 11**).
6. Plants should be ready for harvesting between 14 and 16 days (*see* **Note 12**) after sowing and could be used until day 21, after which they start flowering.

3.3 Protoplast Isolation

Two-week-old plants yield the healthiest protoplasts as well as the highest counts. Ideal leaves will be a deep green, possibly with some lighter stripes along the blade. Light green leaves will result in fewer protoplasts and lower transfection efficiency.

1. In a laminar flow hood, using a clean razor blade, cut *S. viridis* leaves at the base ensuring removal of the collar (Fig. 2a) (*see* **Notes 13** and **14**).
2. Make clean cuts perpendicular to the blade, such that each piece is 2–3 mm long (Fig. 2b). Do not cut the blade lengthwise. Aggressive cutting will trigger a stress response, turning leaves to a dark, moist green. Discard stressed pieces as they reduce the health of the protoplasts (Fig. 2c, d) (*see* **Note 15**).
3. Collect the pieces in a 10 cm untreated plastic Petri dish containing 10 mL of 0.6 M mannitol. Leaves should form slightly more than one layer. If you have leftover leaves, collect in a second dish. Add an additional 10 mL 0.6 M mannitol solution to each dish for at least 10 min (*see* **Note 16**).
4. Back in the laminar flow hood, remove mannitol wash from cut leaves using a serological pipette. Add 12.5 mL of the enzymatic solution to each plate.
5. Place plates in a vacuum desiccator, and keep lids of the Petri dishes slightly ajar (Fig. 2e). Set vacuum between 10 and 15 in Hg. Keep under vacuum for 30 min. Slowly repressurize vacuum (*see* **Note 17**).
6. Place plates on a plate shaker at 25 °C and protect from light using foil. Set the shaker to 40 rpm for 3–4 h. Increase the speed to 80 rpm for an additional 30 min.
7. The remainder of the protoplast isolation should be done in a laminar flow hood. Place a 70 μm filter in a clean 10 cm Petri dish. Pipette 2 mL of W5 buffer onto the filter to prime.
8. Sharply shake the plate with digested leaf pieces in a cross pattern, just enough that the solution does not spill out. The

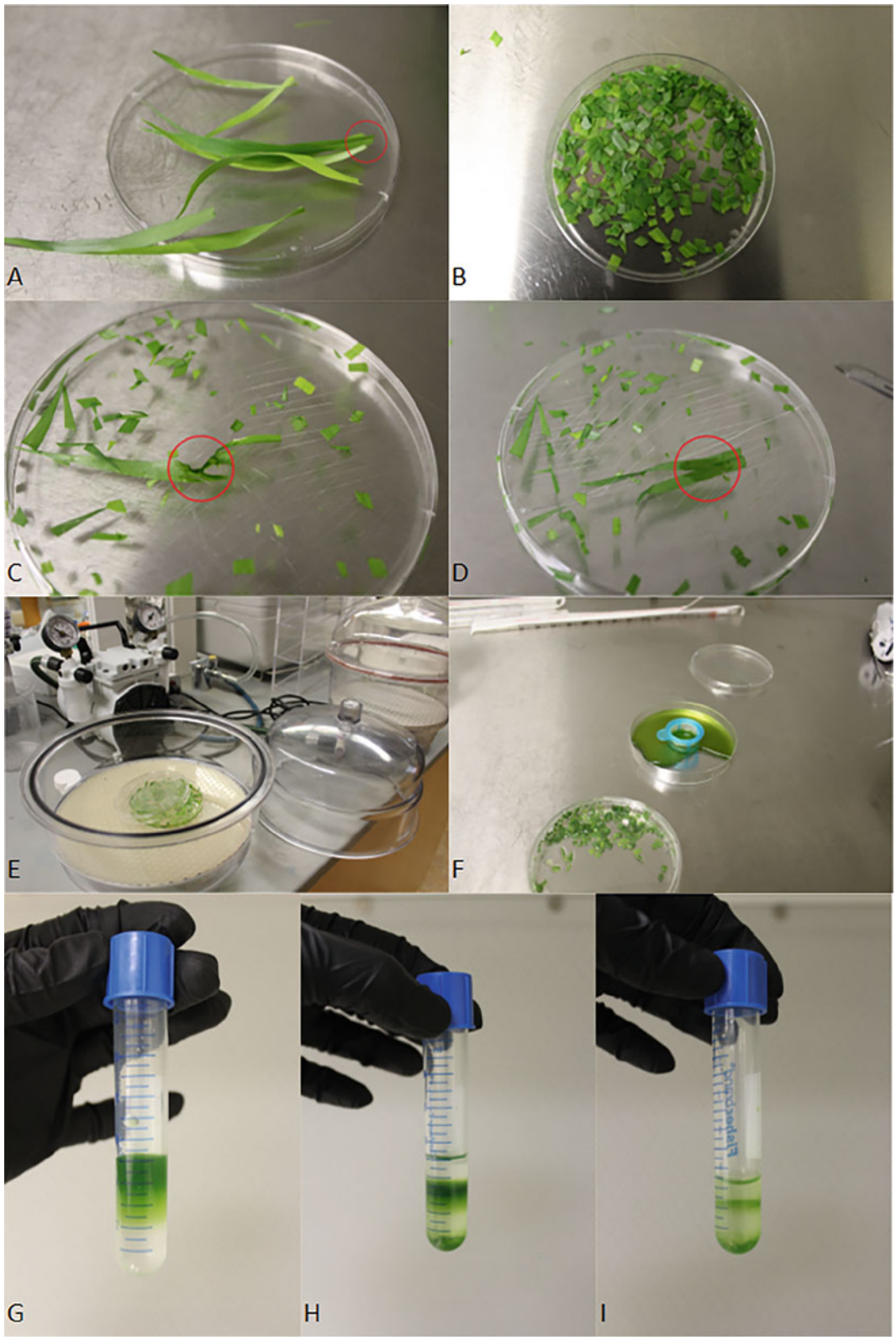

Fig. 2 Cutting *S. viridis* leaves for protoplast extraction. Pictures taken on a 10 cm diameter Petri dish for scale. (**a**) Portion of the leaf to be collected. Circle indicates where to cut in order to remove collar. (**b**) Leaves

solution should be a deep, bright green. Using a serological pipette, slowly transfer liquid to the filter (Fig. 2f).

9. Add 10 mL of W5 buffer to leaves. Shake to release more protoplasts. Transfer liquid to the filter.
10. Repeat **step 9**.
11. Carefully remove the filter from the Petri dish allowing remaining liquid to drip off.
12. Collect liquid using a serological pipette, slowly dispensing into a 50 mL conical centrifugation tube. Dispense along the wall of the conical tube, just above the liquid line, so as to not stress the protoplasts.
13. Centrifuge in a swinging-bucket rotor at 100 rcf for 5 min.
14. Using a serological pipette or a syringe with a blunt tip needle, carefully remove the supernatant.
15. Add 8 mL of W5 and gently invert until the pellet is fully suspended.
16. Repeat **step 13**.
17. In the meantime, add 3 mL of 0.55 M sucrose solution into a 15 mL disposable culture tube.
18. Repeat **step 14**.
19. Add 2 mL of W5 and gently invert until the pellet is fully suspended.
20. Using a serological pipette, slowly transfer the resuspended protoplasts dropwise along the wall of the culture tube just above the sucrose liquid line. There should be two layers, clear and green, respectively (Fig. 2g).
21. Carefully, so as to not mix the layers, place the tube in a centrifuge with a swinging-bucket rotor and turn off powered deceleration.
22. Spin at 500 rcf for 5 min. Deceleration will take an additional 5–15 min.
23. In the meantime, add 5 mL of W5 buffer to a clean 15 mL culture tube.

Fig. 2 (continued) cut down to appropriate size for enzymatic reaction. (**c** and **d**) Examples of leaves to not include in digest. Note the darkening of the leaf as a stress response indicated by the circles. (**e**) Enzymatic reaction ajar in vacuum desiccator. (**f**) Image of debris filtering step. (**g**) Isolating protoplasts from debris using a sucrose gradient. Gradient prior to centrifugation with two distinct layers formed. (**h**) Layering after centrifugation with protoplasts present at the green central layer. (**i**) Sucrose gradient after protoplasts have been transferred into W5 buffer

24. The spin will separate out the protoplasts from debris. Debris will settle to the bottom of the tube, while the protoplasts float in the intermediate layer between two clear layers (Fig. 2h).
25. Using a wide orifice micropipette, transfer the protoplast layer to the 5 mL of W5, 200 μL at a time. Use a slow motion while releasing the micropipette plunger and minimize the amount of the lower clear layer you transfer (Fig. 2i).
26. Repeat **step 13**. Standard deceleration may be used.
27. Repeat **step 14**.
28. Add 5 mL of W5 buffer and gently invert until the pellet is fully suspended.
29. Using a hemocytometer visualized at 10× magnification, calculate the concentration of living protoplasts. Live protoplasts will be spherical and transparent. They commonly have an asymmetrical distribution of smaller spheres within. Dead protoplasts will be dark aspherical clumps (Fig. 3a).
30. Repeat **steps 13** and **14**.
31. Add MMG buffer to protoplast to bring final concentration to 10^6 protoplasts/mL (*see* **Note 18**).
32. Store protoplasts at room temperature in the dark until ready to use. We recommend beginning the transfection immediately. Alternatively, protoplasts may be stored in the dark at 25 °C overnight and then transfected with lower efficiency.

3.4 Low-Throughput Transfection

1. For each transfection, prepare a sterile plastic 15 mL culture tube. Add 10 μg of each plasmid necessary for the transfection (*see* **Note 1**).
2. Gently invert the tube with protoplasts (prepared in Subheading 3.3) to resuspend. Using wide orifice pipette tips, transfer 5×10^5 protoplasts (500 μL) to a 15 mL culture tube with DNA.
3. Pipetting slowly, add 500 μL of the 40% PEG 4000 solution to the protoplasts and DNA. Tightly seal the caps for the culture tubes and gently invert once. Immediately begin a timer set for 10 min and place the tube in the dark (*see* **Note 19**).
4. At the end of 10 min, add 3.5 mL of W5 buffer to each sample. Tightly seal caps for the culture tubes and gently invert twice.
5. Centrifuge at 100 rcf for 5 min. In the meantime, label a 24-well untreated culture plate.
6. Remove the supernatant using a micropipette.
7. Using a wide orifice micropipette tip, suspend the pellet in 1 mL of the W5 buffer and transfer into the appropriate well in the culture plate.

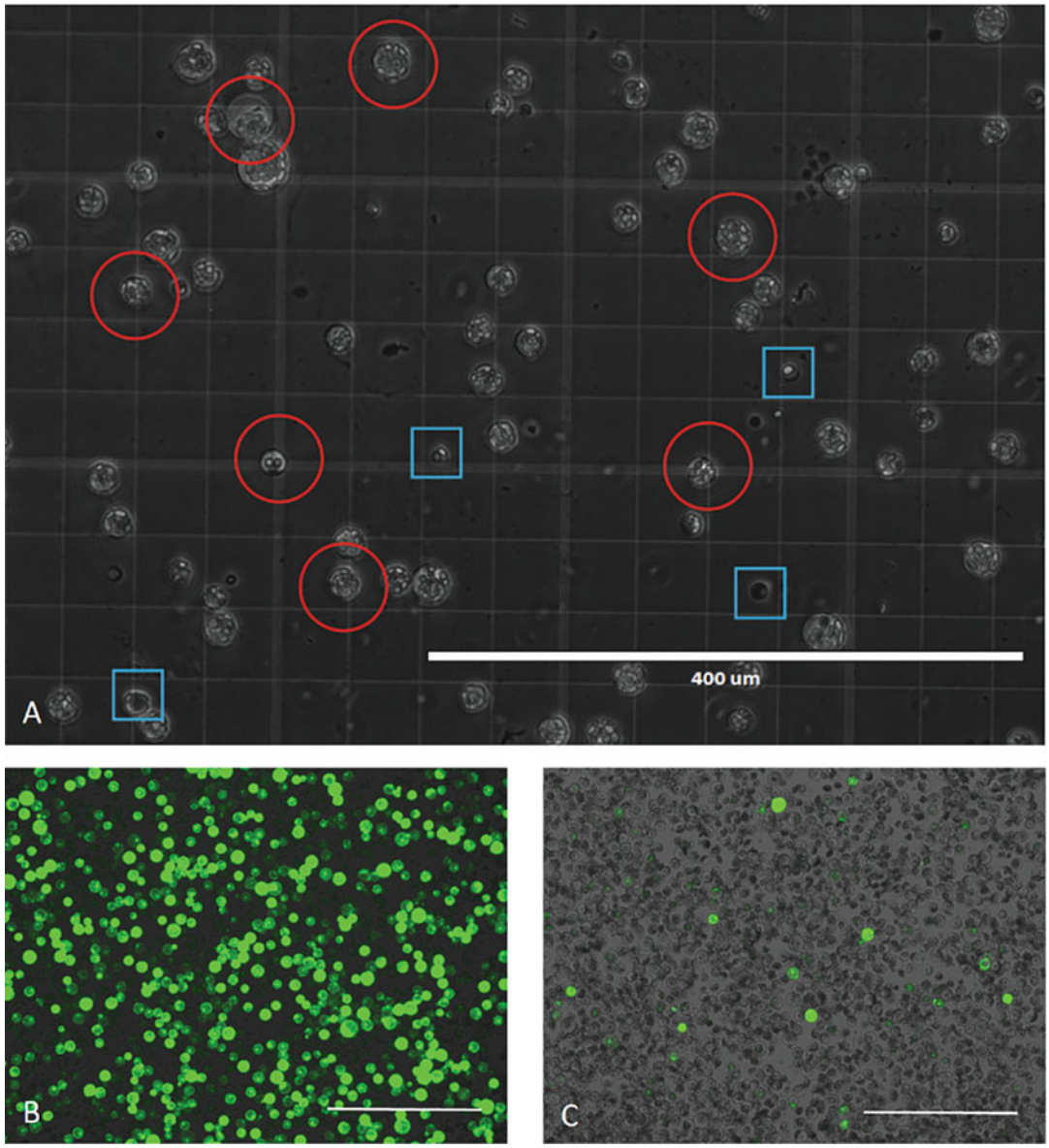

Fig. 3 (**a**) Microscope image of isolated protoplasts after sucrose gradient. Red circles indicate some examples of healthy protoplasts. Blue squares indicate some examples not to be counted. (**b** and **c**) Side-by-side comparison of transformation efficiency by brand of midi-prep kit. These images are representative figures of transformations done in triplicate on the same batch of protoplasts using the same settings on image gathering using the microscope. All conditions were held constant except for the brand of midi-prep used to collect plasmid DNA. The right figure demonstrates what a successful protoplast transformation should look like with a GFP-expressing control. Here, GFP is driven by the PbUbiquitin promoter. Approximate scale bar: 0.4 mm

8. Store the plate undisturbed in the dark at room temperature for 16–24 h. Visualize under 10× magnification. The percentage of protoplasts positive for GFP can be used to estimate the transfection efficiency (Fig. 3b, c).

3.5 96-Well Format High-Throughput Transfection

1. Preload a round-bottom 96-well tissue culture plate with all DNA plasmids (2 μg in 20 μL H_2O) intended for transfection (*see* **Note 20**).
2. Weigh out PEG 4000 for a 40% weight/volume solution into a 15 mL or 50 mL Falcon tube. Prepare 100 μL/transfection of PEG solution rounding up to the nearest mL, with a transfection representing a preloaded well. Add MMG buffer up to total volume and place on a shaker. As you conduct the protoplast isolation, the solution will dissolve.
3. Conduct protoplast isolation as indicated in Subheading 3.3 for **steps 1–27**.
4. Add 2 mL of MMG buffer to the protoplast pellet and gently invert until the pellet is fully suspended.
5. Using a hemocytometer, calculate the concentration of living protoplasts. Add MMG buffer to protoplasts to bring final concentration to 10^5 cells/50 μL. Store protoplasts at room temperature in the dark until ready to use.
6. Preload three polystyrene reagent reservoirs/basins with the protoplast suspension, PEG solution, and W5 buffer by decanting (*see* **Note 21**).
7. Using a multichannel pipette and large orifice tips, aliquot 50 μL of protoplasts (10^5 cells) into each preloaded well (*see* **Note 22**).
8. Using a multichannel pipette and large orifice tips, add 50 μL of PEG solution into each preloaded well containing protoplasts. Slowly pipette up and down three times to mix solutions.
9. Incubate the plate in the dark on the bench for 10 min.
10. Using a multichannel pipette and large orifice tips, add 100 μL W5 solution. Slowly pipette up and down three times to mix solutions.
11. Centrifuge the plate at 100 rcf for 5 min.
12. Using a multichannel pipette and regular orifice tips, remove the supernatant (~150–170 μL).
13. Using a multichannel pipette and large orifice tips, add 150 μL W5 buffer. Slowly pipette up and down three times to mix solutions.
14. Repeat **steps 13** and **14**.

15. Using a multichannel pipette and large orifice tips, add 100 µL W5 buffer. Slowly pipette up and down three times to mix.
16. Store the plate undisturbed in the dark at room temperature for 16–24 h.

3.6 Reporter Gene Detection

The following protocol was developed using a luminometer and dual luciferase assay (*see* **Note 23**). To carry out the protocol, a luminometer with at least two injectors is recommended. Promega kits E1910, E1960, and E1980 were used for detecting firefly and *Renilla* luciferase activity *in planta*.

1. Decant (20 µL × number of transfection reactions) of 1× PLB buffer into a polystyrene reagent reservoir.
2. Centrifuge the 96-well plate with the transfected protoplasts at 300 rcf for 5 min.
3. Using a multichannel pipette and regular orifice tips, remove the supernatant.
4. Using a multichannel pipette and regular orifice tips, add 20 µL of 1× PLB to each well. Pipette up and down three times to resuspend the pelleted cells.
5. Place the 96-well plate on a shaker for 15 min at 50 rpm (*see* **Note 24**).
6. While passive lysis is occurring, prepare the firefly and *Renilla* luciferase substrates per the manufacturer's instructions.
   (a) LARII: Resuspend the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II.
   (b) 1× Stop & Glo Buffer: Add 50× Stop & Glo substrate to the required amount of Stop and Glo Buffer (0.2 mL of 50× substrate to 10 mL buffer).
7. Transfer ~20 µL of lysate to a polystyrene luminometer plate equipped for your luminometer.
8. Wash the injectors with 70% ethanol solution.
9. Prime the injectors with the prepared LARII and 1× Stop & Glo (*see* **Note 25**).
10. Set a program such that the one injector first dispenses 100 µL of LARII per well, followed by 3 s of shaking, followed by 10 s of luminescence detection.
11. Injector 2 should then dispense 100 µL of 1× Stop & Glo solution per well, followed by 3 s of shaking, followed by 10 s of luminescence detection (*see* **Note 26**).

12. Once the entire plate has been read, Relative Light Units (RLUs) will be generated for firefly and *Renilla* luciferase. These values can be used to calculate fold change or can be used as raw units for comparison.

3.7 qRT-PCR Screening

3.7.1 RNA Isolation

1. Using a micropipette, briefly pipette up and down to suspend the transfected protoplasts (*see* **Note 27**).
2. Using the same tip, transfer the W5 buffer and protoplasts into a labeled 1.7 mL microcentrifuge tube. This should collect approximately 5×10^5 protoplasts.
3. Centrifuge the protoplasts at 100 rcf for 5 min.
4. Remove the supernatant using a micropipette, pipetting slowly to not disturb the pellet (*see* **Note 28**).
5. In a chemical fume hood, add 750 μL of TRIzol reagent to each pellet. Briefly vortex each sample to suspend the pellet.
6. Incubate at room temperature (22–26°°C) for 5 min.
7. In a chemical fume hood, add 150 μL of chloroform to the TRIzol mixture. Thoroughly mix the sample by vortexing (*see* **Note 29**).
8. Centrifuge the tubes at 12,000 rcf (4 °C) for 5 min.
9. The sample will have separated into distinct aqueous and organic phases. In a chemical fume hood, using a micropipette, carefully transfer the aqueous phase to a new tube, ensuring that none of the organic phase is transferred. Discard the organic phase into an appropriate hazardous waste container (*see* **Note 30**).
10. Continuing in the fume hood, add an additional 450 μL of chloroform. Vortex to create an emulsion as in **step 7**.
11. Repeat **step 8**.
12. The aqueous phase will separate from the organic phase as in **step 9**, but both phases will be colorless. Following the same procedure, transfer the aqueous phase to a new tube (*see* **Note 31**).
13. Add 4 μL of 5 μg/μL RNase-free glycogen to each sample (20 μg total), pipetting up and down to mix.
14. Add 450 μL of 100% isopropanol and vortex to mix well. Incubate on ice for 5 min.
15. Centrifuge the tubes at 12,000 rcf (4 °C) for 15 min.
16. Decant and discard the supernatant. About 50–100 μL of liquid will remain.
17. Add 1000 μL of 75% ethanol to the sample.
18. Repeat **step 8**.

19. Discard the supernatant using a micropipette removing as much as possible without disturbing or removing the pellet.
20. Allow the pellet to dry at room temperature with the lid open for at least 30 min. The pellet will become translucent in that time.
21. Resuspend and dissolve the pellet in 25 μL of RNase-free water. Immediately place and store the samples on ice.
22. Measure the RNA concentration using a UV spectrophotometer. You can expect 25–70 ng/μL of RNA. These samples may be stored at −80°°C.
23. Treat the samples with TURBO DNase per the manufacturer's instructions.

3.7.2 qRT-PCR

Use filtered micropipette tips for setting up the qRT-PCR. We recommend setting up each reaction in triplicate or greater.

1. Spray and wipe down all equipment and benchtop with RNase removing spray (such as RNase Away).
2. Utilizing the NEB Luna Universal One-Step RT-qPCR Kit (NEB E3005), a master mix is set up for each primer set to be screened (*see* **Note 32**). A 1× reaction consists of 6 μL of Luna Universal One-Step Reaction Mix (2×), 0.6 μL of Luna WarmStart RT Enzyme Mix (20×), 0.5 μL each of the forward and reverse primers (10 μM), and 3.4 μL of nuclease-free H_2O (*see* **Note 33**). The master mix should be kept on ice.
3. Load the master mix(es) onto a 96-well PCR plate with 11 μL per well.
4. Thaw RNA samples and load 1 μL per well (20–100 ng), switching tips with each transfer (*see* **Note 34**).
5. Seal plates with optically clear adhesive film. Using a roller or the back of a marker, carefully press down along three of the four edges of the film to ensure a proper seal. Press across the entirety of the film with dragging toward the remaining edge to seal each well and push out any air pockets. Seal the final edge as the other three.
6. Briefly centrifuge the plate.
7. Place the plate in the thermocycler. Set protocol to 55 °C for 10 min, 95 °C for 1 min, 40 times cycle through 95 °C for 1 min, 60 °C for 30 s, and a plate read. Add a melt curve step at the end of the cycle.

3.7.3 Data Analysis

1. First, calculate the ΔC_t by taking the difference of the experimental C_t value and the housekeeping gene C_t value.
2. Calculate the $\Delta\Delta C_t$ by taking the difference of the ΔC_t of the experimental and the ΔC_t of the untreated condition. Fold change can be calculated by $2^{-\Delta\Delta C_t}$.

3.8 Example 1: Guide RNA Selection for Overexpression of S. viridis Endogenous Developmental Genes

PTAs allow for robust, programmable induction of gene expression in a variety of systems allowing for investigation of overexpression phenotypes, therapeutic development, and generation of novel gene circuits. Nonetheless, sgRNA choice impacts PTA activity in an unpredictable, nonadditive manner [22]. We applied the low-throughput protoplast transfection and qRT-PCR protocols to investigate the PTA activity profiles of sgRNA targeting CLV3 and MYB21, two key developmental regulators in *S. viridis*.

1. Ten (five each) (Fig. 4a, b) sgRNA expression plasmids were designed to target upstream of the CLV3 and MYB21 TSS. These plasmids were collected by midi-prep along with plasmids expressing GFP, Cas9-GCN, and anti-GCN-ScFv-VP64 (*see* Subheading 3.1).
2. Protoplasts were transfected in PEG 4000 solution for 10 min with 10 μg of one of the sgRNA expression plasmids and 10 μg of each of the remaining three plasmids (*see* Subheadings 3.3 and 3.4).
3. Sixteen hours post-transfection, the protoplasts were collected and lysed in TRIzol reagent. RNA was isolated from the protoplasts via chloroform isolation (*see* Subheading 3.7.1).
4. The RNA was immediately used in a qRT-PCR reaction with primers measuring the expression for CLV3, MYB21, and GRAS (as a housekeeping gene from [25]) (*see* Subheadings 3.7.2 and 3.7.3) (*see* **Note 33**).

Figure 4c shows the results of the sgRNA screening analysis described above. Each sgRNA induces expression to a variable degree solidifying the need to screen multiple guides when designing a PTA. It is noteworthy that several sgRNAs exhibit a repressing phenotype, possibly through competition with binding of endogenous transcription factors. Initializing protoplast transfection prior to the generation of transgenic plants helps minimize the screening efforts required downstream and allow for a more thorough search of design space.

4 Notes

1. We purified our water using the Millipore Milli-Q Direct Water Purification System.
2. You may double or triple this step in a single tube if you are extracting protoplasts from multiple plates. If you are having trouble isolating healthy protoplasts, the enzymatic solution may be filtered to remove potential contaminants. The filtration step has not been necessary in our hand.

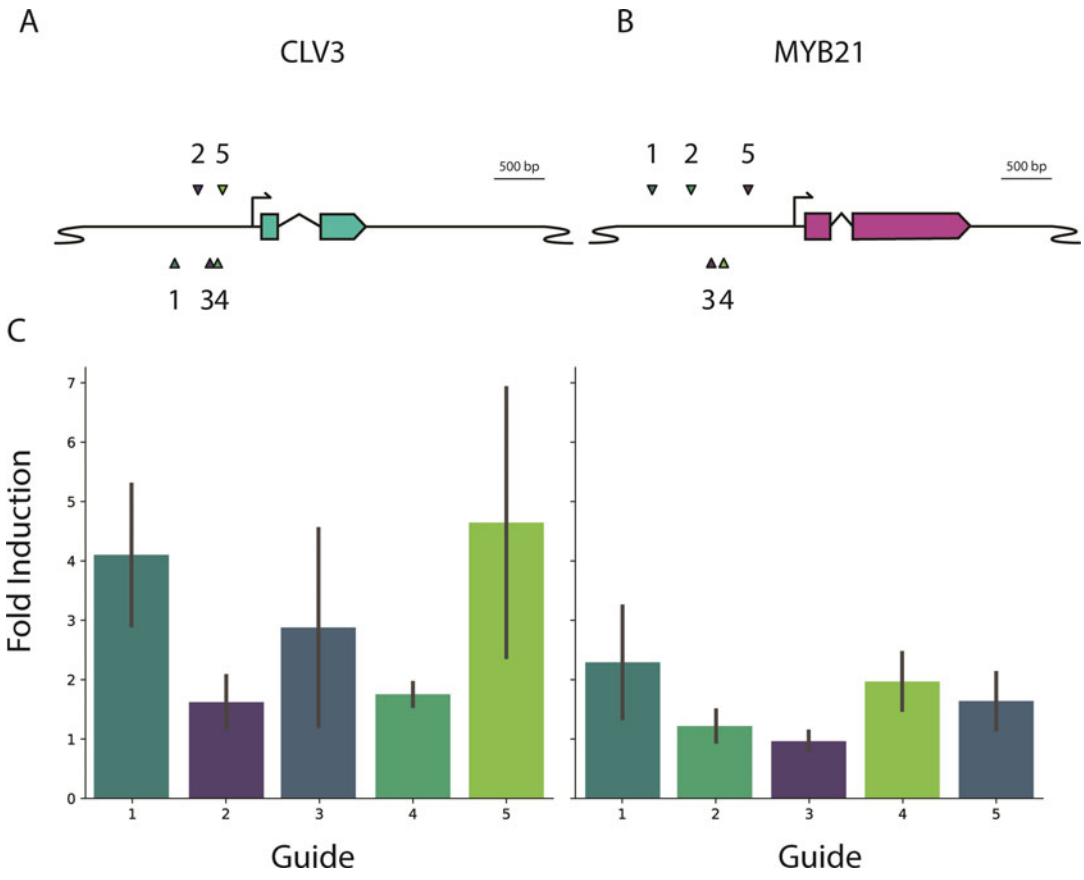

Fig. 4 (**a**) Map of CLV3 locus with PTA guide positions relative to coding sequence. Locations are to scale. (**b**) Map of MYB21 locus with PTA guide positions relative to coding sequence. Locations are to scale. (**c**) Induction of CLV3 and MYB21 using PTAs with guides targeting upstream of each TSS. Five guides were designed for each gene and individually used with the dCas9-Suntag-VP64 PTA. Fold induction was calculated relative to the no guide control. Experiments were performed in triplicate. Error bars represent one standard deviation

3. You may purchase premade wide orifice tips or make your own as follows. Spray a pair of scissors with 70% ethanol and wipe until dry. Using a multichannel pipette, pick up tips and cut end such that an approximately 1 mm orifice is left. Eject tips back into the rack. Autoclave tips at 121 °C and 15 psi for at least 30 min.
4. We recommend that each transfection include a constitutive (in our hands GFP driven by pvUbi, 35S, and CmYLCV promoters are effective) GFP plasmid as a control.
5. It is recommended that culture preparations are done in a laminar flow hood or biosafety cabinet.
6. You may pause here by freezing the pellet down at −20 °C for at least 2 weeks.

7. midi-prepped plasmids will be transfected at higher rates than mini-prepped plasmids. It is recommended that midi-prepped plasmids are only compared to other midi-prepped plasmids, and mini-prepped plasmids only compared to other mini-prepped plasmids.
8. In our hands, the brand of midi-prep kit plays a role in transfection efficiency. For best results, we recommend the Macherey-Nagel NucleoBond Xtra Midi kit (REF 740410). In a side-by-side comparison, in triplicate, Macherey-Nagel gave >80% transfection, while the previous brand gave 8% (Fig. 3b, c).
9. While you may collect your midi-prep the same day you do the transfection, we recommend that you perform the midi-prep at least 1 day in advance of the protoplast transfection.
10. Freshly collected seeds are dormant. It takes around 6 months of storage at room temperature for the dormancy to break. Make sure you have an idea of the germination rate before you use your seeds.
11. We use Miracle-Gro all-purpose plant food (24-8-16).
12. Because of the fast growing of the seedlings and number of plants we place per pot, some plants may show iron deficiency symptoms such as interveinal yellowing and less often chlorosis of emerging leaves. However, these symptoms do not seem to interfere with protoplast isolation or transfection. If these phenotypes are of any concern, they usually disappear by reducing the number of seeds planted per pot (to around 5–7) or by using larger pots.
13. One gram of leaves is used per protoplast preparation (producing 1.0–1.4 $\times$ 10^6 protoplasts). We only use the first two fully expanded leaves for protoplast isolation. One to two pots will produce enough leaves for a protoplast isolation.
14. Do not collect dead leaves or leaves with dried patches. You may remove the top few millimeters of the blade if that is the only dried portion.
15. You may stack several leaves together before cutting into smaller pieces. We recommend that you do not stack more than five at a time as larger stacks tend to have more stress response.
16. We prepare an aliquot of 15 mL 0.8 M mannitol, then dilute the mixture to 0.6 M by adding 5 mL H_2O with every preparation.
17. The vacuum step improves the efficiency of protoplast isolation but may be bypassed with an additional 30 min at **step 8** of the protoplast isolation.

18. We commonly collect protoplasts for counting immediately prior to transferring to the sucrose gradient and assume 100% collection. We found that we lose a negligible amount during the gradient and subsequent wash. This methodology saves time as counting can be done during the slow gradient separation step but is not recommended until you are comfortable with isolating protoplasts from the gradient.
19. If necessary, break up into smaller batches as incubating for longer than 10 min increases the risk of the protoplasts dying.
20. If a plasmid or set of plasmids, e.g., GFP/luciferase/Cas9/sgRNA, will be transfected in all wells, we recommend preparing a master mix.
21. Many reagent reservoirs hold ~15–25 mL total volume. If your protoplast suspension is >10 mL total volume, we recommend keeping the protoplasts in the original tube.
22. If you did not transfer protoplasts to a reagent reservoir, use a single channel pipette and large orifice tips to preload a 96-well plate.
23. Approved DLR luminometers can be found at the following website: https://www.promega.com/products/pm/dlready-luminometers/dlready-validated-luminometers/.
24. Lysate containing PLB and cells can be stored at −20 °C for up to 1 month and −80 °C for up to 1 year.
25. The 1× Stop & Glo solution will stick to plastic over time. It is important to thoroughly wash injectors before and after use of this solution.
26. The program should carry out this set of operations on a per well basis. For example, well 1 should execute injector 1 dispensing 100 μL LARII + shaking + detection followed by injector 2 dispensing 100 μL 1× Stop & Glo + shaking + detection before well 2 has this set of operations executed.
27. You may alternatively use columns designed for the extraction of RNA from small samples (we use Macherey-Nagel REF 740990). We have found these to be effective for efficient extraction and downstream usage.
28. You may pause here by flash freezing the protoplasts in liquid nitrogen and storing them at −20 °C.
29. Ensure that the vortexed sample appears as an emulsion of the aqueous and organic phases. It is normal for separation of the phases to begin shortly after vortexing, but it is important to generate a thorough emulsion during the vortexing.
30. Many TRIzol reagents include a dye that separates with the organic phase. Tilting the tube at a 45° angle during the duration of the transfer allows for the final few microliters of

the aqueous phase to collect and be more easily extracted. The total amount transferred will be ~450 μL.

31. The total transferred will be ~450 μL.
32. The master mix should be made up to 10% more reactions than will be performed (i.e., for 100 reactions set up a 110× master mix).
33. Primer design for qRT-PCR may require optimization. We generally design 2–3 pairs targeting across exon junctions with a 70–150 bp amplicon. We validate the primer set through an initial qRT-PCR on untransfected protoplasts looking for a single peak in the melt curve.
34. It is recommended that you dilute the RNA to a single concentration across all samples. This is not necessary with normalization from housekeeping genes.

Acknowledgments

AS and MZ were funded by NIGMS Biotechnology Training grant NIHT32GM008347. MJS and JACM are part of a team supported by the Advanced Plant Technologies program, DARPA Award HR001118C0146. MJS and MZ are supported by USDA NIFA award 2018-33522-28747.

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Chapter 17

High-Throughput Transfection and Analysis of Soybean (*Glycine max*) Protoplasts

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Abstract

With the advent of plant synthetic biology, there is an urgent need to develop plant-based systems that are able to effectively enhance the speed of design-build-test cycles to screen large numbers of synthetic constructs. Thus far, protoplasts have served to fill this need, with cell suspension cultures serving as the primary source tissue to enable high-throughput protoplast experimentation. The possibility to use low-cost food-grade enzymes for cell wall digestion along with polyethylene glycol (PEG)-mediated transfection makes protoplasts particularly suited to automation and high-throughput screening. In other systems for which synthetic biology is well established (model bacteria and yeast), libraries of components, i.e., promoters, 5′ untranslated regions, 3′ untranslated regions, terminators, and transcription factors, serve as the basis for the design of complex genetic circuits. In order for synthetic biology to make similar strides in plant biology, well-characterized libraries of functional genetic parts for plants are required, necessitating the need for high-throughput protoplast assays.

In this chapter, we describe an optimized method for the preparation of soybean (*Glycine max*) dark-grown cell suspension cultures, followed by protoplast isolation, automated transfection, and subsequent screening.

Key words Automation, Cell suspension culture, High-throughput screening, Image processing, Robotics, Synthetic biology

1 Introduction

A primary goal of plant synthetic biology is to utilize engineering tools and approaches to generate crops with improved traits [1–3]. Much effort has been spent on designing plants with improved resistance to biotic (e.g., pathogens) [4] and abiotic (e.g., drought;

Alessandro Occhialini and Mary-Anne Nguyen contributed equally to this work.

Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6_17,

salt stress) stresses [5], as well as plants with increased photosynthetic efficiency [6] and value-added traits (e.g., carotenoids; omega-3 fatty acids) [7, 8].

One important bottleneck for precise metabolic engineering in plants is the current lack of well-coordinated gene expression and a lack of genetic elements to optimize the expression level of each gene of interest in large metabolic engineering constructs. The efficient installation of functional multigene pathways relies on the precise spatiotemporal control of gene expression in which all genes within the pathway are expressed at optimal levels relative to each other. While numerous complex genetic circuits have been installed in microorganisms [9], the installation of similar circuits in plant cells is still at an early stage. Improving research in this direction will accelerate crop improvement for many agronomically important species.

The adoption of Golden Gate modular cloning by the plant synthetic biology community has been an important breakthrough in standardizing parts for fast and precise assembly of a large number of plant transformation vectors [10, 11]. Optimized methods for assembly of multigene constructs using both previously validated and uncharacterized regulatory elements along with new rationally designed synthetic elements have solved the bottleneck of facile cloning of plant expression constructs. However, except for some model plants like *Arabidopsis*, the long life cycle of the majority of crop species makes screening a large number of regulatory elements in whole plants infeasible in terms of both time and money.

In order to accelerate the design-build-test cycle in plant synthetic biology, protoplasts could be used as a powerful single cell model for fast screening of DNA constructs. The removal of the cell wall as a barrier to transformation and the utilization of non-integrating binary vectors make protoplasts an appropriate tool to achieve high transformation frequency and transgene expression within only 24 h [12]. Furthermore, protoplast isolation and transformation methods have been developed for many agronomically important crops [13]. Protoplasts can also be obtained from almost any kind of source tissue representing a valuable proxy for tissue-specific expression [14]. The ability to obtain large numbers of protoplasts from fast-grown liquid cultures further makes them ideally suited for automated, high-throughput screening [15].

In this chapter, we describe an optimized protoplast-based system from soybean (*Glycine max*) suitable for automated, high-throughput PEG-mediated transfection and quantitative analysis using a fluorescent-imaging plate reader. In this method, the production of large amounts of protoplasts suitable for high-throughput screening is achieved by using dark-grown cell

suspension cultures that are subjected to efficient cell wall digestion by inexpensive food-grade enzymes.

2 Materials

Prepare all solutions using ultrapure water (18 MΩ-cm resistivity at 25 °C). Perform all autoclaving for 30 min at 121 °C and a chamber pressure of ~100 kPa. When indicated in the text, perform filter sterilization using 0.22 μm mesh filters. Perform all plant tissue culture and media preparation under sterile conditions. Store media at room temperature (22 °C) in the dark for short-term storage (1 month) and at 4 °C for long-term storage (6–8 months).

2.1 Plant Material and DNA Construct

1. Soybean: Mature seeds of *Glycine max* cv Williams 82 were obtained from the USDA, ARS Soybean Germplasm Collection (Accession: PI 518671).
2. Plasmid DNA: The pICH-mEme plasmid used for protoplast transfection was assembled by Golden Gate cloning using previously described genetic modules [10]. In this plasmid, the expression of a *GFP* gene (encoding for mEmerald, a synthetic variant of green fluorescent protein (GFP) from *Aequorea victoria*, FPbase ID: AD4BK) is under the control of the double (2×) 35S promoter from the CaMV (*Cauliflower mosaic virus*) fused to the TMV (*Tobacco mosaic virus*) Ω leader, while the CaMV 3′UTR polyadenylation signal is used as a terminator. For construction of pICH-mEme, the fluorescent reporter expression cassette was inserted into *Bsa*I cloning sites of the pICH47732 backbone plasmid [10]. Pure plasmid preparations (~1 μg/μL) were obtained by alkaline lysis and column purification using the commercial ZymoPURE II Plasmid Midiprep Kit (Cat #: D4200). Plasmids were resuspended in sterile ultrapure water, and the DNA concentration was determined using a NanoDrop™ OneC UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.). The ratio of absorbance at 260/280 and 230/260 was used to estimate DNA purity. Pure plasmid preparations with 260/280 and 230/260 values of ~1.8 and ~2.0–2.2 were used.

2.2 Stock Solutions and Media for Soybean Tissue Culture

1. MS macro (10×): 206 mM NH_4NO_3, 188 mM KNO_3, 15 mM $MgSO_4$, and 12.5 mM KH_2PO_4. For 1 L of solution, dissolve 16.5 g NH_4NO_3, 19 g KNO_3, 3.7 g $MgSO_4{\cdot}7H_2O$, and 1.7 g KH_2PO_4 in water. Autoclave, let cool, and store at 4 °C.
2. MS micro (1000×): 100 mM H_3BO_3, 100 mM $MnSO_4$, 30 mM $ZnSO_4$, 1 mM Na_2MoO_4, 0.1 mM $CuSO_4$, 0.1 mM $CoCl_2$, and 5 mM KI. For 1 L of solution, 6.2 g H_3BO_3, 22.2 g

$MnSO_4 \cdot 4H_2O$, 8.6 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $Na_2MoO_4 \cdot 2H_2O$, 0.025 g $CuSO_4 \cdot 5H_2O$, 0.024 g $CoCl_2 \cdot 6H_2O$, and 0.83 g KI in water. Autoclave, let cool, and store at 4 °C.

3. Calcium (100×): 300 mM $CaCl_2$. For 1 L of solution, dissolve 44 g $CaCl_2 \cdot 2H_2O$ in water. Autoclave, let cool, and store at 4 °C.
4. Iron (100×): 11 mM Na_2EDTA and 10 mM $FeSO_4$. For 1 L of solution, solubilize 3.73 g Na_2EDTA and 2.78 g $FeSO_4 \cdot 7H_2O$ in water (*see* **Note 1**). Autoclave, let cool, and store at 4 °C in the dark (*see* **Note 2**).
5. Miller-I: 441 mM KH_2PO_4. For 1 L of solution, dissolve 60 g KH_2PO_4 in water. Autoclave, let cool, and store at 4 °C.
6. BY-2 vitamins: 0.3 mM thiamine-HCl and 55.5 mM myo-inositol. For 1 L of solution, dissolve 0.1 g of thiamine-HCl and 10 g myo-inositol in water. Autoclave, let cool, and store at 4 °C.
7. 2,4-D (1 mg/mL): Purchased from PhytoTech Labs (product ID: D295).
8. Seed germination medium: For 1 L of medium, dissolve 20 g sucrose, and add 100 mL of 10× MS macro and 1 mL of 1000× MS micro in water. Adjust the pH to 5.7 using 1 M KOH. Add 3 g of Phytagel and sterilize by autoclave. Let the medium cool down to ~50 C. Pour medium in sterile Magenta™ GA-7 boxes (*see* **Note 3**).
9. Callus induction medium (CI): For 1 L of medium, dissolve 30 g sucrose, and add 100 mL of 10× MS macro, 1 mL of 1000× MS micro, 10 mL of 100× calcium, 10 mL of 100× iron, 3.5 mL of Miller-I, and 10 mL of BY-2 vitamins into 500 mL of ultrapure water. Bring the volume to 1000 mL using ultrapure water, and adjust the pH to 5.7 using 1 M KOH. Sterilize by autoclave. Cool down the medium to ~50 °C and then add 2.64 mL of 1 mg/mL of 2,4-D (final concentration of 2.64 mg/L). Store the medium at room temperature in the dark.
10. Callus induction solid medium (CIS): Add 2 g of Phytagel in 1 L of CI liquid medium before autoclaving. Sterilize by autoclave, let cool the medium to ~50 °C, and then add 0.2 mL of 1 mg/mL 2,4-D solution (final concentration of 0.2 mg/L). Pour medium in 100 × 20 mm (diameter × height) Petri dishes and store the medium at room temperature in the dark (*see* **Note 4**).

1. Mannitol (1 M): For 1 L, dissolve 182.2 g mannitol in water (*see* **Note 5**). Filter sterilize and store at room temperature.

2.3 Stock Solutions and Media for Isolation and Transfection of Protoplasts

2. MES (500 mM, pH 5.7): For 100 mL, add 9.76 g of 2-(*N*-morpholino)ethanesulfonic acid (MES) in water. Adjust the pH to 5.7 using 1 M NaOH. Filter sterilize and store at room temperature.
3. KCl (1 M): For 100 mL, add 7.4 g of KCl in water. Autoclave and store at room temperature.
4. KOH (1 M): For 100 mL, add 5.6 g KOH in water. Autoclave and store at room temperature.
5. $CaCl_2$ (1 M): For 100 mL, add 11.1 g of $CaCl_2$ in water. Autoclave and store at room temperature.
6. $MgCl_2$ (1 M): For 100 mL, add 9.5 g of $MgCl_2$ in water. Autoclave and store at room temperature.
7. NaCl (4 M): For 100 mL, add 23.37 g of NaCl in water. Autoclave and store at room temperature.
8. Rohament® CL (cellulase): Liquid formulated fungal cellulase enzyme for hydrolyzing non-starch polysaccharides (*see* **Note 6**).
9. Rohapect® UF (pectinase): Liquid formulated pectinase product used for depectinization and cross-flow membrane filtration (*see* **Note 6**).
10. Rohapect® 10 L (pectinase/arabinase): An enzyme cocktail commercialized for juice clarification (*see* **Note 6**).
11. Sucrose (23%): Add 23 g of sucrose in 100 mL of water. Filter sterilize and store at room temperature.
12. Protoplast isolation buffer: 600 mM mannitol, 1 mg/mL bovine serum albumin (BSA), 20 mM KCl, 10 mM $CaCl_2$, 4.6 mM β-mercaptoethanol, 20 mM MES (pH 5.7). For the preparation of 100 mL of protoplast isolation buffer, add 60 mL of 1 M mannitol, 100 mg of BSA, 4 mL of 500 mM MES (pH 5.7), 2 mL of 1 M KCl, 1 mL of 1 M $CaCl_2$, 32 μL β-mercaptoethanol. Adjust the final volume to 100 mL with water. Adjust the pH to 5.7 using 1 M KOH. Filter sterilize (*see* **Note 7**).
13. Enzyme solution: Add 960 μL of Rohament® CL, 880 μL of Rohapect® 10 L, and 140 μL of Rohapect® UF into 20 mL of protoplast isolation buffer. Filter sterilize (*see* **Note 7**).
14. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7). For the preparation of 100 mL of W5 solution, add 3.84 mL of 4 M NaCl, 12.5 mL of 1 M $CaCl_2$, 0.5 mL of 1 M KCl, 0.4 mL of 500 mM MES (pH 5.7). Adjust the volume to 100 mL with water. Adjust the pH to 5.7 using 1 M KOH. Filter sterilize.
15. Wash solution (WS): 600 mM mannitol, 20 mM KCl, 4 mM MES (pH 5.7). For the preparation of 100 mL of WS solution,

add 60 mL of 1 M mannitol, 2 mL of 1 M KCl, 0.8 mL of 500 mM MES (pH 5.7). Adjust the volume to 100 mL with water and adjust the pH to 5.7 using 1 M KOH. Filter sterilize.

16. Washing incubation (WI): 600 mM mannitol, 4 mM KCl, 4 mM MES (pH 5.7). For the preparation of 100 mL of WI solution, add 60 mL of 1 M mannitol, 0.4 mL of 1 M KCl, 0.8 mL of 500 mM MES (pH 5.7). Adjust the volume to 100 mL with water and adjust the pH to 5.7 using 1 M KOH. Filter sterilize.
17. MaMg solution: 400 mM mannitol, 15 mM $MgCl_2$, 4 mM MES (pH 5.7). For the preparation of 100 mL of solution, add 40 mL of 1 M mannitol, 1.5 mL of 1 M $MgCl_2$, 0.8 mL of 500 mM MES (pH 5.7). Adjust the volume to 100 mL with water and adjust the pH to 5.7 using 1 M KOH. Filter sterilize.
18. PEG solution: 25% PEG 4000 (w/v), 200 mM mannitol, 100 mM $CaCl_2$ (*see* **Note 7**). For the preparation of 10 mL of PEG solution, add 2.5 g of PEG 4000 (*see* **Note 8**), 2 mL of 1 M mannitol, 1 mL of 1 M $CaCl_2$, 3.5 mL of water (*see* **Note 9**). Add water up to the final volume of 10 mL. Filter sterilize.

2.4 Equipment and Supplies

1. Autoclave.
2. Chemical fume hood.
3. Laminar flow hood.
4. Growth chamber.
5. Incubator with shaker.
6. Desiccator.
7. Centrifuge with a swinging-bucket rotor.
8. Vortex.
9. Olympus BX60 Microscope.
10. Hemacytometer.
11. 25–30 cm Potts-Smith forceps (curved and straight tip).
12. 12.7 cm surgical design surgeon grade scalpel.
13. Carbon steel surgical scalpel blade (type 10#).
14. ZymoPURE II Plasmid Midiprep Kit (Zymo Research).
15. NanoDrop™ OneC UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.).
16. Tecan EVO robotic system.
17. BioTek Cytation 5 Cell Imaging Multi-Mode Reader.
18. MilliporeSigma™ Milli-Q™ Ultrapure Water Systems.
19. 99.5% extra pure ethanol.
20. 70% ethanol.

21. Commercial bleach (6% sodium hypochlorite).
22. 12 M HCl.
23. Beakers: 50 mL, 100 mL, 200 mL, 500 mL, and 1 L.
24. Graduate cylinders: 50 mL, 100 mL, 200 mL, 500 mL, and 1 L.
25. Magenta™ GA-7 vessels size 7.6 × 7.6 × 10.2 cm.
26. 250 mL Pyrex® Erlenmeyer flask.
27. 100 × 20 mm (diameter × height) Petri dishes.
28. Deep 96-well plates (1.2 mL well volume).
29. 96-well fluorescent screening plates (370 μL well volume).
30. Culture tubes: 15 and 50 mL conical Falcon tubes.
31. Serological pipettes: 5 mL, 10 mL, 25 mL.
32. Nylon mesh filter: 425 μm, 100 μm, 40 μm.
33. 3 M micropore tape.
34. Poly-(2-hydroxyethyl methacrylate) (polyHEMA).
35. Automation conductive tips for a Tecan EVO robotic system, 200 μL and 1000 μL volume.

3 Methods

3.1 Surface Sterilization of Soybean Seeds and Germination

1. Wash 80–100 mature seeds for 2 min in a flask containing 70% ethanol. Let the seeds air-dry completely on a sterile filter paper for ~60 min (*see* **Note 10**).
2. Transfer washed seeds into clean 100 × 20 mm (diameter × height) Petri dishes (~50 seeds per plate) and then transfer in a desiccator placed in a chemical fume hood. Put the lids slightly tilted on the Petri dishes to allow seeds to be efficiently exposed to chlorine vapors.
3. Place a clean 200 mL beaker with 100 mL of commercial bleach in the center of the desiccator surrounded by plates containing seeds. Slowly add 3.5 mL of 12 M HCl into the beaker (*see* **Note 11**).
4. Seal the desiccator immediately. Let it stand overnight (~16 h) in the chemical hood.
5. After sterilization, open the desiccator, close the Petri dishes, and transfer them immediately in sterile conditions in a laminar flow hood.
6. Under the laminar flow hood, remove lids and let the seeds stand for at least 30 min to completely remove the excess chlorine gas (*see* **Note 12**).

7. Dispense four to six sterile soybean seeds per Magenta box containing seed germination medium and incubate for ~3 weeks in a growth chamber at 24 °C under white light (~100 μmol m^{-2} s^{-1}) for 16 h day and 8 h night photoperiod.

3.2 Generation of Soybean Dark-Grown Cell Suspensions

1. Use 20-day-old soybean plants grown in vitro. Perform all procedures of callus induction and cell suspension culture production in sterile condition in a laminar flow hood.
2. Cut fresh leaf tissue into ~0.5 × 0.5 cm long pieces using a scalpel and place them, adaxial side down, into a Petri dish containing CIS medium. Place ~13–16 leaf disks per Petri dish for a total of 20 dishes (Fig. 1a) (*see* **Note 13**) and seal the dish using a 3 M micropore tape.
3. To induce callus production, incubate the leaf material for ~3 weeks at 24 °C under white light for 16 h day and 8 h night photoperiod (Fig. 1b).
4. After 3 weeks, excise callus grown from leaf tissue using a scalpel and place callus pieces into a new Petri dish containing fresh CIS medium. At this point, the callus proliferation is performed in a growth chamber at 24 °C under complete darkness (*see* **Note 14**).
5. Subculture calli into fresh CIS medium every 2 weeks for a minimum number of three subcultures, necessary to get enough proliferated calli (Fig. 1c).
6. For initiation of cell suspension culture, chop 1 g of fresh callus using a sterile spatula.
7. Next, transfer the callus into a 250 mL Erlenmeyer flask containing 50 mL CI liquid medium and incubate in the dark at 28 °C under gentle shaking (80 rpm) for 1 week.
8. In order to obtain homogeneous cell suspension cultures, let the cells settle in the flask and replace the supernatant with 40 mL of fresh CI liquid medium.
9. Repeat **step 8** every week for a total of ~5 weeks.
10. Filter the homogeneous cell suspension culture through a 425 μm nylon mesh filter to remove large cell aggregates.
11. Transfer 5 mL of filtrated cells into a new flask containing 45 mL CI liquid medium.
12. Place the flask in the dark at 28 °C under gentle shaking (80 rpm) (Fig. 1d–f).
13. After the first filtration, perform the subculture 5 mL of cell suspension to 45 mL of fresh CI liquid medium once a week. The cell suspension culture can be maintained for at least 6–8 months (*see* **Note 15**).

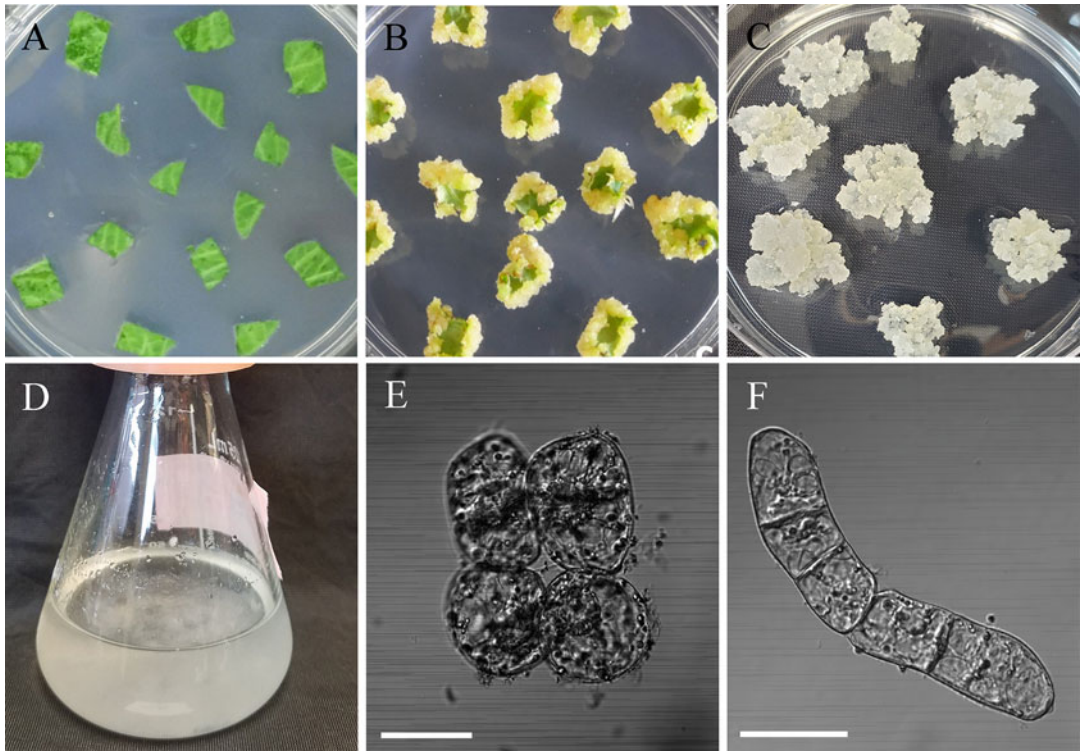


Fig. 1 (**a**) Leaf disks plated on CIS media. (**b**) 3-week-old calli grown on leaf disks exposed to light. (**c**) Fast proliferating dark-grown calli. (**d**) Established dark-grown cell suspension culture. (**e**) Round and oval shape cell types. (**f**) Elongated cell type. Scale bar: 40 μm

3.3 Protoplast Isolation

1. Add 5 mL of soybean cell suspension culture into a new 250 mL flask containing 45 mL of CI liquid medium.
2. Incubate the culture for 4 days in the dark at 24 °C under gentle shaking (80 rpm).
3. Transfer the 4-day-old cell suspension culture into a sterile 50 mL Falcon tube.
4. Let the cells settle at room temperature for 15 min. Remove the supernatant using a serological pipette.
5. Resuspend the cells in 20 mL of enzyme solution.
6. Set the tube at room temperature in a laminar flow hood for 45–60 min in total darkness.
7. Place the tube on its side on a shaker at 24 °C for 60 min under gentle shaking (80 rpm).
8. Remove the undigested cell aggregates by filtering the suspension through a 100 μm nylon filter and then through a 40 μm nylon filter into 50 mL Falcon tubes.
9. Centrifuge at 100 × *g* for 3 min at room temperature using a centrifuge with a swinging-bucket rotor.

10. Aspirate the supernatant by pipetting.
11. Gently resuspend the cell pellet in 10 mL of WS. Slowly add the WS along the walls of the Falcon tube without disturbing the pellet (*see* **Note 16**).
12. Prepare the sucrose gradient by gently adding 10 mL of 23% sucrose to the bottom of the Falcon tube containing protoplasts, taking care not to disturb the two phases (*see* **Note 17**). After this step, the top fraction containing protoplasts and a bottom sucrose fraction are obtained.
13. Separate intact protoplasts by centrifuging the tube at 100 × *g* for 3 min at room temperature using a swinging-bucket rotor.
14. Collect intact protoplasts located at the interface layer using a P1000 manual pipette (~2–3 mL) and transfer into a new sterile 15 mL Falcon tube (*see* **Note 18**).
15. Add 10 mL of WS to intact protoplasts collected from **step 14**.
16. Centrifuge the tube at 100 × *g* for 4 min at room temperature. Remove the supernatant and gently resuspend the protoplast pellet in 3 mL of W5 solution (*see* **Note 19**).
17. Incubate protoplasts on ice for 30 min. During this time, count 10 μL of protoplasts using a hemacytometer.
18. Centrifuge protoplasts again for 3 min at 100 × *g*.
19. Remove the supernatant and resuspend protoplasts with an appropriate amount of MaMg solution to get a final concentration of 10^6 cells/mL.

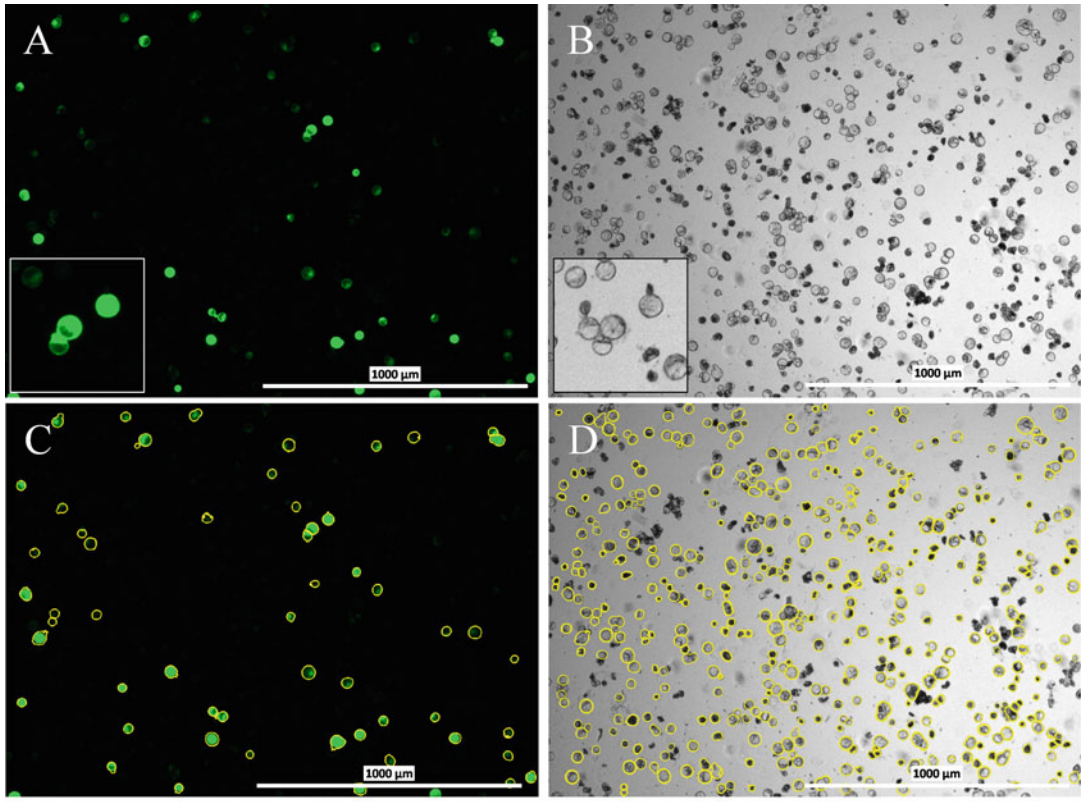
3.4 High-Throughput Protoplast Transfection Using a Tecan EVO Robotic System

1. Load all necessary materials and solutions on the robotic platform: (1) deep 96-well plate (*see* **Note 20**), (2) 96-well fluorescent screening plate (*see* **Note 21**), (3) plasmid (1 μg/μL stock concentration), (4) protoplasts (10^6 cells/mL stock density in MaMg), (5) 25% PEG, (6) W5 solution, and (7) WI solution.
2. All pipetting and manipulation occurring in this section are carried out by the Tecan Freedom Evo 150 system as programmed.
3. Pipette 10 μL of plasmid (10 μg total) into each well in a deep 96-well plate (*see* **Note 22**).
4. Transfer 100 μL of protoplasts (10^5 cells) into each well containing plasmid.
5. Add an equal volume of 25% PEG solution (110 μL) into each well containing plasmid and protoplasts.
6. Shake at 1500 rpm using a plate shaker for 10 s to mix components (*see* **Note 23**).
7. Incubate at room temperature for 15–20 min with no agitation to allow transfection of protoplasts.

8. Add 780 μL of W5 buffer to each well and shake at 1500 rpm for 10 s to terminate reaction.
9. Incubate protoplasts at room temperature and in the dark for 1 h to allow the protoplasts to settle on the bottom of the well (*see* **Note 24**).
10. Aspirate and discard 700 μL of supernatant, leaving ~300 μL of volume containing protoplasts in the well (*see* **Note 25**).
11. Pipette 600 μL WI buffer into each well and shake at 1000 rpm.
12. Incubate protoplasts at room temperature and in the dark for an additional hour to allow protoplasts to settle.
13. Aspirate and discard 700 μL of supernatant, leaving ~300 μL in each well.
14. Transfer the 300 μL of transfected protoplasts contained in the deep well plate into a clean 96-well fluorescent screening plate.
15. Incubate the plate in the dark at room temperature for 24 h until image analysis (*see* **Note 26**).

3.5 Data Collection and Analysis Using BioTek Cytation 5 Cell Imaging Multi-Mode Reader

1. Move a 96-well fluorescent screening plate to the multi-mode plate reader associated with the robotic system. The Gen5 Data Analysis software from BioTek is used to acquire, select, and analyze a large number of images of each well of the plate automatically (*see* **Note 27**).
2. Using the program interface, select the image of choice followed by the "analyze" tab function.
3. Within the "analyze" tool, select cellular analysis and choose the channel of interest (e.g., in this case brightfield or GFP).
4. Under the "calculated metrics" tab, select and add object circularity.
5. Under the "subpopulation analysis" tab function, select circularity and then add condition. Circularity greater than 0.7 of roundness is chosen as the first condition for the analysis (*see* **Note 28**).
6. Size is added as another condition and is set between 20 and 70 μm diameter (*see* **Note 29**).
7. Select all conditions are met and then the apply condition function.
8. Cell count results based on all conditions applied before are displayed. The same analysis can be applied to all selected images within the experiment file.
9. Follow **steps 4–8** for all channels of interest (e.g., in this case brightfield and GFP).



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Count GFP positive (n1)	Count tot. (n2)	Trans. efficiency (%)
56	449	12.5

Fig. 2 Fluorescent images showing transfected soybean protoplasts expressing GFP under the control of an experimental promoter (**a** and **b**). Transfected protoplasts and total cells are selected (yellow circles, **c** and **d**, respectively) by using the data analysis software. The counts of GFP-positive protoplasts (n1: 56) and total protoplasts (n2: 449) along with the transfection efficiency (12.5%) are indicated (**e**). The GFP (**a** and **c**) and brightfield (**b** and **d**) signals are shown. Scale bar: 1000 µm; 200 µm (inserts)

10. Calculate the transfection efficiency using the cell count from GFP channel and total brightfield cell count under the data reduction tab within an experiment file. An example of data analysis is shown in Fig. 2.

4 Notes

1. Make sure to dissolve Na_2EDTA completely before adding $FeSO_4$.
2. Wrap the bottle in aluminum foil; the iron stock solution is light sensitive.
3. Use Magenta™ GA-7 plant tissue culture vessels size 7.6 × 7.6 × 10.2 cm.
4. Use deep Petri dishes of 10 cm diameter and 2 cm height.
5. Warm at 50 °C to provide complete solubilization of the mannitol stock solution.
6. The use of food-grade instead of lab-grade enzymes is critical to reduce the cost for high-throughput screening in protoplasts. In our lab, the best results have been achieved using the enzyme cocktail Rohament CL (cellulase), Rohapect UF (pectinase), and Rohapect 10 L (pectinase/arabinase) from AB Enzymes.
7. Make this solution fresh the day you perform the extraction.
8. Best results have been obtained using PEG 4000 from Sigma-Aldrich Cas: 25322–68-3.
9. PEG is difficult to dissolve. For this purpose, add all components into the Falcon tube, warm the PEG solution at ~40–50 °C, and vigorously vortex for ~5 min. Repeat this procedure again if the PEG is not completely dissolved.
10. Washing seeds in 70% ethanol is necessary to remove surface waxes and dust improving the following sterilization procedure.
11. The beaker in the center of the desiccator provides homogeneous sterilization of the seeds.
12. Use sterile seeds immediately or seal the Petri dish with Parafilm before storing at room temperature. Sterile seeds may be used for up to 3 months.
13. Twenty dishes of tissue will guarantee enough callus for the following procedures of cell suspension culture preparation.
14. To avoid light, place plates in a box covered with aluminum foil.
15. Soybean suspension cultures double in cell volume in 3 days as described previously by Sultana et al. [13]. In order to guarantee healthy and vigorous cell growth, it is necessary to start a new cell suspension culture from fresh callus every 6–8 months.
16. It is important not to resuspend cells by pipetting up and down; instead, gently invert the tubes to resuspend them.

17. Keep the pipette perpendicular to the ground to prevent bubbles from forming at the bottom of the pipette.
18. Place the tip a few millimeters above the interface layer and release pipette plunger with decent force to pull cells away from interface. Be sure not to dip the tip into the sucrose phase.
19. If cells do not pellet well, there is likely sucrose in the solution. In this case, discard as much supernatant as possible, resuspend in maximum volume of W5, and centrifuge again.
20. Use a deep 96-well plate with conical-well bottom format (1.2 mL of maximum volume of well). The plates can be treated with polyHEMA to avoid any protoplasts to stick on the bottom of the well. For this procedure, prepare 20 mg/mL of polyHEMA solution dissolving 2 g of poly-(2-hydroxyethyl methacrylate) in 100 mL 95% ethanol by rotating several hours at 65 °C. After that, add 100 μL of polyHEMA solution per well, shake at 1500 rpm for 15 s, and air-dry overnight at 37 °C. The coating takes 24 h before plates can be used for protoplast transfection. PolyHEMA-coated plates can be stored indefinitely at room temperature.
21. Use a dark 96-well plate with flat bottom suitable for fluorescent microscopy (370 μL of maximum volume of well).
22. The volume can be adjusted according to the concentration of plasmid used. However, use a minimum plasmid concentration of 1 μg/μL to keep the maximum volume of 10 μL.
23. Place the deep well plate on the plate shaker at the beginning of the transfection experiment to avoid any unnecessary move during this process.
24. Manually place aluminum foil to cover the plate for incubation steps. Manually remove the aluminum foil before aspirating.
25. Leaving 300 μL of solution ensures protoplasts to remain undisturbed.
26. Manually place aluminum foil to cover the plate until the analysis using the plate reader.
27. Time-lapse images and images at different magnifications can be taken.
28. Most protoplasts are near perfect circles, but a minimum roundness value of 0.7 was selected to include some allowance of shape deformation.
29. This size parameter range is sufficient to exclude debris while accommodating standard protoplast sizes.

Acknowledgments

This study was supported by funding from the Tennessee Soybean Promotion Board and USDA Hatch Grants.

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Kan Wang and Feng Zhang (eds.), *Protoplast Technology: Methods and Protocols*,
Methods in Molecular Biology, vol. 2464, https://doi.org/10.1007/978-1-0716-2164-6,

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