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# Optimized protocols for protoplast isolation, transfection, and regeneration in the Solanum genus for the CRISPR/Cas-mediated transgene-free genome editing

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**Abstract**

The Solanaceae family includes the largest flowering crops such as tomatoes, potatoes, and eggplants. Consumer demand has led to massive development of plants in the Solanum genus, and many different Solanum varieties are now available on the market. The recent advances in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-based genome editing have allowed laboratories and smaller crop production companies to utilize the technology in various crops. The traditional transformation method in crops involves the use of Agrobacterium, which is considered the most efficient method for introducing exogenous genetic materials in target plants. The Agrobacterium-mediated transformation method has been also established in the Solanaceae family, enabling CRISPR/Cas-based genome editing in crops like tomatoes, potatoes, and eggplants. However, the Agrobacterium-mediated approach inevitably accompanies the insertion of exogenous DNA into the plant genome and often causes the formation of chimera that require further propagation steps. Alternatively, the CRISPR/Cas components can be introduced into protoplasts in the form of DNA for transient expression or a mixture of protein and RNA to avoid genomic insertion of foreign materials. The protoplast transformation approach involves processes including protoplast preparation, transfection, and regeneration, which require a comprehensive understanding and greater technical mastery of the tissue culture phase. Here we highlight the current research advances in protoplast transformation and discuss how to optimize the procedures of protoplast isolation, transfection, and regeneration for efficient and reproducible CRISPR/Cas-based genome editing in the genus Solanum.

**Keywords** Protoplast, Transient expression, Regeneration, Gene editing, CRISPR/Cas9

## Introduction

The genus *Solanum* comprises a huge collection of more than 1500 species, including important economic plants such as tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*), and eggplants (*Solanum melongena*). The genome sizes of tomato diploid, potato diploid, and eggplant haploid are estimated to be 950 Mb, 844 Mb, and 1.21 Gb, respectively [1, 2]. These plants have a relatively compact gene size, which makes them attractive targets for genetic engineering. Tomatoes, in particular, have become a popular model organism for scientific research due to their many fundamental

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biological evens including abundant nutritional constituents both primary and secondary metabolites, abiotic and biotic stress responses, typical developmental growth of fruit vegetable, and the most importantly their relatively short life cycle [3]. Short life cycle of model plant allows phenotypic and genetic observation over multiple generations quickly, which is required for rapid experimental turnaround. The *Agrobacterium*-mediated transformation method has been extensively developed in plants and is well-established for tomatoes and potatoes. This technique has enabled the production of genetically engineered plants such as the pioneering Flavr Savr tomato with delayed softening and improved resistance to environmental stressors [4, 5]. In potatoes, genetic modification has led to the development of varieties with desirable traits, such as lower acrylamide content to ensure food safety and the development of firm-cooking potatoes known as Amflora, which contains pure amylopectin starch [6, 7]. In addition, recent studies have shown the potential of CRISPR/Cas9 technology in generating mutant tomato plants for crop improvement [8, 9]. Most transgenic *Solanum* reports including the application of CRISPR/Cas9 have been employed stable gene transfer method mediated by *Agrobacterium tumefaciens*.

In the era of genome editing, an alternative modality has been devised for the delivery of the CRISPR-Cas system. Specifically, CRISPR ribonucleoprotein (RNP) complexes or plasmids harboring the CRISPR-Cas system can be directly introduced into the protoplasts, which is the unicellular state achieved through the removal of the cell wall, utilizing a method known as transient transfection [10]. There are multiple advantages to genome editing using RNP with protoplasts, including the absence of transgene insertions (DNA-free approach), rapid degradation of RNPs leading to reduced off-targeting and lower mosaicisms, the lack of necessity for codon optimization in the target plants by using Cas9 protein, and the elimination of a high level of DNA construction steps with a binary vector to insert T-DNA for expressing Cas9 and gRNA in the target plants [11]. Especially, the absence of transgene insertions allows for mitigating concerns related to genetically modified organisms (GMOs). In the case of utilizing plasmid vectors instead of RNP for transfection with protoplast, we can still anticipate the absence of T-DNA insertions, and minimal mosaicisms by expressing Cas9 and gRNA transiently [12]. And the strong advantage of plasmid vector for Cas9 and gRNA expression is that it is the most accessible method, and most of researchers and crop developers are able to use the plasmid vector.

The RNP delivery method entails protoplast isolation, protoplast transfection, and protoplast regeneration into reproductive plants. The bottleneck in plant genome editing with RNP lies in the protoplast treatment and tissue culture processes, necessitating specialized expertise in tissue culture and being inherently time-consuming. Recent studies have demonstrated that efficient gene editing is achievable by employing an optimized protocol to introduce CRISPR/Cas9-RNP complexes into tomato or potato protoplasts [11, 13]. Although previous studies have reported high editing efficiency in protoplast transformation, the regeneration of shoots from RNP-transfected protoplasts remains a challenging technical bottleneck due to low survival rates until the generation of whole transgenic plants [11, 13]. Several previous reports have documented successful regeneration from the non-transformed protoplasts of cultured tomatoes [14, 15]. Moreover, recent studies have demonstrated protoplast regeneration in transformed tomatoes with CRISPR/Cas9-induced mutations [10, 16, 17]. However, genetic modifications of other important *Solanum* species such as potatoes, eggplants, and bell peppers are still challenging due to the low efficiency of the transfection and regeneration [13, 18]. Given the reported variability in regeneration and low mutation rates, an optimized protocol needs to be developed to achieve both high editing efficiency and high reproducibility in target plants.

The establishment of a technique for transgene-free genome editing and proficient protoplast regeneration within the genus *Solanum* holds the potential to facilitate precise genome modifications. Consequently, a comprehensive examination of experimental parameters concerning protoplast isolation, protoplast transfection, and subsequent regeneration within the *Solanum* genus was undertaken to delineate the optimal conditions conducive to successful genome modifications.

## Protoplast transformation for transgene-free CRISPR/Cas-based gene editing

The gene editing process involving protoplasts comprises three primary phases: Protoplast isolation, protoplast transfection, and protoplast regeneration. Protoplast isolation is a fundamental procedure wherein plant cells undergo enzymatic or mechanical treatment for the removal of cell walls. This process yields denuded cells utilized across diverse domains of plant research, encompassing genetics, biotechnology, and cell biology [19–22]. Protoplast transfection serves as a potent tool in plant genetic engineering, enabling the introduction of foreign genes, elucidation of gene function, and the generation of transgenic plants with desired traits [14, 23]. These techniques provide researchers with the means to investigate the functional aspects of specific genes and introduce new genetic materials into plant cells, thereby facilitating the development of improved and novel plant varieties [10, 16]. Protoplast regeneration encompasses the

division of protoplasts, the synthesis of new cell walls, and their eventual development into fertile plants [14, 24]. This technique finds extensive application in the generation of transgenic plants, achieved either through particle bombardment of callus or Agrobacterium-mediated transformation. Overall, protoplast isolation and regeneration techniques play an important role not only for plant scientists and breeders seeking to improve plants and advance plant biology, but also in plant biotechnology for the production of genetically modified plants with desirable traits such as increased resistance to pests or diseases, improved flavor, or longer shelf life.

In addition to protoplast isolation and regeneration, the selection of the CRISPR-Cas delivery system serves another critical technical aspect for establishing an efficient crop genome editing procedure. The use of a pre-made CRISPR/Cas9 ribonucleic acid protein complex with protoplasts is considered the most advanced and efficient method for CRISPR-Cas system transfection [13, 25]. The utilization of a pre-made RNP complex eliminates the need for cells to transcribe and translate the Cas9 protein, thereby enabling faster and more efficient editing and enhancing editing efficiency [26]. Transient expression of CRISPR/Cas9 is important to avoid prolonged exposure and minimize the risk of unintended mutations [27]. Therefore, pre-made RNP can minimize off-target effects by mitigating continuous Cas9 protein expression, thereby reducing cellular stress and potential unintended effects on cell physiology. Additionally, it simplifies the experimental workflow by eliminating the need to clone Cas9 and guide RNA into a vector, saving time and resources in the experimental setup. These advantages contribute to the efficiency, precision, and broad applicability of the CRISPR/Cas9 system in various experimental settings [28].

## Considerations for high-efficiency protoplast isolation

To enhance the efficiency of protoplast transfection and regeneration, it is crucial to generate healthy and viable protoplasts. Some essential considerations for the effective isolation of protoplasts are outlined in Table 1 and described below. Optimal isolation results are typically attained by utilizing fresh and actively growing tissues, such as young leaves or hypocotyls, as their cell walls are thinner and more susceptible to enzymatic digestion [12, 29].

In protoplast isolation methods, the efficiency of enzymatic cell wall digestion is crucial. Complete removal of the cell wall is necessary without compromising the viability of the protoplast. To efficiently separate protoplasts, the dissolution conditions of fresh plant tissue, enzyme solution (e.g., cellulase, pectinase, macerozyme), mannitol, and calcium chloride play a major role. Cellulase is an enzyme that hydrolyzes cellulose, the primary component of plant cell walls. The recommended concentration is typically 1.5–2% (w/v), but higher concentrations of cellulase may be required to efficiently digest cellulose-rich cell walls in tomato and eggplant tissues. The concentration range of cellulase can be optimized for the specific cell wall composition of these plant species [12, 30–33]. Macerozyme is a complex enzyme mixture containing cellulase, hemicellulase, and pectinase activities. Hemicellulase and pectin lyase specifically target hemicellulose and pectin, respectively, which are components of plant cell walls. The inclusion of these enzymes, along with cellulases, helps break down a wide range of cell wall components, ensuring thorough digestion and release of protoplasts [34, 35]. Osmotic equilibrium is imperative for maintaining the structural and functional integrity of protoplasts. Mannitol or sorbitol is employed to sustain osmotic balance as an osmotic substance, thereby preventing osmotic shock to the protoplasts [36]. Consequently, these osmotic substances are consistently utilized in all subsequent procedures, encompassing the washing of protoplasts after enzymatic digestion, transfection, and regeneration, until the formation of callus. Calcium ions play a pivotal role in stabilizing the plasma membrane of protoplasts, thereby contributing to the facilitation of the fusion process [37]. Calcium chloride is also utilized to induce the fusion of isolated protoplasts. In this context, calcium ions aid in the establishment of bridges between adjacent protoplasts, thereby facilitating their fusion. Optimal execution of the cell wall lysis step necessitates precise adjustment of enzyme concentrations, incubation time, mannitol concentration, and shaking speed, tailored to the characteristics of the specific plant species and tissue types, ensuring optimal outcomes. This optimization process aims to maximize both the yield and viability of the protoplasts. Through careful optimization of these parameters, the attainment of a high yield of viable protoplasts becomes feasible for subsequent analysis and manipulation [24].

The procedure commences with the excision of fresh plant tissue, which is then finely cut into small pieces, approximately 0.5 to 1 mm in size (Fig. 1A). Subsequently, these tissue fragments are immersed in an enzyme solution designed to facilitate the digestion of cell walls, leading to the subsequent release of protoplasts (Fig. 1B, C). The enzyme solution employed for efficient protoplast isolation from tomatoes typically comprises 0.75% (w/v) Macerozyme R-10, 1.5% (w/v) Cellulase Onozuka R-10, 0.6 M mannitol, 10 mM $CaCl_2$, 0.1% (w/v) BSA, 10 mM MES, with the pH adjusted to 5.8 [11, 16, 23]. The tomato tissue is fully immersed in the enzyme solution and subsequently incubated at room temperature in the dark for

**Table 1** Enzyme solution for protoplast isolation in previous studies

| Species | Tissue source | Predigestion | Enzyme composition | Digestion buffer | Conditions | Yield | Reference |
|---|---|---|---|---|---|---|---|
| Tomato *(solanum lycopersicum)* | Leaves | Sliced | 2% cellulase R-10, 0.5% macerozyme R-10 | 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM $KH_2PO_4$, 0.5 mM $Na_2HPO_4$, 0.5 mM KCl, 0.4 M sucrose-K3 solution, micronutrients | 12 h, overnight, 27 °C, dark | 575,000 ± 153,292.7 (86.91%) | Horvath [29] |
| Tomato *(solanum lycopersicum, Solanum pennellii, Solanum pimpinellifolium)* | Leaves | Sliced | 1.5% Cellulase RS, 0.75% Macerozyme R-10 | 0.6 M mannitol, 10 mM MES, 10 mM $CaCl_2$, 0.1% BSA | 16 h, 25 ℃, dark, | $1.0 \times 10^6$ protoplasts/ml | Nicolia et al. [11], Liu et al. [3] |
| Tomato *(Solanum peruvianum)* | Leaves, Stems | Sliced | 1.5% Cellulase RS, 0.75% Macerozyme R-10 | 0.6 M mannitol, 10 mM MES, 10 mM $CaCl_2$, 0.1% BSA | 4–5 h, dark, 60-80 rpm | $4.8 \times 10^6$ | Lin et al. [16] |
| Tomato *(Solanum esculentum)* | Roots | Sliced | 5% Myrothecium cellulase, 0.02 M phosphate buffer | 0.6 M Sucrose | 2 h, 27 ℃ | Not disclosed | Cocking et al. [34] |
| | Leaves | Sliced | 0.75% Cellulase, 0.1% macerozyme | 0.3 M mannitol, 170 mg/L $KH_2PO_4$, 440 mg/L $CaCl_2{\cdot}2H_2O$, 1500 mg/L $KNO_3$, $MgSO_4{\cdot}7H_2O$, 0.3 M sucrose, vitamins, 1.0% polyvinylpyrolidine, 5 uM MES | 4–6 h, 28 ℃, 60 rpm | $6.6 \times 10^6$/g | Shahin [46] |
| | Leaves | Sliced | 3% Meicelase-P, 0.1% Macerase | 13% mannitol, 9% Sorbitol, CPW salt | 12–14 h, 27 °C, 45 rpm | $3 \times 10^4$/ml | Randall et al. [39] |
| | Roots | Sliced | 2% cellulase, 2% macerozyme, 0.1% pectolyase | 500 mM sorbitol, 10 mM $CaCl_2$, 0.5% BSA, 5 mM DTT, 0.5% PVP, 5 mM MES | 2 h, 27 ℃, 60 rpm | $1 \times 10^5$ Cell·ml$^{-1}$ | Shin et al. [40] |
| Tomato *(Lycopersicon hirsutum, Solanum esculentum)* | Leaves | Sliced | 0.6% cellulase, 0.1% pectinase | 9% Mannitol, 1.0 mM NaCl, 0.2 mM KCl, 0.2 mM $CaSO_4$, 3 mM MES | 8 h, dark | Not disclosed | Rueda et al. [42] |
| Potato *(Solanum tuberosum)* | Tubers | Sliced | 0.2% Pectolyase, 0.5% Rhozyme, 1% Cellulase R10 | 0.5% BSA, 10% mannitol | 4–5 h, dark | $7.5 \times 10^4$ to $5 \times 10^5$/g | Jones et al. [45, 58] |
| | Tubers | Sliced | 4% cellulose R-10, 0.8% macerozyme R-10, 1% hemicellulase | 0.55 M mannitol, 2 mM CaCl2, 1 mM $KH_2PO_4$, 10 mM MES, 1 mM $MgCl_2$, 50 mM Tris buffer | 18–20 h, 29 ℃, dark, 180 rpm | $1.6 \times 10^5$ protoplasts/g fresh weigh | Laimbeer et al. [43] |
| | Leaves | Sliced | 1% Cellulase R-10, 0.5% Macerozyme R-10 | 0.5 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, 15% sucrose, 0.1% BSA | 6 h, dark, 40 rpm | $1.6 \times 10^6$ protoplasts/g fresh weigh | Moon et al. [67] |
| | Leaves | Sliced | 0.5% Cellulase R-10, 0.5% Macerase R-10 | 5 mM $CaCl_2$, 0.01 M MES, 0.5 M sucrose | 15–16 h, 28–30 ℃, | $2.4 \times 10^6$ to $4.6 \times 10^6$ | Konovalova et al. [44] |

**Table 1** (continued)

| Species | Tissue source | Predigestion | Enzyme composition | Digestion buffer | Conditions | Yield | Reference |
|---|---|---|---|---|---|---|---|
| Eggplant *(Solanum melongena L. and Solanum insanum L.)* | Leaves | Sliced | 0.067% Macerozyme R-10, 0.33% Meicelase | 0.5 M mannitol, 1/2 MS salts (200 mg/L $NH_4NO_3$), 1% sucrose, 1/2 MS vitamins | 16 h, 25 ℃ | $1\times10^4$ per hypocotyl | NISHIO et al. [37] |
| Eggplant *(Solanum melongena L.)* | Stems | Sliced | 1.5% Cellulase R-10, 0.75% Macerozyme R-10 | 6 mM $CaCl_2$, 1 mM $KH_2PO_4$, 0.5 M Sucrose, 0.05% MES | 3 h, 27 ℃, dark | 4 to $5\times10^4$ protoplasts per ml | Sihachakr et al. [68] |
| | Leaves | Sliced | 1.25% cellulose, 0.4% macerozyme | 0.5 M mannitol, 10 mM $CaCl_2$, 20 mM MES, 1.5 mM $MgCl_2$, 20 mM KCl, 1.5 mM $KH_2PO_4$ | 3 h, 26 ℃ | $1.2\times10^7$/g fresh weight (FW) | Wang et al. [48] |
| | Leaves | Sliced | 0.3% cellulase R10, 0.65% pectinase | 0.3 M mannitol, 2% PVP, 0.2% BSA, 3 mM MES, 1/10 RA salts | Overnight, 25 ℃, dark | $2\times10^5$ $ml^{-1}$ | Yu et al. [18] |
| | Leaves | Sliced | 1.5% Cellulase R-10, 0.5% Macerozyme R-10 | 6 mM CaCl2, 1 mM KH2PO4, 0.5 M Sucrose, 0.05% MES | Overnight, dark | $5\times10^4$ protoplasts per ml | Fournier et al. [20] |

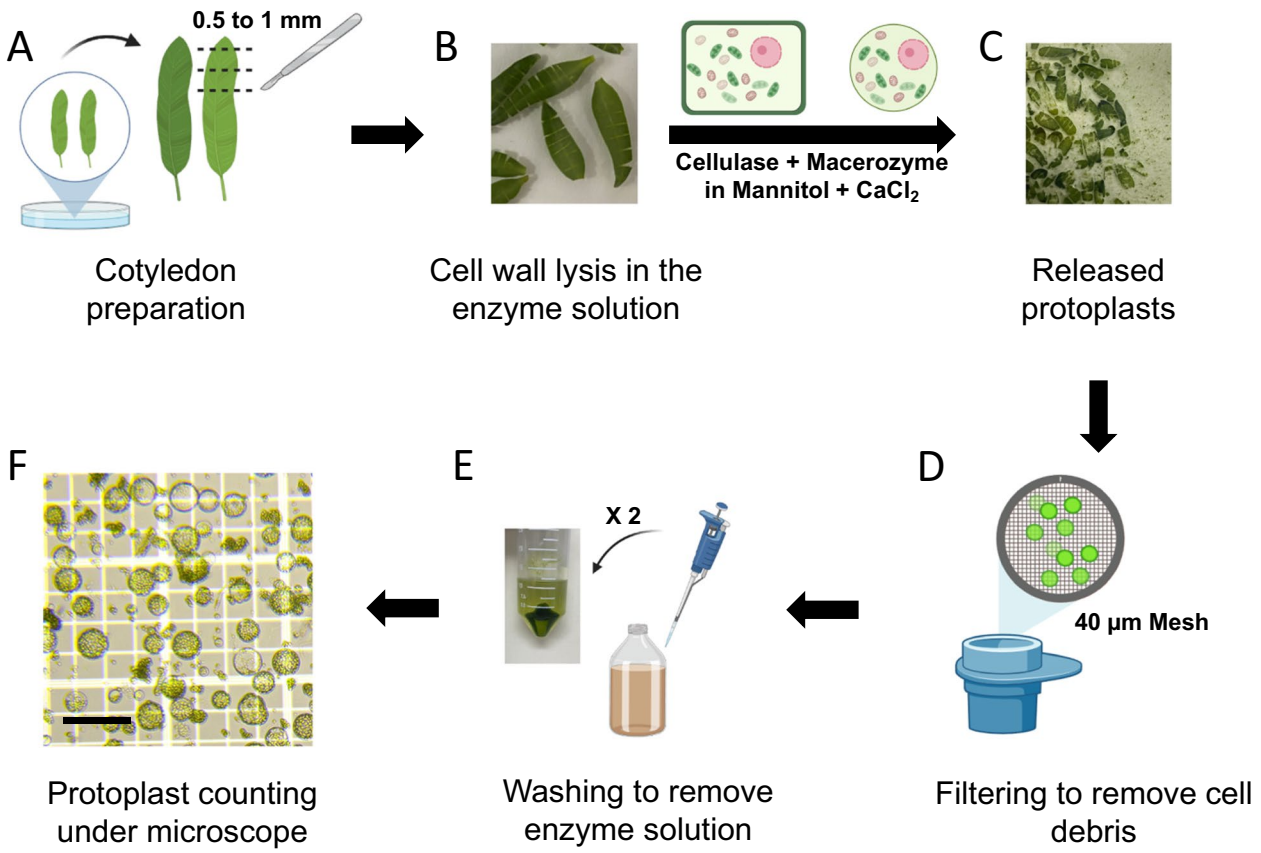



**Fig. 1** Schematic diagram of protoplast isolation from plant cotyledons. **A** Cotyledon preparation by slicing into thin strips (0.5 to 1 mm), **B** Incubation of sliced cotyledons in the digestion solution for 16 h, **C** Confirmation of the release of protoplasts in the digestion solution, **D** Filtration using a 40 μm mesh to remove cell debris, **E** Removal of the enzyme solution by washing with the stabilizing solution twice, **F** Microscopic observation to count protoplasts. Scale bar indicates 50 μm

3 to 5 h with gentle shaking at 50 to 70 rpm. It is noteworthy that the incubation time may vary depending on the specific tissue type and the combination of enzymes employed. The selection of enzymes for protoplast isolation is contingent upon the plant species, as detailed in Table 1. For the extraction of protoplasts from tomato and eggplant, it is generally advisable to utilize cellulase at a concentration of 1.5 to 2% (w/v), in conjunction with additional cell wall lysis enzymes such as macerozyme or pectin lyase [38–42]. 1% (w/v) cellulase was shown to be effective for extracting protoplasts from leaves and tubers in potatoes [43–45]. The isolation buffer for tomato protoplasts needs to be supplemented with 0.6 M sorbitol or mannitol to reduce osmotic shock during the isolation process, as suggested before [11, 23, 46]. Proper control of enzyme concentrations and treatment durations is crucial to minimize damage to the protoplasts and maximize the yield of viable cells. Following cell wall lysis, the reaction mixture is filtered through a 40-μm nylon mesh to eliminate undigested tissue and large debris (Fig. 1D). Subsequently, the filtrates are subjected to low-speed centrifugation (e.g., 100×*g*) for 5 to 10 min, forming a pellet consisting of protoplasts. Protoplasts, being denser than the buffer solution, can be easily separated in this manner. The isolated protoplasts are washed in a stabilizing solution containing 0.6 M sorbitol or mannitol at least twice to remove residual enzymes (Fig. 1E) and examined under microscope (Fig. 1F). As the isolation process can cause stress in the cells, it is imperative to minimize mechanical stress to avoid damage to the protoplasts. During the filtering step, gentle pipetting is highly recommended to mitigate excessive mechanical stress in the cells.

## Protoplast transfection for transgene-free gene editing

Protoplast transfection is a well-known scientific method employed for the introduction of exogenous DNAs into plant cells, enabling their transient expression with no need for genomic integration [11]. During the process, protoplasts are exposed to foreign DNA and subsequently incubated under controlled conditions to

facilitate DNA uptake [47]. The success of transfection relies on the species of target plants and the types of foreign genetic materials used, requiring optimization of factors such as DNA or RNA concentration, incubation time, and temperature.

The efficacy of protoplast transfection depends on the selected approach and shows variability. Numerous transfection conditions have been extensively documented, each of which is associated with distinct efficiencies [24]. Among the widely embraced methods for introducing foreign DNA into eukaryotic cells is transfection through polyethylene glycol (PEG) [11]. PEG, a water-soluble polymer, forms complexes with DNA, thereby enhancing its cellular uptake [48]. The success rates of the PEG-mediated protoplast transfection typically fall within the range of 30 to 50% [23]. According to the Wang et al. [48], PEG-mediated transfection system of eggplant protoplasts, the transformation efficiency was increased until the PEG concentration was reached to 40% and then slightly decreased. An optimal transfection efficiency of approximately 53% was observed at 40% of PEG concentration in the eggplant [48].

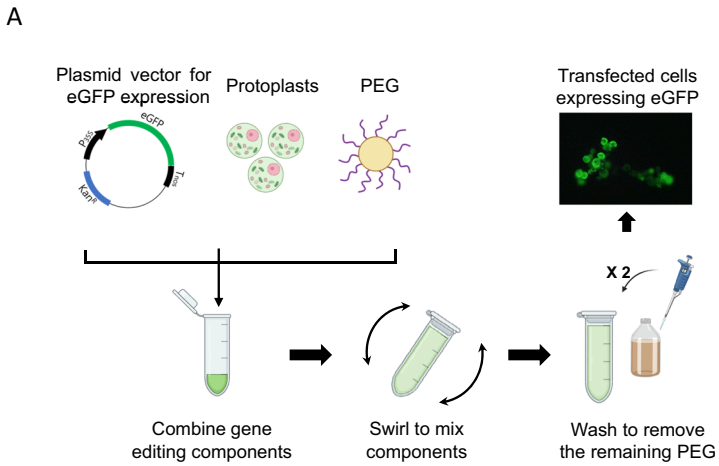
A common protocol for PEG-based transfection with nucleic acids is described below (Fig. 2A). The DNA or RNA intended for transfection is purified and subsequently dissolved in an appropriate buffer solution (40% PEG, 0.2 M Mannitol, 100 mM $CaCl_2$). Protoplasts extracted from fresh plant tissues as described earlier (Fig. 1) are resuspended in a solution containing PEG and the purified nucleic acids and gently mixed (Fig. 2A). The mixture of protoplasts-PEG-DNA is incubated at room temperature with swirling for 10 to 15 min. The transfected protoplasts are washed twice with a buffer solution (5 M NaCl, 1 M $CaCl_2$, 1 M KCl, 1 M MES) to mitigate the potential toxicity of PEG to the cells, and then plated onto tissue culture plates or dishes containing complete media, facilitating their growth and expansion. Introduction of foreign genetic materials can be confirmed by visualizing of marker proteins such as fluorescent proteins (Fig. 2A). We found that the concentration of PEG affects the viability of transfected protoplasts. As the concentration of PEG increases from 10 to 40%, the fraction of viable cells tends to decrease (Fig. 2B). The vitality was assessed using 0.01% fluorescein diacetate (FDA) staining and visualization under a fluorescence microscope. As the PEG concentration increased from 10 to 40%, there was a tendency for the fraction of viable cells to decrease (Fig. 2B). Notably, at a 40% PEG concentration, approximately 20% of healthy protoplasts were observed in tomato protoplasts under the previously described transfection conditions. Therefore, it is crucial to carefully consider and determine the optimal PEG concentration for both protoplast transfection efficiency and cell viability.

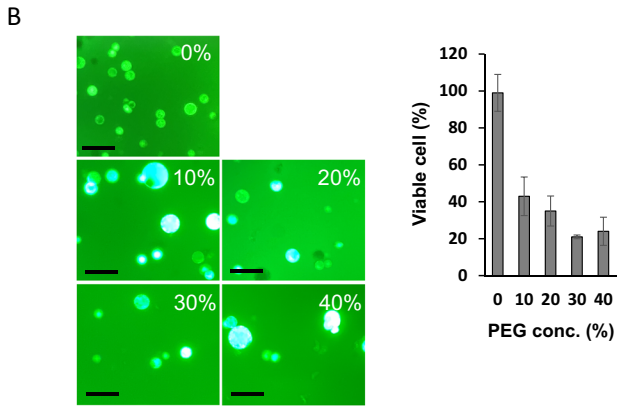
The DNA-free genome editing method employing CRISPR/Cas9 RNPs has emerged as a viable alternative to conventional DNA-based approaches [11]. Protoplasts can be transfected in a completely DNA-free manner by introducing a complex of protein and RNA [16]. This strategy involves the direct delivery of preassembled Cas9 protein and gRNA to the target cells, circumventing the possibility of DNA integration into the genome [26, 49]. Furthermore, the RNP complex delivered into the cell is less stable and subsequently degrades by cellular enzymes after inducing mutations in the target gene. This degradation helps prevent off-target mutagenesis, resulting in low off-target rates [50]. To create a pre-made CRISPR/Cas-gRNA RNP complex, both components can be easily obtained by ordering commercial supplements. The Cas protein can be obtained from bacterial expression, and the gRNA can be transcribed in vitro [51, 52]. The gRNA synthesized in vitro can be modified and used for versatile purposes [53, 54].

Notably, the PEG-mediated transfection has been attempted to deliver CRISPR/Cas9 RNPs for genome editing in *Solanum* genus, including potatoes (*S. tuberosum*) and tomatoes (*S. lycopersicum*) [13, 23]. In their study, Andersson et al. [13] performed the PEG-mediated transfection on potato protoplasts to introduce CRISPR/Cas9 RNPs targeting the granule-bound starch synthase (GBSS) gene. The CRISPR/Cas9 RNPs successfully induced mutations in the GBSS gene, yielding transgene-free edited plants with altered starch contents [13]. In their study, Naing et al. [17] also utilized PEG-mediated delivery to introduce CRISPR/Cas9 RNPs targeting the phytoene desaturase (PDS) gene into tomato protoplasts. This resulted in the production of albino plants with reduced pigment contents [17]. Despite the limited

(See figure on next page.)

**Fig. 2** PEG-based protoplast transfection for gene edition. **A** An overview of the PEG-based protoplast transfection procedure. Transfection can be confirmed by visualizing a marker protein, GFP. **B** The viability of tomato protoplasts was observed under a fluorescence microscope. Protoplasts with green fluorescence were viable. Scale bars indicate 50 μm. Protoplasts exhibiting a perfect round shape were counted as viable protoplasts ($n = 6$; $^*p < 0.05$). **C** Comparison of PEG-mediated protoplast transfection and Agrobacterium-mediated transfection targeting the ALS2 gene in tomato cells using the CRISPR/Cas9 system. The target sequences are in bold, and the edited regions are in red. The black underline indicates the gRNA sequences, and the red letters indicate the PAM sequence

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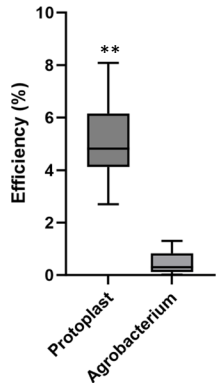


| ALS2 target | CTATTACAGGTCAAGTGCCAAGG | Number of cases | Efficiency (%) |
|---|---|---|---|
| Protoplast | CTATTACAGGTCAAGTGCCA | 190505 | |
| | CTATTACAGGTCA----CCA | 5466 | |
| | CTATTACAGGTCAAGT-CCA | 1517 | |
| | CTATTACAGGTCAAGT----- | 634 | |
| | CTATTACAGGT------CCA | 571 | |
| | CTA-TACAGGTCAAGTGCCA | 12 | |
| | CTATT-CAGGTCAAGTGCCA | 6 | |
| | CTATTACAGGTCAAGTG-CA | 5 | |
| | CTATTACAGGT-AAGTGCCA | 3 | |
| | CTATTACAGGTC-AGTGCCA | 3 | |
| | CTATTACAGGTCAAGTGACCA | 964 | |
| | CTATTACAGGTCAAGTGTCCA | 825 | |
| | CTATTACAGGTCAAGTCTCCA | 2 | 5.25 |
| Agro | CTATTACAGGTCAAGTGCCA | 47836 | |
| | CTATTACAGGTCA----CCA | 55 | |
| | CTATTACAGGTCAA---CCA | 50 | |
| | CTATTACAGGTC-----CCA | 23 | |
| | CTATTACAGGT-------CA | 12 | |
| | CTATTACAGGTCAAGT-CCA | 12 | |
| | CTATTACAGGT------CCA | 10 | |
| | CTATTACAGGT--------A | 10 | |
| | CTAT----------CCA | 8 | |
| | CTA-TACAGGTCAAGTGCCA | 4 | |
| | CTATTACAGGTCAAGT---- | 3 | |
| | CTATTACAGGTCAAGTGGCCA | 10 | |
| | CTATTACAGGTCAAGTGCCCA | 8 | |
| | CTATTACAGGTCAAGTGTCCA | 4 | 0.44 |

**Fig. 2** (See legend on previous page.)

number of cases studied, the utilization of PEG-mediated transfection as a delivery technique for CRISPR/Cas9 RNPs presents numerous benefits including exceptional efficiency, minimal toxicity, and ease of use, as described before in this report. In our previous study, a highly efficient gRNA was selected to edit the herbicide-related gene ALS2 in tomatoes [55, 56]. Using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/), a sequence of 20 bp in the 5 direction from the protospacer adjacent motif (PAM, 5′-AGG) sequence in the target gene of the tomato genome was selected as 5′-CTATTACAGGTC AAGTGCCA-3′. According to previous research Yu et al. [56], the corresponding guide RNA (gRNA) targets the allele to produce the ALS2 protein with reduced susceptibility to herbicides by correcting the amino acid at position P197 [56]. With the same gRNA, we found that the PEG-mediated protoplast transfection is approximately twelve times more efficient than the *Agrobacterium*-mediated transfection, supporting the superior editing efficiency of this method (Fig. 2C). The experimental results of ALS2 gene transfection via PEG-mediated protoplasts revealed a maximum value of 8 and an average value of 4.8. The value depicted in Fig. 2C is a measurement of 5.25 within the box plot range, displaying diverse patterns. In contrast, the experimental results from the *Agrobacterium*-mediated transfection method displayed a maximum value of 1.3 and an average value of 0.7. Various editing patterns were observed within the box plot range, with a value of 0.44. These findings suggest that the protoplast transfection method might exhibit higher efficiency in gene editing compared to the *Agrobacterium*-mediated transfection method.

Electroporation is another widely employed technique for protoplast transfection with exogenous DNA. In this method, a transient electric field is applied to the protoplasts to temporarily induce pores in the cell membrane, allowing the entry of foreign DNA [57, 58]. Jones et al. employed electroporation to investigate the factors influencing transient gene expression in protoplasts derived from various potato tissues, such as leaves, tubers, and suspension cells. They suggested that the most favorable field strength depends on the protoplast size, and the optimal field strength varies in response to the application of electrical pulses, ranging from 150 to 250 V/cm, highlighting the roles of each parameter in successful electro-transfection [58]. As the effects of these factors may also vary depending on the plant species, the quality of the starting protoplasts, and the specific conditions used in the transfection, the optimization of the electroporation process is crucial for maximizing transfection efficiency while minimizing cell damage [59, 60].

Electroporation has been successfully employed for delivering CRISPR/Cas9 components into protoplasts, with a demonstrated transfection efficiency ranging from 20 to 30% [61]. A protoplast electro-transfection protocol for the *Solanum* genus is outlined as follows [58, 62]. The purified DNA to be transfected is dissolved in a buffer solution (1 M Mannitol, 0.3 M $MgCl_2$, 1 M MES) that is compatible with the protoplasts. Subsequently, 10 to 20 μg/ml (final concentration) of the DNA is mixed with the isolated protoplasts (approximately $2 \times 10^5$ cells/ml) in a tube or cuvette. Electrical DC pulses of 50 μs duration at 500 to 800 V/cm are applied to the mixture of protoplasts and DNA using an electroporator [62]. The transfected protoplasts are then washed twice with a buffer solution (5 M NaCl, 1 M $CaCl_2$, 1 M KCl, 1 M MES) and plated onto tissue culture dishes containing complete media for cell growth and expansion. Commonly used protoplast culture media are described later in the protoplast regeneration section.

## Plant regeneration from transfected protoplasts

Protoplast regeneration is the process of reconstructing plant cells from isolated protoplasts (Fig. 3). The mechanism of regeneration varies depending on the tissue types or plant species [24]. In certain cases, regeneration involves the activation of stem cells or the reprogramming of existing cells into a less differentiated state, facilitating tissue repair and regeneration [63]. Regeneration is commonly achieved by sequentially growing protoplasts in two distinct media before initiating the general plant callus growth and regeneration process: the protoplast culture medium and the callus-inducing medium. Specialized media known as protoplast culture medium and callus-inducing medium are formulated with essential nutrients and growth factors that promote cell division and cell wall reconstitution [14, 23]. Similar to other plant tissue culture media, the Murashige and Skoog (MS) medium or Kao and Michayluk (KM) medium, a balanced salt solution, is commonly employed to constitute the both medium. MS medium has been employed for providing essential inorganic nutrients including nitrogen, phosphorus, potassium, and micronutrients [24]. Additionally, vitamins such as thiamine, pyridoxine, and nicotinic acid are introduced to support plant tissue growth and development. Carbohydrates, such as sucrose or glucose, are included as an energy source in most cases, and glutamine, asparagine, and proline may be added to serve as additional nitrogen sources for protein synthesis. The pH of medium is typically maintained around 5.5 to 6.5, with buffering agents like MES or MOPS added to stabilize the pH.

The first distinct medium for protoplast regeneration, the protoplast culture medium, may contain additional components such as 0.3 to 0.8 M of mannitol to provide osmotic support, maintain cell integrity, and

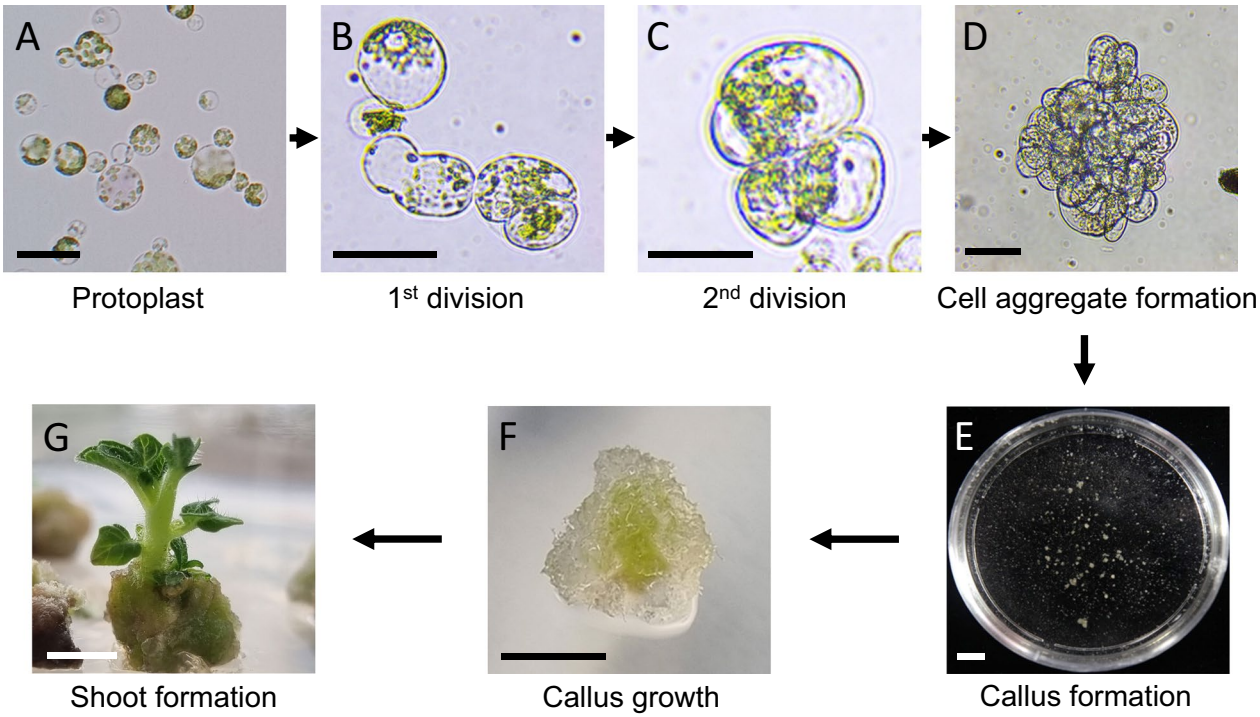


**Fig. 3** Plant regeneration from the tomato protoplasts. **A** Tomato protoplasts freshly isolated from cotyledons, **B** Protoplasts during first cell divisions in 7 days after isolation in the protoplast culture medium, **C** Protoplasts during second division after 14 days in the protoplast culture medium, **D** Cell aggregate formation after 3 weeks in the protoplast culture medium, **E** Callus formation after 1 months on the callus induction medium, **F** Callus growth for 2 months on the callus growth medium, **G** Shoot regenerated on the shoot induction medium for 2 months. Scale bars, 50 µm **A**–**D** and 1 cm **E**–**G**

promote cell division [39, 46]. Plant growth regulators are also included to facilitate cell division and regeneration. Among the most commonly used plant growth regulators are Indole-3-acetic acid (IAA) or 1-Naphthaleneacetic acid (NAA) as auxins, and zeatin as cytokinins, added to the medium at a concentration of 0.1 to 1.0 µM to promote cell division and differentiation [24, 64, 65]. The protoplast regeneration process is challenging and requires careful manipulation of various factors, including nutrient concentrations, plant growth regulators, and environmental conditions [24]. Additionally, the composition of the media needs to be optimized for specific plant species or cell types, as presented in Table 2 [23, 24, 66]. The solution used for efficient protoplast regeneration from tomatoes usually consists of 3.62 *g*/L KM medium, 3% sucrose, 0.5 M mannitol, 2 mg/L 2,4-D, 0.5 mg/L BAP, and 10% KM vitamin solution, with the pH adjusted to 5.8.

Once the protoplasts are successfully cultured and maintained in the culture medium, they are transferred to the callus-inducing medium, which supports cell proliferation and development of pluripotent callus. For callus induction of the protoplasts from tomato leaves, the MS medium is typically supplemented with 1 or 2 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L of IAA. The optimal concentration of 2,4-D may vary depending on the *Solanum* species (Table 2). For eggplants, add 0.2 or 1.0 mg/L of 2,4-D and 1 mg/L of NAA to the medium. Additionally, 1 mg/L of kinetin or 0.5 mg/L of benzyladenine (BA) may be included to promote callus induction [67, 68]. The concentration of cytokinin is typically lower than that of auxins. The medium is typically solidified with 0.8% (w/v) of agar and maintained at pH 5.7. Other additives such as sucrose, vitamins, and amino acids can also be incorporated into the medium to support the growth and differentiation of the callus. The composition of the media used during the leaf-to-callus transition in three different *Solanum* species—tomatoes, potatoes, and eggplants—is similar (Table 2). The regenerated cells are examined to confirm the presence of transfected DNA by PCR and sequencing.

## Concluding remarks

In the era of CRISPR/Cas9-based genome editing, protoplast transformation plays a key role in efficient crop engineering and breeding. Given the significant market shares of tomatoes, potatoes, and eggplants in the vegetable industry, it becomes essential to develop

**Table 2** List of the protoplast regeneration media used in previous studies

| Species | Protoplast culture medium | Callus Induction medium | Callus growth medium | Shoot Induction medium | Rooting Induction medium | Reference |
|---|---|---|---|---|---|---|
| Tomato *(solanum lycopersicum)* | Microcalli proliferation medium (3.62 *g*/L KM medium, 3% Sucrose, 9% Mannitol), 2 mg/L 2,4-D, 0.5 mg/L BAP, 10% KM Vitamin Solution | 4.4 *g*/L MS(Murashige & Skoog) medium containing vitamins, Myo-Inositol 60 *g*/L, 2 mg/L 2.4-D, 0.5 mg/L 6-BAP, 30 *g*/L Sucrose, 8 *g*/L Plant Agar | 4.4 *g*/L MS(Murashige & Skoog) medium containing vitamins, 2 mg/L 2.4-D, 0.5 mg/L 6-BAP, 30 *g*/L Sucrose, 8 *g*/L Plant Agar | 4.4 *g*/L MS(Murashige & Skoog) medium containing vitamins, 0.5 mg/L a-NAA, 2 mg/L 6-BAP, 30 *g*/L Sucrose, 30 *g*/L Plant Agar | 4.4 *g*/L MS(Murashige & Skoog) medium containing vitamins, 1 mg/L IBA, 30 *g*/L Sucrose, 8 *g*/L Plant Agar | |
| Tomato *(Solanum peruvianum)* | Microcalli not transferred | None | None | 4.405 *g*/L MS medium with vitamins, 30 *g*/L sucrose, 6 *g*/L phyto agar, e 0.1 mg/L IAA and 0.75 mg/L zeatin | None | Lin et al. [10] |
| Tomato *(Solanum esculentum)* | MS salts without $NH_4NO_3$, 1900 mg/L $KNO_3$, 1000 mg/L myo-Inositol, 20 mg/L Casein hydrolysate, 2 mg/L Thiamine-HCl, 2 mg/L Giycine, 0.5 mg/L Nicotinic acid, 1 mg/L 2.4-D, 0.5 mg/L IAA, 0.5 mg/L 6-BAP, 10 *g*/L Sucrose, 80 *g*/L Mannitol, 5000 mg/L Glucose | MS salts without $NH_4NO_3$, 1900 mg/L $KNO_3$, 1000 mg/L myo-Inositol, 20 mg/L Casein hydrolysate, 2 mg/L Thiamine-HCl, 2 mg/L Giycine, 0.5 mg/L Nicotinic acid, 1 mg/L 2.4-D, 0.5 mg/L IAA, 0.5 mg/L 6-BAP, 10 *g*/L Sucrose, 80 *g*/L Mannitol, 5000 mg/L Glucose | MS(Murashige & Skoog) medium containing vitamins, 1 mg/L IAA, NAA, 0.4% Bacto Agar, | MS(Murashige & Skoog) medium containing vitamins, 2 mg/L Zeatin, 30 g/L Sucrose | MS(Murashige & Skoog) medium containing vitamins, 10 *g*/L sucrose, 7 *g*/L Bacto agar | Randall et al. [39] |
| Potato *(Solanum tuberosum)* | Microcalli not transferred | MS(Murashige & Skoog) medium containing vitamins, 20 *g*/L Sucrose, 0.05 mg/L 6-BAP, 10 *g*/L Bacto agar | Macro A and micro A stocks, iron elements A, carbohydrate A, vitamins A, 1 *g*/L BSA, 1.0 mg/L NAA, 0.4 mg/L BAP, and other organics, | 30 *g*/L sucrose, 0.01 mg/L NAA, 2.0 mg/L zeatin, 0.1 mg/L Gibberellic acid (GA3), 4.0 *g*/L plant agar, 10 *g*/L sucrose, 0.01 mg/L NAA, 2.0 mg/L zeatin, 0.1 mg/L GA3, 2.5 *g*/L gelrite | None | Moon et al. [67] |
| Eggplant *(Solanum melongena L.)* | KM medium, 0.5 mg/L Zeatin, 0.2 mg/L 2.4-D, 1 mg/L NAA, 0.05% MES, 6.3% Glucose | MS medium, 7 *g*/L Agar, 20 *g*/L Sucrose, vitamin, 1–4 mg/L 6-BAP, 0.2 mg/L IAA, 1–4 mg/L Zeatikn | MS medium, 7 *g*/L Agar, 20 *g*/L Sucrose, vitamin, 1–4 mg/L 6-BAP, 0.2 mg/L IAA, 2 mg/L Zeatikn, 0.5 mg/L P, 0.5 mg/L NAA | None | None | Sihachakr et al. [68] |
| Eggplant *(Solanum melongena L ,Solanum insanum L.)* | 1/2 MS salts (200 mg/L $NH_4NO_3$), 1 mg/L NAA, 1 mg/L 2.4-D, 1 mg/L Kinetin | MS salts (200 mg/L NH4NO3), 1 mg/L NAA, 1 mg/L 2.4-D, 0.2 M mannitol, 3% Sucrose, 0.8% Agar | MS salts (200 mg/L NH4NO3), 1 mg/L NAA, 1 mg/L 2.4-D, 0.2 M mannitol, 3% Sucrose, 0.8% Agar | 1/3 MS, 1% Sucrose | MS medium, 1 mg/L NAA | Nishio et al. [37] |
| Eggplant *(Solanum melongena L.)* | KM medium, 0.5 mg/L Zeatin, 0.2 mg/L 2.4-D, 1 mg/L NAA | KM medium, 0.5 mg/L Zeatin, 0.2 mg/L 2.4-D, 1 mg/L NAA | KM medium, 2 mg/L 6-BAP, 0.1 mg/L 2.4-D | MS solid medium, 20 *g*/L Sucrose, vitamins, 2 mg/L Zeatin, 0.1 mg/L IAA | None | Fournier et al. [20] |

comprehensive protocols for protoplast isolation, transfection, and regeneration, customized to the specific requirements of the *Solanum* genus. The success of crop breeding greatly depends on technical proficiency, and understanding the processes of protoplast transformation and fine-tuning the parameters affecting editing efficiency would help achieve desired outcomes.

### Abbreviations

| | |
|---|---|
| ALS | Acetolactate synthase |
| CRISPR | Clustered regular interspaced short palindromic repeats |
| Cas9 | CRISPR-associated protein 9 |
| gRNA | Guide RNA |
| PAM | Protospacer adjacent motif |
| NGS | Next-generation sequencing |
| MS | Murashige and Skoog basal medium |
| IAA | Indole-3-acetic acid |
| NAA | 1-Naphthaleneacetic acid |
| PEG | Polyethylene glycol |

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### Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Competing interests
The authors declare that they have no competing interests.

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