

Article

A Stable and Rapid Protoplast-Based CRISPR/Cas9-Ribonucleoprotein Complex Screening System for Octoploid Strawberry (*Fragaria* × *ananassa*)

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a simple and powerful tool, which enables gene knockout or insertion of new gene cassettes. This method has been applied to various plants and is used for crop improvement. Cultivated strawberry (*Fragaria* × *ananassa*), a member of the Rosaceae family, is a high-value horticultural crop. However, its complex octoploid genome poses challenges for precise genome editing in polyploids. This study aimed to establish a protoplast-based, DNA-free genome-editing approach in the cultivated octoploid strawberry. We optimized the culture conditions and enzyme combinations to enable efficient protoplast isolation from the fully developed leaves. The highest protoplast yield was obtained with a Murashige and Skoog medium containing 1% sucrose and 2 mg/L 6-benzylaminopurine (BA), along with enzymatic digestion using 2% Viscozyme, 1% Celluclast, and 1% Pectinex. Transient transfection conditions were optimized using a green fluorescence protein (GFP) plasmid with the highest expression efficiency (up to 52.5%) observed using 40% PEG 4000 and 20 min incubation. Under these conditions, Cas9 ribonucleoproteins (RNPs) targeting the *FaPDS* and *FaPG1* genes were introduced, and guide RNA (gRNA) screening was conducted by targeted deep sequencing. In conclusion, this study successfully demonstrated protoplast isolation and DNA-free CRISPR/Cas9 genome editing in cultivated strawberry. The optimized protoplast-based system provides a valuable platform for functional genomics and molecular breeding efforts in octoploid strawberries.

Keywords: *Fragaria*; strawberry; protoplast; CRISPR/Cas9; gRNA screening



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1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a genome-editing tool derived from an adaptive immune mechanism of bacteria, which protects against bacteriophage infection [1–3]. In this system, foreign DNA molecules are captured and integrated into the CRISPR array, which subsequently guides Cas9 endonuclease to induce a double-strand break (DSB)

at the corresponding target sequence [4]. These breaks are repaired through homology-directed repair or nonhomologous end joining. This process can result in small insertions or deletions (InDels), which may cause frameshift mutations. This system facilitates insertion of a specific gene cassette or targeted disruption of gene function. CRISPR/Cas9-mediated genome editing has been successfully used in model plants, such as *Arabidopsis thaliana* and *Nicotiana benthamiana* [5–7]; in major crops including rice (*Oryza sativa*) [5,8], tomatoes (*Solanum lycopersicum*) [9], grape (*Vitis vinifera* L.) [10,11], and chili peppers (*Capsicum annuum* L.) [12]; and in polyploid crops such as wheat (*Triticum aestivum* L.) [13] and potatoes (*Solanum tuberosum* L.) [14]. However, its application in polyploid species is more challenging than that in diploid crops because a guide RNA (gRNA) must be designed to simultaneously target multiple and/or specific alleles of the target gene [15].

Cultivated strawberry (*Fragaria* × *ananassa*) is a highly crucial horticultural species among the Rosaceae members. It is highly valued for its varied flavors and beneficial effects. Cultivated strawberry is an octoploid species ($2n = 8\times = 56$), originating from interspecific hybridization between *Fragaria virginiana* and *Fragaria chiloensis* [16]. The sub-genomic complexity of cultivated strawberries has been proposed from a combination of four diploid progenitor species comprising four distinct sub-genomes (A, B, C, and D), two of which have been identified [17]. The genetic complexity and polyploidy of cultivated strawberries pose challenges for traditional cross-breeding methods and often prolong the development of new varieties. Therefore, genetic engineering may be an efficient alternative for introducing new traits and enabling functional genomic studies on strawberries. However, the genome complexity and genotype-dependent low transformation efficiency of the octoploid strawberry, along with its recalcitrant nature, present significant challenges for establishing a stable *Agrobacterium*-mediated transformation system. Moreover, to avoid transgene integration and comply with non-genetically modified organism regulations, eliminating *Agrobacterium*-delivered gene cassettes from edited plants is necessary. A few genome-editing efforts have been reported in octoploid strawberry using *Agrobacterium*-mediated methods [18–20]; however, none have used a protoplast-based approach.

Protoplasts are plant cells in which the cell wall has been enzymatically removed, leaving only the plasma membrane and cytoplasm. Transient transformation systems using protoplasts have been widely applied to evaluate gene expression [21], determine the subcellular location of proteins [22], and assess genome-editing efficiency. In particular, protoplasts can serve as a DNA-free genome-editing platform by enabling the delivery of CRISPR/Cas9 packages in the form of ribonucleoproteins (RNPs) without integrating foreign DNA [23]. However, protoplast-based genome editing has not been reported in octoploid strawberry, and existing protocols for protoplast isolation require more than 10 h of enzymatic digestion [24,25], which limits their practical application. Therefore, a rapid and efficient transient assay method based on protoplasts is required for cultivated strawberries.

Genome-editing efficiency in plants is remarkably influenced by the efficiency of gRNA and target gene structure [23,26,27]. Therefore, pre-validating efficiency based on gRNA target sites before transformation is essential. In vivo screening using protoplasts is necessary to accurately determine the effectiveness of genome editing within cells [28,29]. Although previous studies have reported gene-editing efficiency using protoplasts of the wild strawberry *Fragaria vesca*, DNA-free gene-editing studies using protoplasts of the octoploid strawberry *Fragaria* × *ananassa* have not yet been reported [30].

In this study, we aimed to establish a rapid and efficient platform for protoplast isolation and polyethylene glycol (PEG)-mediated transfection for the cultivated strawberry with a focus on enabling the screening of gRNA activity for CRISPR/Cas9-based genome editing. To efficiently isolate protoplasts from fully developed leaves of in vitro-grown

plants, we optimized the culture medium composition and enzyme concentrations. Additionally, we evaluated the plasmid DNA quantity, type of PEG, and transfection time for optimal transformation efficiency. Subsequently, a protoplast-based transient expression system involving plasmid DNA and RNP delivery was developed and applied to determine the genome-editing activity of candidate gRNAs.

2. Results

2.1. Effects of Sucrose Concentration and Cytokinin Type on Plantlet Morphology and Protoplast Yield

Preliminary experiments showed that young leaves from non-hormone-treated in vitro runner-propagated plants were unsuitable for direct use in protoplast isolation owing to low yield (Supplementary Figure S1). To assess the effect of the culture medium composition on the protoplast yield, in vitro-propagated plants were sub-cultured on four media (Table 1). Plants grown under each condition showed distinct phenotypes (Figure 1a). Those cultured on kinetin-supplemented media developed roots, elongated petioles, and broad, dark-green leaves. In contrast, plants on BA-supplemented media formed multiple compact shoots without root development. Notably, small, thin leaves were prominently induced on MS medium supplemented with vitamins, 10 g/L sucrose, and 7 g/L plant agar, supplemented with 2.0 mg/L BA (1S + BA). Protoplasts were isolated from young true leaves of plants grown in each test medium. Although protoplast isolation was achieved for all groups, the number of protoplasts obtained significantly varied depending on the type of medium (Figure 1b). The highest protoplast-isolation efficiency (30.4×10^5 protoplasts/g·FW) was obtained using leaves from plants cultivated on 1S + BA medium (Figure 1b, Supplementary Figure S2). The applicability of gRNA screening was tested by assessing protoplast viability using a reproducible protoplast assay. Protoplasts were incubated with Evans blue solution, which stains dead but not living cells. All samples exhibited viability rates > 94.7%, with the highest viability of 96.2%, and no significant differences were detected among the treatments (Supplementary Figure S2). These results indicate that variations in the type and concentration of hormones and carbon sources used in the culture medium can affect the efficiency of protoplast isolation from in vitro-cultured strawberry plants. Consequently, all subsequent experiments were conducted using plants cultured on 1S + BA medium.

Table 1. Composition of the media used for in vitro growth of octoploid strawberries.

Media	Purpose	Basal Medium	Sucrose (g/L)	Hormone(s)	Agar (g/L)	pH
MS	In vitro propagation	1/3 MS (including MS vitamins)	20	-	7	5.8
2S + BA	Multi-shoot induction	1/3 MS (including MS vitamins)	20	2 mg/L BA	7	5.8
2S + Kn		1/3 MS (including MS vitamins)	20	2 mg/L Kinetin	7	5.8
1S + BA		1/3 MS (including MS vitamins)	20	2 mg/L BA	7	5.8
1S + Kn		1/3 MS (including MS vitamins)	20	2 mg/L Kinetin	7	5.8

2.2. Effect of Enzyme Concentration on Protoplast Yield

To further improve the efficiency of protoplast isolation under the 1S + BA condition, we assessed additional extraction conditions using both the standard enzyme mixture and a relatively high-concentration enzyme solution to facilitate relatively rapid release of protoplasts from strawberry tissues. As the protoplast yield and extent of cell-wall digestion can vary depending on the enzyme concentration, two combinations of Viscozyme,

Celluclast, and Pectinex (VCP) were tested. Combination 1 comprised 2% Viscozyme, 1% Celluclast, and 1% Pectinex (V2:C1:P1), and combination 2 comprised 1% Viscozyme, 0.5% Celluclast, and 0.5% Pectinex (V1:C0.5:P0.5). V2:C1:P1 demonstrated the highest protoplast yield at 8.79×10^6 protoplasts/g·FW. In contrast, combination 2 yielded 3.04×10^6 protoplasts/g·FW. The protoplast yield using V2:C1:P1 was approximately 2.9-fold higher than that obtained using V1:C0.5:P0.5 (Figure 2a,b, Supplementary Figure S3). To evaluate whether the increased yield was accompanied by a loss of protoplast quality, viability was assessed using Evans blue staining. Combination 2 resulted in a higher average viability (94.48%) than that achieved by combination 1 (87.32%), indicating that relatively high enzyme concentrations led to slightly reduced membrane integrity owing to relatively extensive digestion (Supplementary Figure S3). Cell yield is a critical factor for transient assays. Therefore, we used the V2:C1:P1 enzyme mixture for the subsequent experiments.

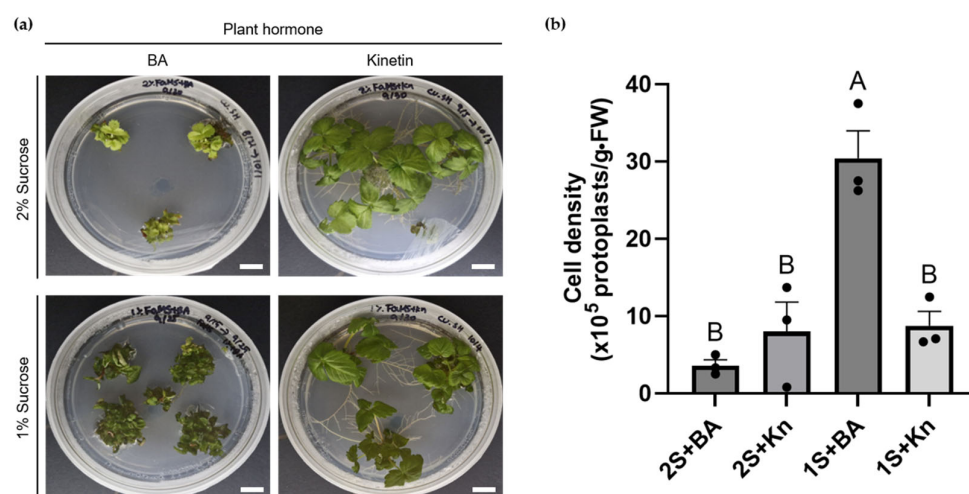


Figure 1. Effects of culture-medium composition on strawberry growth and protoplast yield. (a) Morphological characteristics of strawberry plants cultured in vitro on MS medium supplemented with 1% or 2% sucrose and 2 mg/L BA or kinetin (Kn). Scale bar, 1 cm. (b) Protoplast yield from leaves of plants grown on each medium type. Data are presented as mean $\pm$ standard error (SEM) ($n = 3$). $p < 0.05$ (one-way ANOVA). Values sharing the same letter are not significantly different.

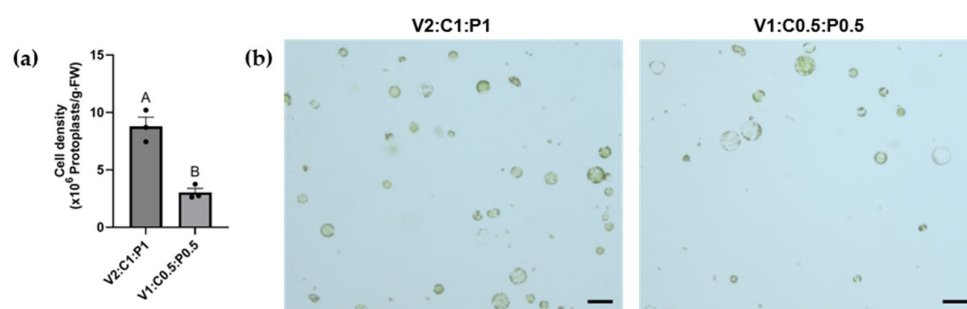


Figure 2. Effect of enzyme concentration on protoplast yield from in vitro-cultivated strawberries. (a) Comparison of protoplast yield after 4 h of digestion using two types of enzyme mixtures from leaves of plants cultured for 8 weeks on 1S + BA medium. Data are presented as mean $\pm$ SEM ($n = 3$). $p < 0.05$ (two-tailed t -test). Values sharing the same letter are not significantly different. (b) Light microscopic images of protoplasts isolated using an enzyme mixture. Scale bar, 50 μ m.

2.3. Optimization of Transfection Conditions Based on the Efficiency of GFP Expression

To enable efficient transfection and RNP screening in strawberry protoplasts, transfection conditions were optimized using a GFP-expressing plasmid. The optimal parameters for plasmid DNA concentration, PEG type, and incubation time were established to de-

velop a reliable transient expression system. To determine the optimal DNA concentration, strawberry protoplasts were transfected with a GFP-expressing plasmid at concentrations ranging between 10 and 50 μg using PEG 4000 for 20 min, and the transfection efficiency was evaluated by monitoring GFP fluorescence 72 h post-transfection. Protoplasts transfected with 30 μg GFP plasmid exhibited the maximum GFP expression (42.0%), which was 3.23-fold (13.0%), 1.96-fold (21.4%), 1.36-fold (30.8%), and 1.20-fold (35.1%) higher than that of 10, 20, 40, and 50 μg plasmids, respectively (Figure 3a, Supplementary Figure S4).

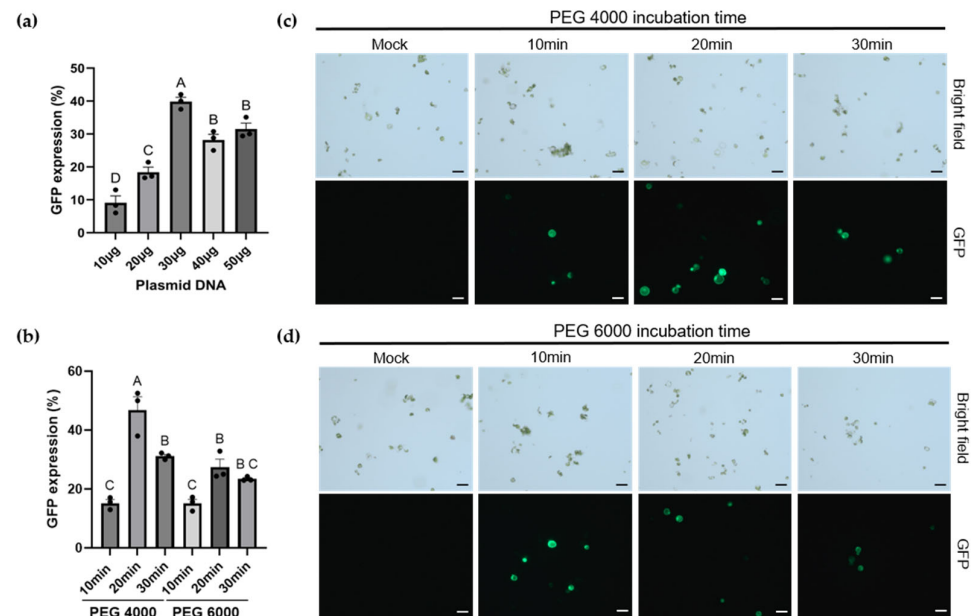


Figure 3. Optimization of transfection conditions. **(a)** Quantification of GFP expression efficiency calculated as the proportion of GFP-expressing cells among the total cell population 72 h after transfection using 10, 20, 30, 40, or 50 μg plasmid DNA. Data are presented as mean $\pm$ SEM ($n = 3$). $p < 0.05$ (one-way ANOVA). Values sharing the same letter are not significantly different. **(b)** GFP expression efficiency after transfection using PEG 4000 or PEG 6000, and incubated for 10, 20, and 30 min. Data are presented as mean $\pm$ SEM ($n = 3$). $p < 0.05$ (one-way ANOVA). Values sharing the same letter are not significantly different. **(c,d)** Representative fluorescence microscopic images of protoplasts transfected with PEG 4000 or 6000 after incubation for 10, 20, and 30 min and subsequent incubation in the dark for 72 h. Scale bar, 50 μm .

To improve the transfection efficiency in strawberry protoplasts, different PEG types commonly used as transfection reagents and various transfection durations were tested to identify the optimal conditions. Using PEG 4000, the maximum efficiencies of GFP expression were 17.05%, 52.5%, and 32.2% at incubation times of 10, 20, and 30 min, respectively. In comparison, PEG 6000 yielded efficiencies of 17.2%, 32.86%, and 24.29%, at the same time points, respectively (Figure 3b–d, Supplementary Figure S4). The efficiency of GFP expression did not significantly vary between PEG 4000 and PEG 6000 treatments at 10 or 30 min of incubation. However, the highest proportion of GFP-expressing protoplasts was observed at 20 min incubation with PEG 4000, yielding a mean value approximately 1.71-fold higher than that of PEG 6000 under the same conditions. Additionally, under the optimized conditions of 30 μg plasmid DNA, PEG 4000, and a 20 min transfection, protoplasts exhibited 65.4% viability (Supplementary Figure S5). Subsequently, these conditions were used for gRNA screening experiments.

2.4. In Vitro Validation of the CRISPR–RNP Complex

To design gRNAs targeting *FaPDS* and *FaPG1* in Seolhyang, the target genomic regions were amplified and Sanger-sequenced to identify the presence of mutations (Supplementary Figure S6). Three gRNAs targeting the conserved exon regions were designed for each gene, *FaPDS* and *FaPG1* (Figure 4), and synthesized via in vitro transcription. The activity of the CRISPR–RNP complexes was validated using in vitro cleavage assays. The complexes were incubated with a DNA fragment containing the respective target region. Control samples treated with only Cas9 showed a single band with no cleavage following PCR amplification; however, samples treated with the complexes demonstrated target-specific cleavage, resulting in two distinct DNA fragments (Supplementary Figure S7).

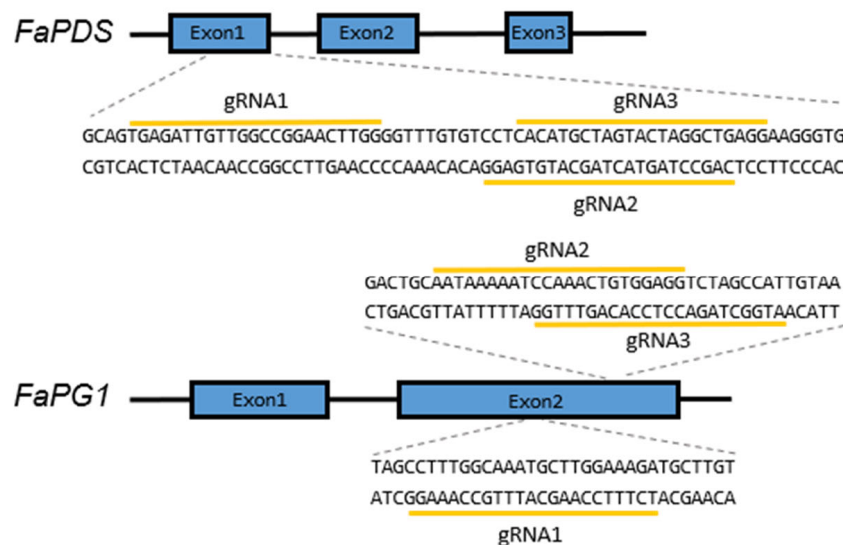


Figure 4. Design of sgRNAs targeting *FaPDS* and *FaPG1* in octoploid strawberries. The exons and introns of *FaPDS* and *FaPG1* are indicated by blue boxes and black lines, respectively. Each gRNA target site is indicated as orange within the DNA sequence.

2.5. gRNA Screening for Targeted Mutagenesis

Using optimal protoplast transfection conditions and CRISPR–RNP complexes, the effectiveness of the CRISPR–Cas9 system for genome editing in strawberry was evaluated. Strawberry protoplasts were transfected with CRISPR–RNP and harvested after 72 h of incubation. Targeted deep sequencing was used to determine InDel frequencies at each gRNA target site. For *FaPDS*, InDel frequencies were up to 0.2% for gRNA1, 0.4% for gRNA2, and 0.4% for gRNA3 (Figure 5a). Analysis of the InDel types revealed that most edits comprised 1–8 bp insertions or deletions 3 bp upstream of the protospacer adjacent motif (PAM) region (Figure 5b). For *FaPG1*, the InDel frequencies were 0.3% for gRNA1, 1.2% for gRNA2, and 1.4% for gRNA3. Notably, an analysis of the InDel types indicated that most edits comprised 1–7 bp insertions or deletions (Figure 5c,d). Overall, CRISPR–RNP-mediated editing of *FaPDS* and *FaPG1* in strawberry protoplasts resulted in InDel frequencies ranging from 0.2% to 1.4%. These results indicate that stable strawberry protoplasts provide a reliable platform for evaluating the efficiency of CRISPR, which supports its use in precise genome-editing applications in strawberries.

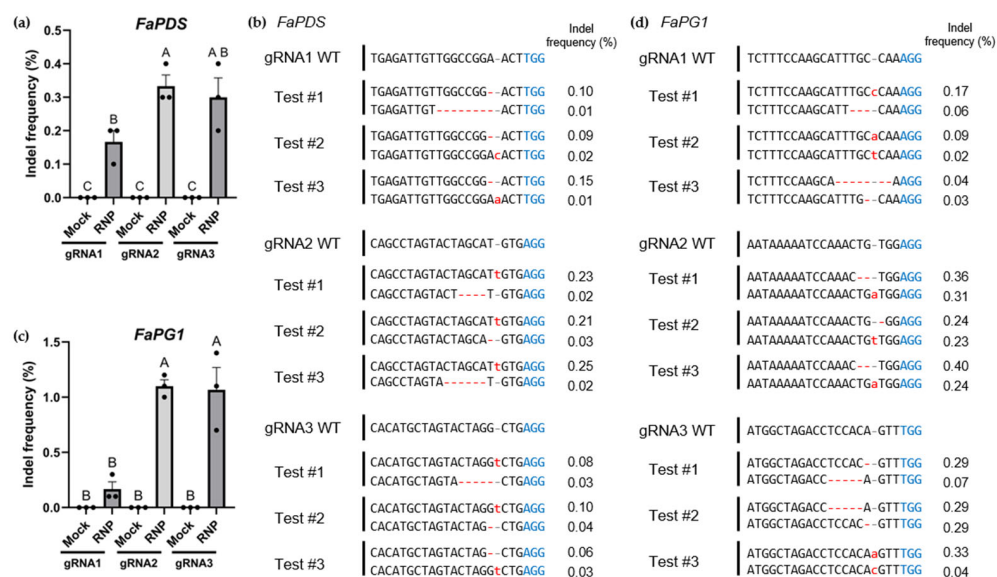


Figure 5. gRNA screening of *FaPDS* and *FaPG1* in *Fragaria × ananassa* via RNP transfection and analysis by targeted deep sequencing. (a) InDel frequencies at the gRNA target sites in *FaPDS* determined following RNP delivery to protoplasts. (b) InDel pattern of each gRNA for the *FaPDS* target regions. The PAM sequence is shown in blue, and the InDel nucleotides are shown in red. (c) InDel frequencies at the gRNA target sites in *FaPG1* determined after RNP delivery to protoplasts. (d) InDel pattern of each gRNA for the *FaPG1* target regions. The PAM sequence is shown in blue, and InDel nucleotides are shown in red. a and c Data are presented as mean ± SEM ($n = 3$). $p < 0.05$ (one-way ANOVA). Values sharing the same letter are not significantly different.

3. Discussion

Genome-editing studies are limited in strawberries owing to genome complexity, technical barriers, and genotype-specific constraints [18–20,30]. For the cultivated octoploid strawberry, designing gRNAs to target conserved sub-genome regions across all homoeologous loci is critical for achieving complete gene knockouts. Additionally, genotype-dependent regeneration and low transformation efficiency may further influence the development of genome-edited strawberries [15]. Efficient genome editing in plants depends on the use of highly active gRNAs. Protoplast-based transient expression assays provide a rapid and robust platform for evaluating gRNA activity in plants [27,31]. Pre-screening enables the identification of functional gRNAs before proceeding with time-consuming transformation, thereby enhancing the overall efficiency of CRISPR/Cas9-based genome-editing workflows [32]. These assays are particularly valuable in polyploid species, like strawberries, in which empirical pre-validation of gRNA activity helps in ensuring functional editing across multiple sub-genomes. Furthermore, the protoplast-based transient transformation system developed in this study offers practical advantages for integrating genome editing in strawberry breeding and functional genomic pipelines. Transient gene editing in protoplasts enables rapid assessment of gene function and single-cell transcriptomic and metabolomic changes [33,34]. Based on these advantages, this platform can support the identification of candidate genes associated with fruit quality, disease resistance, and early selection of edited events through analysis of their editing patterns. In this study, a protoplast-based gRNA screening system was developed and optimized for the octoploid strawberry cultivar ‘Seolhyang’ (Supplementary Figure S8). Optimization focuses on key variables associated with protoplast isolation and PEG-mediated transfection, including plantlet cultivation, enzyme concentrations for protoplast isolation, type of transfection reagent, and incubation time for transfection. Editing efficiency and accuracy for various

gRNAs were evaluated by targeted deep sequencing, which confirmed their ability to induce DSBs and editing patterns at the target sites.

Only a few studies have conducted protoplast extraction from fully developed leaves of the octoploid strawberry (Table 2). Previous protocols required more than 10 h of enzymatic digestion [24,25]. In contrast, our study optimized the plant culture medium (1S + BA) and enzyme composition (V2:C1:P1), enabling efficient recovery of protoplasts at a high yield of 8.79×10^6 protoplasts/g-FW after only 4 h of digestion. This result supports previous findings that plant growth conditions and enzyme mixtures influence protoplast yield and transfection efficiency [35–37]. While sufficient protoplasts were obtained after 4 h of digestion in this study, further research comparing yields across a finer time gradient will be necessary to determine the precise optimal digestion time. Protoplast yield, viability, and transformation efficiency can vary depending on the cultivar. Therefore, these factors should be optimized before genome editing. Optimizing PEG-mediated transfection conditions is essential for improving the accuracy and efficiency of gRNA screening. The transfection efficiency peaked at 30 µg and showed a decreasing trend at higher concentrations. This suggests that excessive amounts of plasmid DNA may hinder efficient uptake, potentially due to the formation of large aggregates or suboptimal DNA-PEG interactions, rather than simply inducing cytotoxicity. Compared with the maximum transfection efficiency of 60% in *F. vesca* protoplasts [30], our study achieved up to 52.5% efficiency, demonstrating that high-level transfection is also feasible in octoploid strawberry. The GFP transfection efficiency observed in this study may appear relatively low compared to previous reports in model plants such as *Arabidopsis* [21], where efficiencies often exceed 60% when measured at the peak expression window (12–24 h). In this study, however, GFP fluorescence was assessed at 72 h post-transfection to align with the timeline used for gene editing efficiency analysis. Given that transient protein expression and protoplast viability naturally decline over prolonged incubation, this delayed observation likely accounts for the lower apparent efficiency. Nevertheless, these results demonstrated that the transfection protocol was sufficient to deliver functional RNP complexes for screening purposes.

Table 2. Comparison of reported methods for strawberry protoplast isolation and transfection.

Plant Material	Genotype	Enzyme Concentration	Digestion Time	Yield of Protoplasts/g-FW	Maximum Transformation Efficiency	Reference (s)
<i>Fragaria</i> × <i>ananassa</i>	77101	1.0% Cellulysin, 0.1% Macerozyme	15–18 h	$3.3 \times 10^6 \pm 1.1 \times 10^6$	-	[24]
<i>Fragaria</i> × <i>ananassa</i>	Chandler	1% Cellulysin, 0.1% Macerace 1.0% cellulase R-10, 0.4%	18 h	-	-	[25]
<i>Fragaria vesca</i>	Hawaii	macerozyme R-10, 0.02% pectinase Y-23 2% Viscozyme,	15 h	3.25×10^5	60%	[30]
<i>Fragaria</i> × <i>ananassa</i>	Seolhyang	1% Celluclast, 1% Pectinex	4 h	8.79×10^6	52.5%	This study

The InDel frequency of *FaPDS*-targeting gRNAs was <0.4%, whereas that of *FaPG1*-targeting gRNAs reached a maximum of 1.4%. The relatively high efficiency of the latter may be attributed to factors such as chromatin accessibility, GC content, and secondary structure of the target site [32]. In polyploid crops, including strawberries, multiple homoeologous copies of each gene are common, and all relevant loci- or sub-genome-specific targets need to be edited for functional knockout. This requirement significantly enhances

the complexity of genome editing in polyploid species [15,38]. In this study, *FaPDS* genes were targeted at four copies, representing a multiple-targeting approach. Consequently, its editing efficiency was lower than that of *FaPG1*, which was targeted at a single copy. Therefore, when multiple targeting is required, using guide RNAs specific to each chromosome may result in relatively high genome-editing efficiency. In polyploid crops like the octoploid strawberry, distinguishing gene editing events across different sub-genomes (A, B, C, and D) is a significant challenge. In this study, we leveraged the whole-genome sequencing data of the target cultivar [39] to design gRNAs targeting conserved regions, ensuring robust editing across sub-genomes. However, conventional short-read deep sequencing has limitations in assigning mutations to specific sub-genomes due to high sequence similarity. To precisely dissect the contribution of each sub-genome to the edited phenotype, future studies should incorporate long-read sequencing technologies (e.g., PacBio or Nanopore) or sub-genome-specific PCR amplification strategies. This will be essential for fine-tuning traits in complex polyploid genomes. While this study focused on RNP delivery to establish a DNA-free editing protocol, the developed high-throughput screening system is also applicable to plasmid-mediated editing. This compatibility indicates that the platform can serve as a versatile tool for validating various editing strategies beyond RNP complexes. It is important to note that this study focused on establishing a rapid screening system to evaluate the editing activity of RNPs in protoplasts. While successful gene editing was confirmed, the regeneration of whole plants from these protoplasts remains a challenge. Future studies will focus on optimizing culture conditions to achieve successful regeneration and obtain gene-edited plants.

In this study, we successfully established a protoplast-based transient-assay platform for gRNA screening to improve genome-editing efficiency. Nonetheless, the present work is confined to transient assessment of genome editing in strawberry protoplasts, indicating the need for future studies aimed at generating stable genome-edited plants. Future efforts should focus on improving the regeneration efficiency of edited cells into whole plants. However, regeneration is highly cultivar-dependent and remains a major challenge for developing genome-edited strawberry cultivars. Establishing cultivar-specific regeneration protocols is critical for converting transient edits into stable genotypes. Combining this gRNA screening platform with efficient regeneration systems can remarkably accelerate breeding efforts in genome-edited strawberries.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

The strawberry cultivar Seolhyang was used as the study material (Figure 6). Runners were obtained from mother plants provided by the Jeonbuk State Agricultural Research and Extension Services (Iksan-si, Republic of Korea). The apical parts of runners were trimmed, leaving 3–4 fully developed leaves, and transferred into 50 mL tubes containing 1% Clorox and 1% Tween 20 for surface sterilization by shaking for 10 min and then rinsing five times with sterile distilled water. The shoot tips were cultured on Murashige and Skoog medium [40] containing vitamins (Duchefa Biochemie B.V., Haarlem, The Netherlands), while retaining 1–2 fully developed leaves near the apical meristem to ensure a virus-free status. Shoots were incubated in a growth chamber at 24 ± 1 °C, 50% relative humidity, and a 16/8 h light/dark photoperiod for 4 weeks. Grown shoots were transferred to a plant growth regulator (PGR)-containing medium, and protoplasts were subsequently extracted [24,25]. We tested four different hormone-supplemented media: MS medium with vitamins, 10 or 20 g/L sucrose, and 7 g/L plant agar, supplemented with either 2.0 mg/L 6-benzylaminopurine (BA) or 2.0 mg/L kinetin (Table 1). Shoots were tested in each medium and cultured under a 16/8 h light/dark photoperiod at 25 °C for 8 weeks.

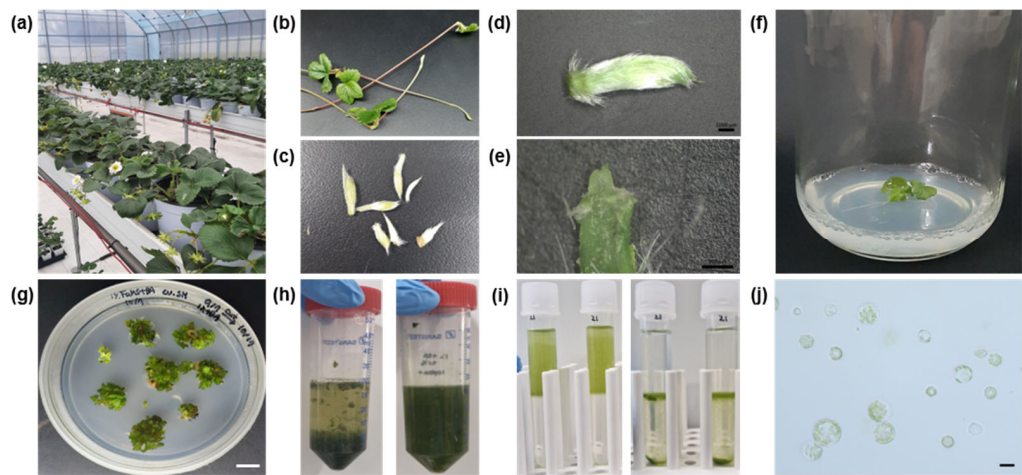


Figure 6. Overview of plant-material preparation and protoplast isolation from *Fragaria × ananassa*. (a) Greenhouse cultivation of octoploid strawberry plants. (b,c) Runners harvested from mother plants. (d) Trimmed runner tips prepared for surface sterilization. Scale bar, 1 mm. (e) The apical meristem region used for in vitro runner propagation. Scale bar, 500 μ m. (f) In vitro-cultured shoots grown for 2 weeks. (g) Plants after 8 weeks of growth on 1S + BA medium. Scale bar, 1 cm. (h) Enzymatic digestion of fully developed strawberry leaves. The left image shows untreated leaf tissue before enzyme incubation, and the right image shows tissue degradation after 4 h of enzymatic digestion. (i) Purification of protoplasts using 21% sucrose gradient. (j) Purified protoplasts observed using a light microscope. Scale bar, 20 μ m.

4.2. Protoplast Isolation and Purification

Young leaves (approximately 1.5 g) were collected from 8-week-old plants grown in vitro, in a hormone-containing medium. Leaves were cut into 1 mm strips using a surgical blade (HFE-SB10; FEATHER, Osaka, Japan) and transferred into an enzyme solution (Table 3). Vacuum infiltration was performed at 0.08 MPa for 40 min using a desiccator, followed by incubation in the dark and shaking at 30 rpm for 4 h to digest the cell wall and release protoplasts. The enzyme-digested suspension was filtered through 100 and 70 μ m nylon meshes and washed with an equal volume of W5 solution (Table 3). Protoplasts were resuspended in fresh W5 solution and isolated on a 21% (*w/v*) sucrose gradient by swing-out centrifugation at 120 $\times$ *g* for 7 min. After centrifugation, intact protoplasts were harvested from the middle layer and incubated in W5 solution for 1 h at 4 $^{\circ}$ C.

Table 3. Composition of solutions used for protoplast isolation from octoploid strawberries and transfections.

Solution	Composition
V1 enzyme	1% Viscozyme, 0.5% Celluclast, 0.5% Pectinex, 90 g/L Mannitol, 1.06 g/L 2-(N-morpholino)ethanesulfonic acid (MES), and Cell and Protoplast Wasing (CPW) salts (27.2 mg/L KH ₂ PO ₄ , 101 mg/L KNO ₃ , 246 mg/L MgSO ₄ ·7H ₂ O, 0.16 mg/L KI, 0.025 mg/L CuSO ₄ ·5H ₂ O, 1480 mg/L CaCl ₂ ·5H ₂ O); pH 5.8
V2 enzyme	2% Viscozyme, 1% Celluclast, 1% Pectinex, 90 g/L Mannitol, 1.06 g/L MES, and CPW salts; pH 5.8
W5	154 mM NaCl, 125 mM CaCl ₂ ·H ₂ O, 5 mM KCl, 5 mM glucose, and 1.5 mM MES; pH 5.6
MMG	4 mM MES, 0.4 M mannitol, and 15 mM MgCl ₂ ; pH 5.7
WI	4 mM MES, 0.5 M mannitol, and 20 mM KCl; pH 5.7

4.3. Assessment of Protoplast Viability

Isolated protoplasts were harvested by centrifugation at 100 $\times$ *g* for 5 min and resuspended in 0.2% (*w/v*) Evans blue solution. Protoplasts were incubated for 10 min in Evans solution, and cell viability was assessed by counting unstained (viable) and blue-stained

(non-viable) cells using an Axio Vert A1 light microscope (Zeiss, Oberkochen, Germany). To ensure accurate quantification, protoplasts were identified based on their intact, spherical morphology. Irregularly shaped structures and cellular debris were strictly excluded from the count.

4.4. Cas9 and gRNA Preparation

The pET28a-Cas9-His plasmid (plasmid #98158; Addgene, Watertown, MA, USA) encoding Cas9 was used to transform *Escherichia coli* BL21 Star competent cells (C601003; Thermo Fisher Scientific, Waltham, MA, USA). Cas9 was subsequently expressed and purified using Ni-NTA agarose resin specific for His-tagged proteins (30210; Qiagen, Hilden, Germany) as previously described [41]. gRNAs were designed using Cas-Designer [42] to target four copies of strawberry phytoene desaturase (*FaPDS*) at 4A, 4B, 4C, and 4D chromosomes and one copy of polygalacturonase (*FaPG1*) genes at 6A chromosome [39]. The target gene sequences, *FvPDS*: NC_020494.1, *FaPDS*: FJ795342.1, and *FaPG1*: AF380299.1, were retrieved from the NCBI database and used as references to design primers for *FaPDS* and *FaPG1*. The primers were designed using Primer3 v.4.1.0 [43]. Genomic DNA was extracted from strawberry leaves using a DNeasy Plant Mini Kit (69106; Qiagen, Hilden, Germany) and then used as a template for polymerase chain reaction (PCR)-based Sanger sequencing (Macrogen, Seoul, Republic of Korea). The target genes were amplified using a 2× Phusion Plus Master Mix (F631L; Thermo Fisher Scientific, Waltham, MA, USA) with specific primers (Supplementary Table S1).

For in vitro transcription of each gRNA, DNA templates were PCR-amplified using Q5 High-Fidelity DNA Polymerase (M0492S; New England Biolabs, Ipswich, MA, USA) with a T7 promoter sequence inserted upstream of the gRNA target site (Supplementary Table S1, Figure S9). The gRNAs were synthesized using T7 RNA Polymerase (M0251L; New England Biolabs), followed by DNase I treatment according to the manufacturer's instructions and purification using a RiboClear kit (303-150; GeneAll Biotechnology Co. Ltd., Seoul, Republic of Korea).

4.5. In Vitro Cleavage Assay

Templates of the *FaPDS* and *FaPG1* target-specific regions for in vitro cleavage were prepared by PCR using a Phusion Plus Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The PCR amplicons were purified using an Expin PCR SV kit (103-102; GeneAll Biotechnology Co., Ltd., Seoul, Republic of Korea). The template, 120 ng of the purified PCR product, was incubated with 1 µg Cas9, 750 ng gRNA, and 1× rCutSmart Buffer (B6004S; New England BioLabs, Ipswich, MA, USA) at 37 °C for 3 h, and held at 8 °C. Subsequently, 4 µg RNase A was added, and the mixture was incubated at 37 °C for 30 min. The products were purified using an Expin PCR SV kit (GeneAll Biotechnology Co., Ltd., Seoul, Republic of Korea). The purified amplicons were mixed with DNA loading dye and analyzed by electrophoresis on a 1.5% agarose gel.

4.6. Protoplast Transfection

RNPs or plasmids were introduced into strawberry protoplasts as previously described [23,30,44], with minor modifications. Strawberry protoplasts were resuspended in MMG solution (Table 3) at a concentration of 4.0×10^5 protoplasts/mL. For each transfection, 250 µL protoplast suspension ($\times 10^5$ protoplasts/g·FW) was used. Protoplasts were transfected with 10–50 µg green fluorescence protein (GFP) plasmid (plasmid #91043; Addgene) using 40% (*w/v*) PEG 4000 or 6000, 0.2 M mannitol, and 0.1 M CaCl₂ and then incubated for 10–30 min at room temperature. For RNP transfection, 80 µg in vitro-transcribed gRNA and 30 µg Cas9 were preassembled at room temperature for 20 min to form RNP

complexes. Protoplasts were transfected by incubation with RNP complexes using 40% (*w/v*) PEG 4000, 0.2 M mannitol, and 0.1 M CaCl₂ for 20 min at room temperature.

Following transfection, the PEG–protoplast mixture was washed thrice with an equal volume of W5 solution. Transfected protoplasts were collected by centrifugation at 100× *g* for 5 min using a swing-out rotor and resuspended in WI solution (Table 3). Cells were incubated in the dark at 25 °C for 72 h. GFP expression was quantified by observing GFP-positive protoplasts using an Axio Vert A1 fluorescence microscope (Zeiss) with an excitation range of 400–650 nm. Transfection efficiency was calculated by analyzing at least five randomly selected fields of view per replicate. To ensure accuracy, the total number of intact spherical protoplasts was counted in the bright-field channel, and the number of GFP-expressing protoplasts was counted in the corresponding fluorescence channel using ZEN (ver 3.6) software (Zeiss).

4.7. Targeted Deep Sequencing

Genomic DNA was extracted from RNP-transfected protoplasts using a DNeasy Plant Mini Kit (69106; Qiagen, Hilden, Germany). The target regions were amplified using a high-fidelity Phusion Plus PCR Master Mix (F631L; Thermo Fisher Scientific, Waltham, MA, USA) for 35 cycles and targeted deep-sequencing primer sets (Supplementary Table S1). Additional rounds of PCR incorporated the multiplexing indices and sequencing adapters into the amplicons. High-throughput sequencing (>7000 reads) was performed on an iSeq 100 platform (Illumina Inc., San Diego, CA, USA) using equal amounts of DNA libraries. Paired-end reads obtained from sequencing were analyzed using Cas-Analyzer [45].

4.8. Statistical Analysis

All data were analyzed using GraphPad Prism v.10.5.0 for Windows (GraphPad Software Inc., Boston, MA, USA). Statistical significance was evaluated using *t*-tests and one-way analysis of variance (ANOVA). All values are presented as mean of three independent experiments ± standard error.

5. Conclusions

In this study, we described an efficient protoplast-isolation and PEG-mediated transfection system and developed a robust and optimized protoplast-based gRNA screening platform for use in cultivated strawberries. Such a platform supports functional genomic research and genome editing by utilizing DNA-free approaches in cultivated strawberries.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants15040565/s1>. Supplementary Table S1: Primers used in this study, Supplementary Figure S1: Preliminary assessment of protoplast isolation, Supplementary Figure S2: Effect of culture medium composition on the morphology of in vitro-cultured strawberry plants, protoplast yield, and viability, Supplementary Figure S3: Representative images and cell viability of strawberry protoplast isolation using different enzyme treatments, Supplementary Figure S4: Fluorescence images of GFP-expressing protoplasts under different transfection conditions, Supplementary Figure S5: Viability test by Evans blue staining after PEG-mediated transfection of strawberry protoplasts, Supplementary Figure S6: Sanger sequencing of the fragments of *FaPDS* and *FaPG1* from *Fragaria × ananassa* cv. Seolhyang, Supplementary Figure S7: In vitro cleavage assay in *Fragaria × ananassa* targeting *FaPDS* and *FaPG1*. Supplementary Figure S8: Schematic of protoplast isolation and transfection in octoploid strawberry, Supplementary Figure S9: Schematic of in vitro transcription for gRNA synthesis.

Author Contributions: Y.K., S.L. and B.-C.K. designed the experiments. Y.K. performed the experiments. J.J. provided the materials and technical support. Y.O. analyzed the strawberry sequence data. Y.K. and B.-C.K. wrote and revised the manuscript. S.L. and B.-C.K. supervised the project and provided scientific advice. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Abbreviations

The following abbreviations are used in this manuscript:

CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
DSB	double-strand break
gRNA	guide RNA
RNP	ribonucleoprotein
GFP	green fluorescent protein
PAM	protospacer adjacent motif
BA	6-benzylaminopurine
Kn	kinetin

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