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A high-efficient protoplast transient system for screening gene editing elements in Salvia miltiorrhiza

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Research Article

Keywords: Medicinal plants, Salvia miltiorrhiza, protoplasts, genome editing

Posted Date: June 27th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3052141/v1

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Version of Record: A version of this preprint was published at Plant Cell Reports on January 23rd, 2024. See the published version at https://doi.org/10.1007/s00299-023-03134-2.

Abstract

Medicinal plants with high-value pharmaceutical ingredients have attracted research attention due to their beneficial effects on human health. Cell wall-free protoplasts of plants can be used to evaluate the efficiency of genome editing mutagenesis. The capabilities of gene editing in medicinal plants remain to be fully explored owing to their complex genetic background and shortfall of suitable transformation. Here, we took the *Salvia miltiorrhiza* as a representative example for developing a method to screen favorable gene editing elements with high editing efficiency in medical plants by a PEG-mediated protoplast transformation. Results indicated that using the endogenous *SmU6.1* of *Salvia miltiorrhiza*to drive sgRNA and the plant codon-optimized Cas9 driven by the promoter *SlEF1α*can enhance the efficiency of editing. In summary, we uncover an efficacious transient method for screening editing elements and shed new light on increasing gene editing efficiency in medicinal plants.

Key Message

A protoplast transient system with high efficiency was devised for the purpose of screening genome editing elements in *Salvia miltiorrhiza*.

Introduction

Since time immemorial, medicinal plants have benefited humans as a prolific source of pharmaceuticals and nutraceuticals. Pesticides and cosmetics industries also rely on medicinal plants' natural compounds. In recent years, interest in medicinal plants and herbal medicines has resurgent. In the World Health Organization report, medicinal plant drugs are used to treat a variety of diseases by many people (Gupta et al., 2020). The therapeutic value of medicinal plants mainly depends on the bioactive compounds, such as alkaloids, terpenes, glycosides, and so on (Zheng et al., 2023). The representative medicinal plants include *Salvia miltiorrhiza* (Li et al., 2018), *Artemisia annua* (Tu 2011), *Taxus genus* (Sabzehzari et al., 2020), *Catharanthus roseus* (van Der Heijden et al., 2004), *Panax* plants (Liu et al., 2020), *Dendrobium* officinale (Li et al., 2022), and *Cannabis sativa* (Kovalchuk et al., 2020).

The herb *Salvia miltiorrhiza Bunge* (Danshen) has been used effectively in the treatment of cardiovascular and cerebrovascular diseases for centuries (Ren et al., 2019). In addition to its significant medicinal value, *S. miltiorrhiza* also has the characteristics of a short life cycle, relatively small genome size, and genetic transferability. Because of this, it has been recognized as an important model medicinal plant (Xu et al., 2016). Wild type *S. miltiorrhiza* is unable to meet the growing demand due to its low content of tanshinones and phenolic acids. Therefore, genetic engineering with S. miltiorrhiza, such as total synthetic biology and gene editing technology, is particularly important for increasing active ingredient yields (Kai et al., 2011).

The CRISPR/Cas9 system contains two parts, CRISPR-associated protein 9 (Cas9) and a single short guide RNA (sgRNA); it identifies a specific site by the complementarity between the sgRNA and the DNA target sequence, which can be used to edit genomes in many species (Gasiunas et al., 2012; Jinek et al., 2012). The CRISPR editing system has been successfully tested in only a few medicinal plants, such as *Artemisia annua* (Zhou et al., 2020), *Dendrobium officinale* (Kui et al., 2016), *Cannabis sativa* (Zhang et al., 2021), *Opium poppy* (Alagoz et al., 2016) and *Salvia miltiorrhiza* (Li et al., 2017; Shi et al., 2022; Zhou et al., 2018). Due to the relatively low editing efficiencies, many medicinal plants still cannot be edited. Scientists are pursuing the high efficiency of plant genome editing through continuous attempts from now and then. In 2013, CRISPR-mediated genome editing was first applied in rice, with an editing efficiency of 7.1-9.4% (Shan et al., 2013). Through optimization, using the endogenous *OsU3* of rice to drive sgRNA and the rice codon optimized Cas9 was driven by the *ZmUBI* promoter to improve the sgRNA and Cas9 expression level. And the efficiency of editing *OsLazy1* and *OsCAO1* genes reached 83.3-91.6% (Miao et al., 2013). Heat stress treatment increased targeted mutagenesis efficiency by five times in Arabidopsis somatic tissues (LeBlanc et al., 2018).

The transcription initiation site of the snRNA U6 promoter is highly conserved, which helps improve the sgRNA homogeneity in plants' genome editing (Shan et al., 2013). The *Arabidopsis* U6 (*AtU6*) promoter has widely expressed sgRNAs heterologously in plant species (Li et al., 2013). In several plant species, the species-specific U6 promoters can enhance editing efficiencies such as soybean, chicory, cotton, and grape (Di et al., 2019; Long et al., 2018; Ren et al., 2021). Thus, it is crucial to characterize optimal endogenous and exogenous U6 promoters. Moreover, applying appropriate promoters to drive Cas9 expression is necessary for efficient genome editing. The cauliflower mosaic virus (CaMV) 35S and ubiquitin promoter have been used to drive the expression of Cas9 in *Arabidopsis* (Ma et al., 2015). Previous studies have used *AtUBQ10* or *2×35S* drive Cas9 to edit the *S. miltiorrhiza* hairy roots efficiently (Zhou et al., 2018). The tomato efficient tissue-specific promoter, ELONGATION FACTOR-1α (*SlEF1α*) promoter, can be used to efficiently drive the adenine base editors (ABEs) and Cas9 in soybean and tomato (Hashimoto et al., 2018; Niu et al., 2023). Moreover, the use of plant codon-optimized Cas9 gene (Cas9p) can significantly affect the editing efficiency of rice (Ma et al., 2015). Researchers have successfully isolated protoplasts from leaf tissues of *Arabidopsis*, rice, and other non-model plants (Yang et al., 2023; Zhu et al., 2017). Protoplasts provide an excellent platform for functional gene characterization in numerous species. Furthermore, it is a powerful tool for CRISPR/Cas9-mediated editing in higher plants. Due to low co-transfection efficiency, protoplasts cannot be easily used to edit herbal medicine plants' genomes (Shao et al., 2023). Thus, it is necessary to develop a practical method for genome editing of *S. miltiorrhiza* based on effective protoplast transient gene expression.

In this work, we reported an effective method for isolating mesophyll protoplasts of *S. miltiorrhiza*. On this basis, high-yield and high-quality protoplasts were recovered from young *S. miltiorrhiza* leaves. Moreover, the protoplasts served as a versatile platform for screening gene editing elements to optimize gene editing in *S. miltiorrhiza*.

Materials and Methods

Plant materials and growth condition

The *Salvia miltiorrhiza Bunge* seeds used in this study were from Shandong, China. *S. miltiorrhiza* was grown in the plant culturing room of the School of Agriculture and Biology, Shanghai Jiao Tong University, at 24±2°C, which was lighted for 16h and dark for 8h.

DNA extraction and S. miltiorrhiza U6 small nuclear RNA promoter clone

Using genomic DNA extracted from *S. miltiorrhiza* leaves as a cloning template, potential *U6* promoters were isolated using a plant DNA kit (CoWin Biotech, Jiangsu, China). Local BLAST search based on *AtU6* promoter (*AtU6-1* and *AtU6-29*) in the *S. miltiorrhiza* genome database (Xu et al., 2016). Identify and clone *S. miltiorrhiza* snRNA U6 were amplified by PCR using 2×Phanta DNA Polymerase (Vazyme Biotech Co., Ltd, Nanjing, China).

Protoplast isolation and viability assessment

The protoplasts were isolated from normally cultured green seedlings and purified using the previous method (Zhang et al., 2011). Healthy young *S. miltiorrhiza* leaves (~1g) were cut into small pieces of 1mm. Immerse the long leaf strips in 20mL of enzymatic hydrolysis solution (Table S1) in the culture plate. Then the culture plate was wrapped in tin foil with a shaker speed of 40rpm at RT. Different durations (2-3h, 4-5h, and 6-7h) were tested. An equal volume of W5 solution (Table S2) was added to the protoplast suspension. The healthy protoplast was separated from the solution by centrifugation at 500rpm for 10min at 8℃. Then resuspend protoplasts with 2mL W5 and microscopic examination for living status using fluorescence microscopy. The protoplasts' viability was detected using a final concentration of 0.01% (w/v) fluorescein diacetate (FDA).

Proplast transformation

Resuspend protoplast particles using MMG solution (Table S3) to obtain approximately densities of approximately 1.0-2.0×10^5cells/mL. PEG-mediated protoplast transformation of *S. miltiorrhiza* has some minor modifications based on previous methods (Yoo et al., 2007). To improve protoplast transformation efficiency, the following steps were implemented 1) Place 10μg of DNA into a 2mL tube. Add and mix 200μL of protoplast suspension by gentle tapping; 2) Slowly add 110μL of 40% PEG-calcium transfection solution (40% PEG4000, 0.6M D-mannitol, 100mM $CaCl_2$) (Table S4). Incubate at 28℃ for 15min; 3) Using 1.8mL W5 solution wash transformed protoplast mixture, then 600rpm for 2min; 4) Remove the supernatant, resuspend protoplasts with 750uL of W5 solution; 5) Incubate in the dark at 22/28/37℃ for 16-24h in the incubator before microscopic examination or Dual-Luciferase Reporter Assay.

Plasmid DNA and vector construction

The pHER-Enhanced Green Fluorescent Protein (EGFP) vector contained the nucleus-targeted EGFP gene driven by a minimal *35S* promoter to detect the transfection ability of protoplasts. PDGB3 alpha 2-dual-luciferase reporter plasmid contains the fLuc (firefly luciferase, an informative protein marker) gene driven by *AtUBQ10* promoter, and the NanoLuc (an engineered super sensitive luciferase, has superior biochemical properties, such as its small size and a 150-fold brighter signal than that of fLuc) gene driven by *2×35S* promoter (Figure 4A).

For constructing knockout (KO) vectors, gRNA targeting NanoLuc was evaluated and designed in the network server CRISPR Direct (http://crispr.dbcls.jp/). Designed gRNA 5'-TAAAGTGATGATCATCCACAGGG-3' was inserted into the SmKO01-SmKO10 vector. SgRNA and *Sp*Cas9/Cas9P were driven by the *AtU6-1*/*SmU6.1*/*SmU6.3* promoter and *2×35S*/*SlEF1a*/*AtUBQ10* promoter, respectively. Finally, the expression cassette was inserted into the linearised pCAMBIA1300 between Kpn and BamH (Cambia, Canberra, and Australia) plant expression vector for the generation of pCAMBIA1300-sgRNA-Cas9 (SmKO01-SmKO10) (Figure. 4B). The One Step Cloning Kit (Vazyme Biotech) was used in the process of construction of plasmids. The primer information is listed in Supplementary Table S1.

Microscopic examination

Use a confocal laser scanning microscope to observe protoplasts (Leica TCS SP5 Germany) and visualized them by a Leica Microsystem LAS AF. GFP was excited at 488nm wavelengths. All fluorescence observation experiments were repeated at least three times independently.

Dual-luciferase and edit efficiency evaluation in *S. miltiorrhiza* protoplast

Protoplast samples were collected into 2mL tubes after 16-24 hours of incubation in the dark. fLUC and NanoLUC activities were analyzed by the Nano-Glo® Dual-Luciferase® Reporter Assay System Kit (Promega). Assume the luminescence values of wild-type untransformed protoplasts serve as a reference for relative fluorescence values. *S. miltiorrhiza* protoplast editing efficiency was quantified by calculating the change in the NanoLUC to fLUC ratio.

Statistical analyses

In all experiments, at least three biological replicates are required. Data are expressed as mean ± standard deviation (SD). A paired two-tailed Student's t-test was conducted to compare the differences between different groups with a significance threshold of $p < 0.05$.

Result

Protoplast isolation from *S. miltiorrhiza* leaves

The 3-week-old *S. miltiorrhiza* leaves were cut into small pieces (~1.0mm) with a tissue culture knife for full digestion (Figure 1A, B). After enzymatic hydrolysis, the protoplasts were released into the solution, and the enzymatic hydrolysate solution turns green. The darker the color is, the more sufficient the

enzymatic hydrolysis is (Figure 1C). After removing impurities such as cell walls and undegraded leaf residues, enrichment and dilute the protoplasts to 1.0-2.0×10^5 cells/mL (Figure 1D). And observe the morphology of protoplasts using a microscope to ensure the integrity of the cell structure of most protoplasts (Figure 1E, F).

Effects of enzymolysis duration on protoplast isolation

In the case of *S. miltiorrhiza* leaves, we found that digestion within 4-5h resulted in the highest protoplast yield and viability (Figure 2, Figure S1D). When enzymolysis lasted 2-3 hours, the protoplast viability was 83.06%, but the protoplast yield was insufficient (Figure 2A-C, Figure S1D). When the enzymatic hydrolysis time is up to 4-5h, the number of protoplasts per unit volume of solution is sufficient, and the vitality reaches 92.70% (Figure 2D-F, Figure S1D). Prolonging the enzymolysis duration to 6-7h destroyed the protoplast structure, precipitation, and decreased vitality to 24.52% (Figure. S1A-D).

Effects of temperature on protoplast transformation efficiency

In this study, *S. miltiorrhiza* protoplast transformation efficiency was examined in relation to incubation time. A 35S:: EGFP plasmid (pHER EGFP) was transfected using the 40% PEG4000 transfection method and incubated for 24 hours at different temperature gradients, the fluorescence of GFP in protoplasts was detected using a confocal microscope (Figure 2. Figure S2A). Transfection efficiencies of 90.31% were achieved at 28℃, comparable to 88.51% at room temperature (RT, 20 ± 2℃) (Figure 2, Figure S2D). Moreover, 71.30% transfection efficiencies were obtained under 37℃ incubation conditions in the *S. miltiorrhiza* leaves protoplasts (Figure S2). Cell aggregation could be easily detected under a confocal microscope in one random visual field (Figure S2).

Identification of U6 promoters in *S. miltiorrhiza*

In order to optimize the CRISPR/Cas9 system in *S. miltiorrhiza*, we conducted a local BLAST search on the *S. miltiorrhiza* genome using *AtU6-1* and *AtU6-29* as queries. Three sequences with characteristic USE motifs and TATA-like boxes were identified with the strongest homology, named *SmU6.1*, *SmU6.2*, and *SmU6.3*, respectively (Figure 4. Supplemental. S1). The promoters *SmU6.1* and *SmU6.3* successfully cloned were used as potential candidate elements to drive sgRNAs in the editing system.

High-performance CRISPR-Cas9 editing for combinatorial elements screening

We constructed a dual-Luciferase reporting system to detect whether CRISPR/Cas9 can work in *S. miltiorrhiza* protoplasts. The NanoLuc was disabled by introducing a mutation to NanoLucM. Knockout of NanoLuc will destroy its Luciferase activity, and firefly Luciferase can be used as an internal control to sensitively quantify the relative editing efficiency through luminescence measurement.

We validated the editing efficiency of different promoters and/or Cas9 by co-transformation with editing vector (SmKO01-SmKO10) and pDual-Luc reporter vector in *S. miltiorrhiza* protoplasts (Figure 5A, B). All sgRNAs were expressed under the *AtU6-1*, *SmU6.1*, or *SmU6.3* promoter. The *2×35S*, *SlEF1a*, or *AtUBQ10*

promoter drove all *Sp*Cas9. And Cas9p was driven by the *SlEF1α* promoter (Figure 5B). The edited protoplasts' fLuc and NanoLuc fluorescence values were detected to examine the relative efficiency of mutations. The results indicate that the relative editing efficiency for protoplasts of *S. miltiorrhiza* under different element combinations is between 13.97% and 33.64%. The *AtU6-1* promoter drives sgRNA, and *AtUBQ10* drives the *Sp*Cas9 resulting in 29.78% CRISPR/Cas9-mediated mutation efficiency in protoplasts (Figure 5C). The endogenous *SmU6.1* promoter to drive sgRNA and *SlEF1α* to drive the *SpCas9* results in higher mutation efficiency. And the editing efficiency has improved to 33.64% using plant codon-optimized Cas9 (Figure 5C).

Discussion

A protoplast is a type of plant cell that is completely or partially removed by mechanical or enzymatic methods. The use of genetic engineering tools can be expanded by using cell wall-free protoplasts (Xu et al., 2021). The complete morphology of protoplasts is the basis for functional verification. To establish a method for separating and purifying the mesophyll protoplasts of *S. miltiorrhiza* effectively, it is necessary to evaluate the effectiveness of several conditions and analyze the yield and vitality of leaf protoplasts.

Protoplast isolation yield and activity are affected by various factors. Including temperature, speed, and duration of enzymatic hydrolysis (Wang et al., 2022). The duration of enzymatic hydrolysis was systematically examined in this study. When the enzymatic hydrolysis time during 4-5h, the number of protoplasts per unit volume of solution is sufficient, and the vitality reaches 92.70%.

Multiple approaches can achieve efficient gene transient transformation and expression in plant species. Particle bombardment can successfully introduce target DNA into rice callus tissue, but its efficiency is low and expensive equipment is required (Bruce et al., 1989). The efficiency of electroporation-mediated methods in plant tissues is also poor. However, the agrobacterium-mediated method is highly efficient and inexpensive (Li et al., 2009). Transient expression analysis of protoplasts can provide high-throughput and rapid plant genes analysis and has been widely applied to characterize gene function (Sheen 2001). Plant protoplasts lose the protection of cell walls, making it easier for foreign genetic DNA to be ingested. Therefore, they are ideal platforms for studying transient gene expression. The transformation efficiency of protoplasts is affected by various factors, including the PEG concentration, plasmid DNA quantity, incubation time, and temperature (Wu et al., 2017).

The protoplast transient expression efficiently and accurately reveals molecular mechanisms (Ma et al., 2020). Using transient transformation of protoplasts, nuclease efficiency and specificity can be assessed quickly and effectively (Nadakuduti et al., 2019). Protoplasts have also been used to validate gene editing systems in different species of plants, particularly those with complex genomes and/or with low editing efficiency (Lin et al., 2018). Previous studies used PEG-mediated protoplast transfection methods: protein degradation analysis and subcellular localization studies of *S. miltiorrhiza* (Zhou et al., 2016).

The CRISPR/Cas9 system has been developed for gene editing and has become a powerful tool for improving plant traits. *AtU6* promoters are commonly used in *S. miltiorrhiza* hair root editing and achieve reasonable editing efficiency (Alagoz et al., 2016). To further improve the editing efficiency of *S. miltiorrhiza*, we make some attempts to make different combinations based on various favorable elements in the gene editing system. The *AtU6-1* promoter drives sgRNA, and *AtUBQ10* drives the *Sp*Cas9 resulting in high mutation efficiency in protoplasts. This is consistent with recent research reports that this gene editor system considerably affects *S. miltiorrhiza* hair root editing (Shi et al., 2021; Shi et al., 2022; Zhou et al., 2018). Moreover, optimizing Cas9 using plant or species codon-optimized is a potential strategy for enhancing Cas9 activity and increasing mutation rate (Ma et al., 2015; Wang et al., 2021). As expected, the endogenous *SmU6* promoter to drive sgRNA and *SlEF1α* to drive the *SpCas9* results in higher mutation efficiency.

In the current case, transformation vectors were rapidly assembled and transiently expressed in model plant cells to achieve high throughput screening (Pfotenhauer et al., 2022). In this work, by editing highly sensitive reporter genes, NanoLuc, we have high-throughput screening for efficient vectors suitable for *S. miltiorrhiza* editing. This also supports the editing of traditional Chinese medicine, which is more difficult.

Conclusion

Protoplasts are small cells that play an important role in plant biology. This study obtained high-yield and -viable protoplasts from the 3-week-old *S. miltiorrhiza* leaf. When determining the effectiveness of protocol separation and transfection, we systematically evaluated the factors that affect the efficiency of protoplast preparation, including enzyme hydrolysis time and culture temperature (Figure 6A). The quantitative analysis of different combinations of editing elements is crucial for improving the efficiency of plant genome editing. Our results also indicated that the endogenous *SmU6.1* promoter drives sgRNA, and SlEF1α drives the optimized Cas9p resulting in the highest mutation efficiency in the *S. miltiorrhiza* protoplast platform (Figure 6B). In summary, a high-efficient protoplast transient system was developed to screen for genome editing elements in *S. miltiorrhiza*. This study establishes a representative example for constructing a component library of a genome editing system. The application of this optimization method can be extended to verify other genome editing systems of *S. miltiorrhiza* and is expected to make an important reference for genome editing research of other traditional Chinese medicine plants.

Declarations

Acknowledgments

This work was funded by: the China Academy of Chinese Medical Sciences (CACMS) Innovation Fund. (CI2021A041003), The Ability Establishment of Sustainable Use for Valuable Chinese Medicine Resources (2060302), National Key R&D Program of China (2018YFA0900600), Shanghai Jiao Tong

University (SJTU) Transmed Awards Research (20190104) and the Bill & Melinda Gates Foundation (grant numbers: OPP1199872 and INV-027291). Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.

Author contributions

Shao J, Zheng J, and Tang K designed the project; Shao J, Peng B, Zhang Y, and Yan X performed most of the experiments; Yao X, Hu X, Fu X, and Li L analyzed the data; Shao J and Peng B wrote this research manuscript. All the authors have read and approved the manuscript.

Data availability statement

All data supporting this study are included in the article.

Conflict of interest The authors declare no competing interests.

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Figures

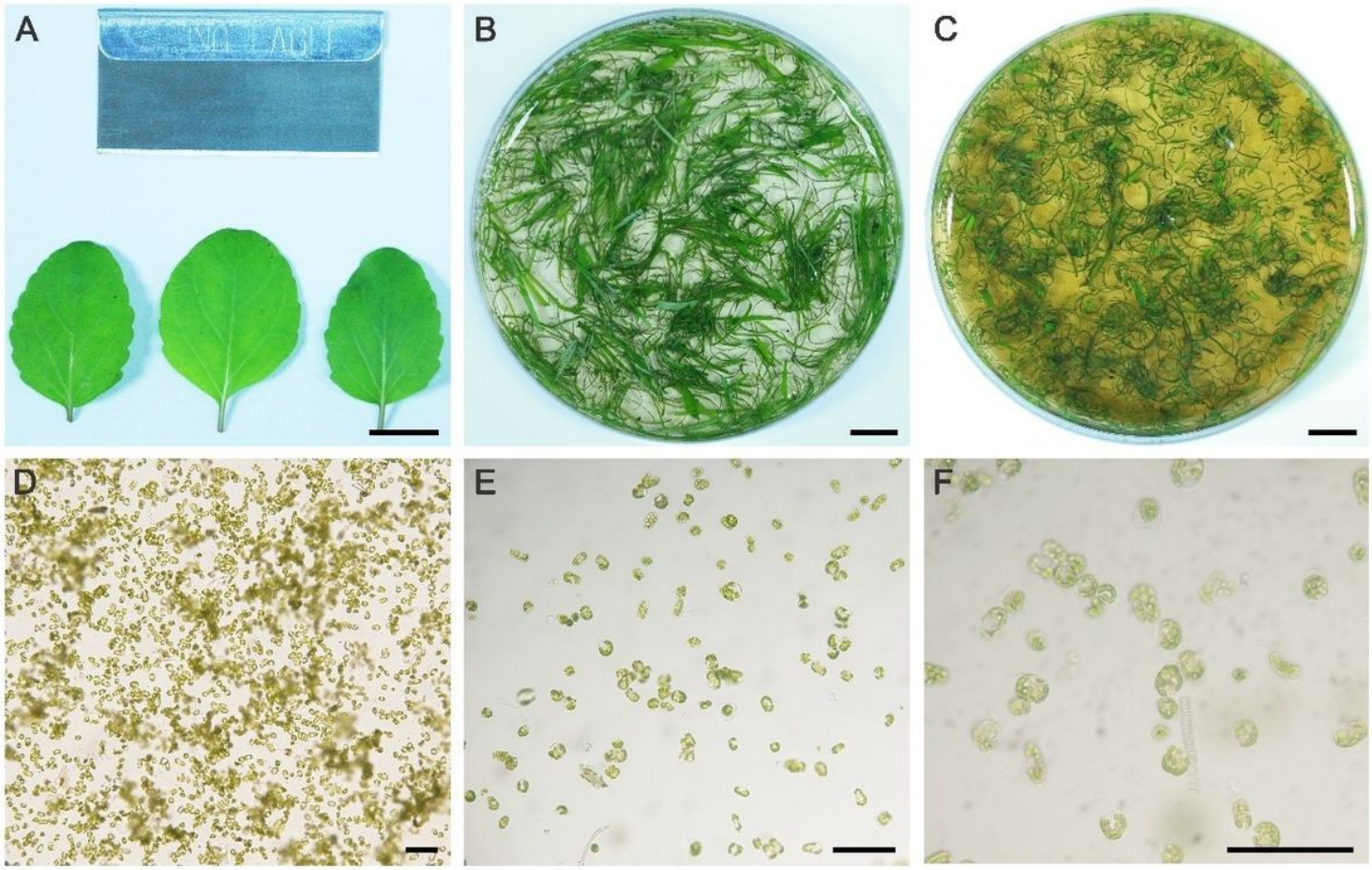


Figure 1

Protoplasts isolation from *S. miltiorrhiza* healthy young leaves. (A) Three-week-old leaves of *S. miltiorrhiza* were used to isolate protoplasts. Scale bar, 1cm. (B) Strip leaves were well immersed in the enzymatic solution. Scale bar, 1cm (C) Cut strips were treated by enzymatic digestion. Scale bar, 1cm (D-E) Protoplasts image obtained under a microscope with a 10×/20×/40× objective. Scale bar, 100μm.

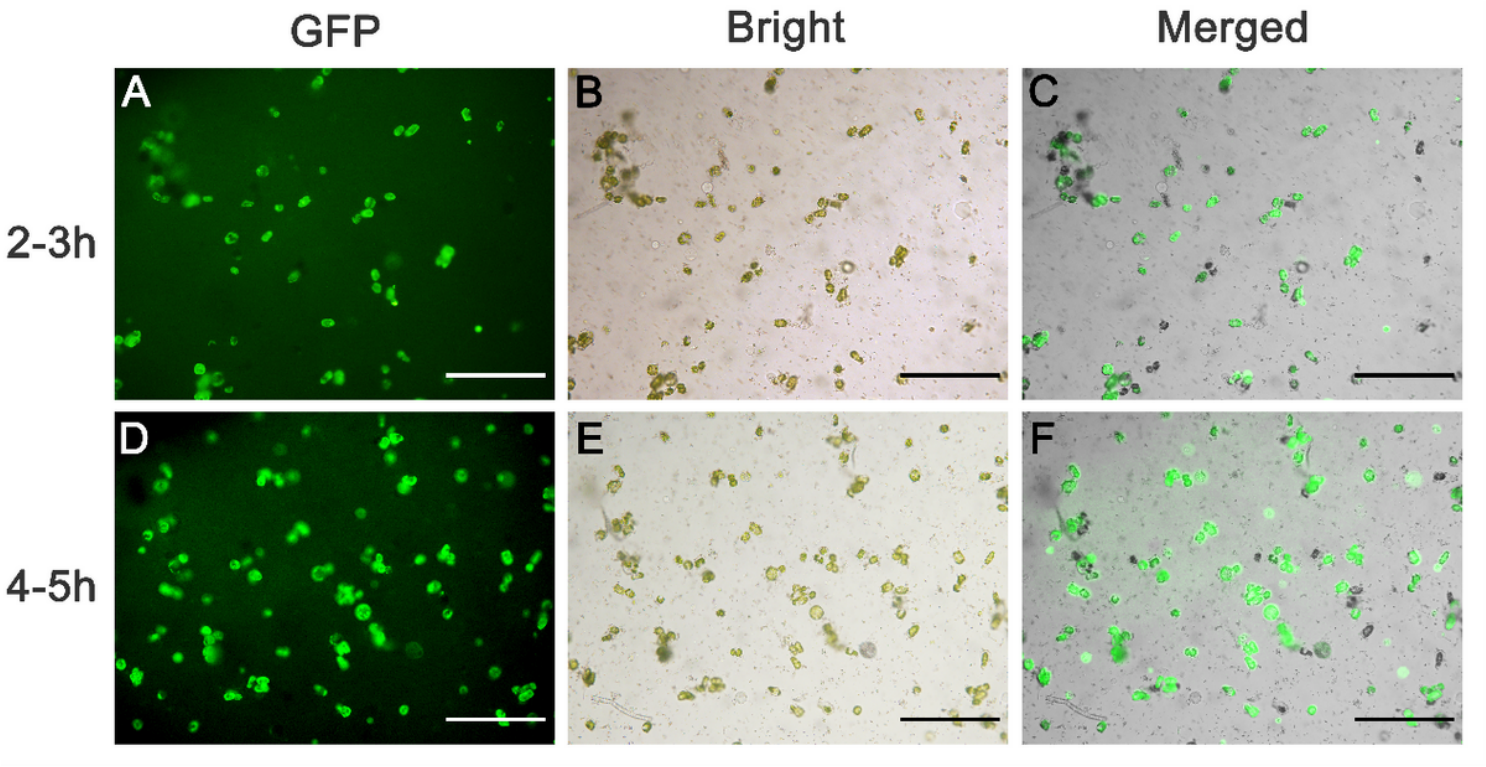


Figure 2

Enzymolysis duration affects *S. miltiorrhiza* protoplasts yield and viability. Image of protoplasts stained with FDA under GFP (A D), bright field (B E), and merged channel (C F), respectively. (A-C) Enzymatic hydrolysis duration 2-3h. (D-F) Enzymatic hydrolysis duration 4-5h. The green fluorescence signals reflect the active cells. Scale bars, 200μm.

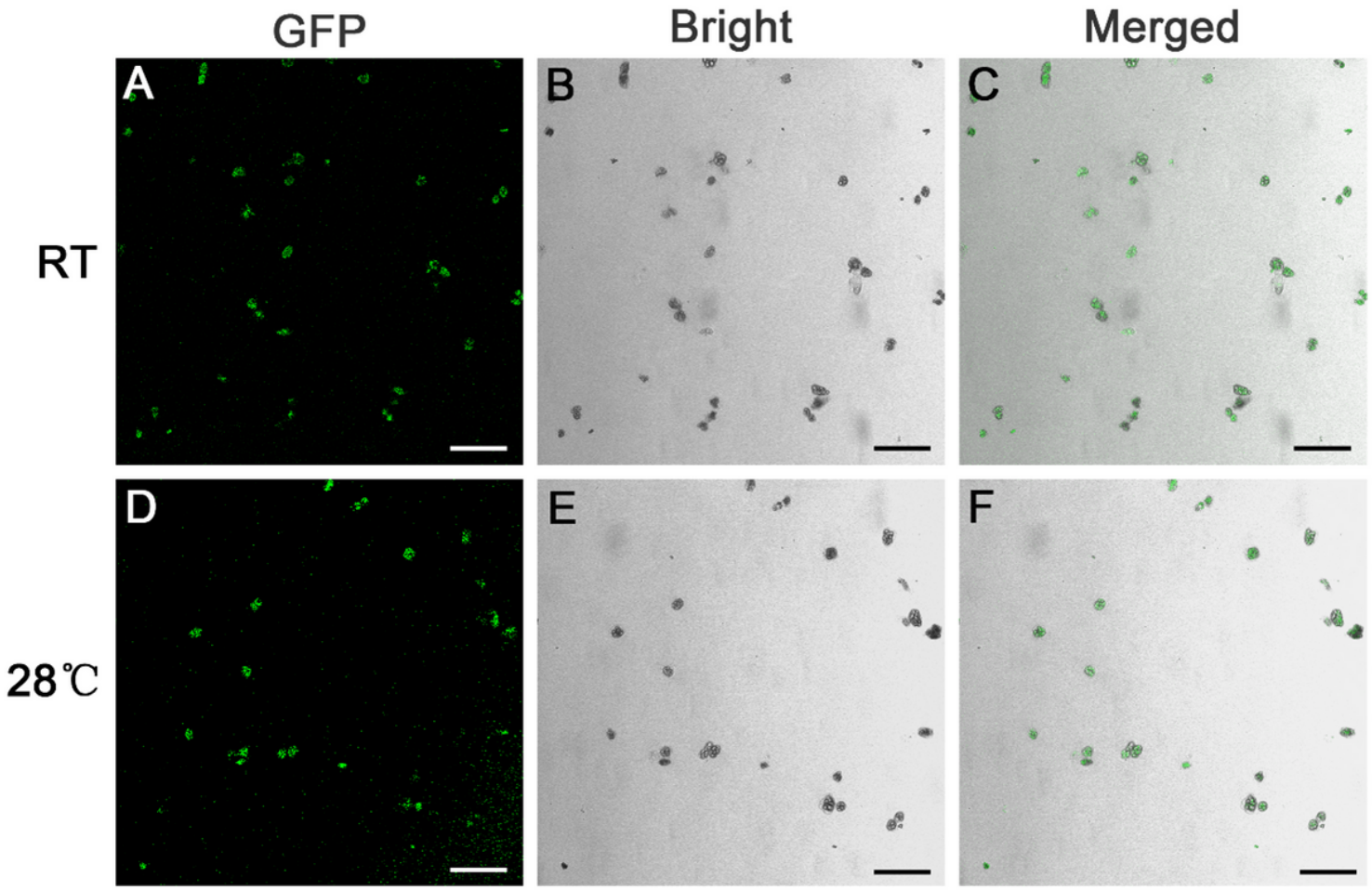


Figure 3

Incubation temperature affects *S. miltiorrhiza* protoplasts transfection efficiencies. The pHER-EGFP was expressed in protoplasts from three-week-old *S. miltiorrhiza*. Protoplasts were imaged under GFP (A, D), bright field (B, E), and merged channel (C, F), respectively. (A-C) Incubation temperature RT. (D-F) Incubation temperature 28℃. Green fluorescent signals reflected the transformed cells. Scale bars, 100μm.

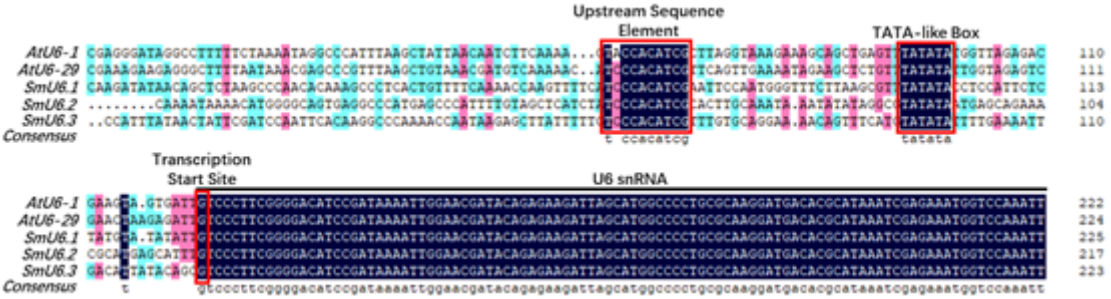


Figure 4

Multiple alignments of U6 and promoter sequences between *S. miltiorrhiza* and *Arabidopsis*. The black line represents the U6 snRNA transcript.

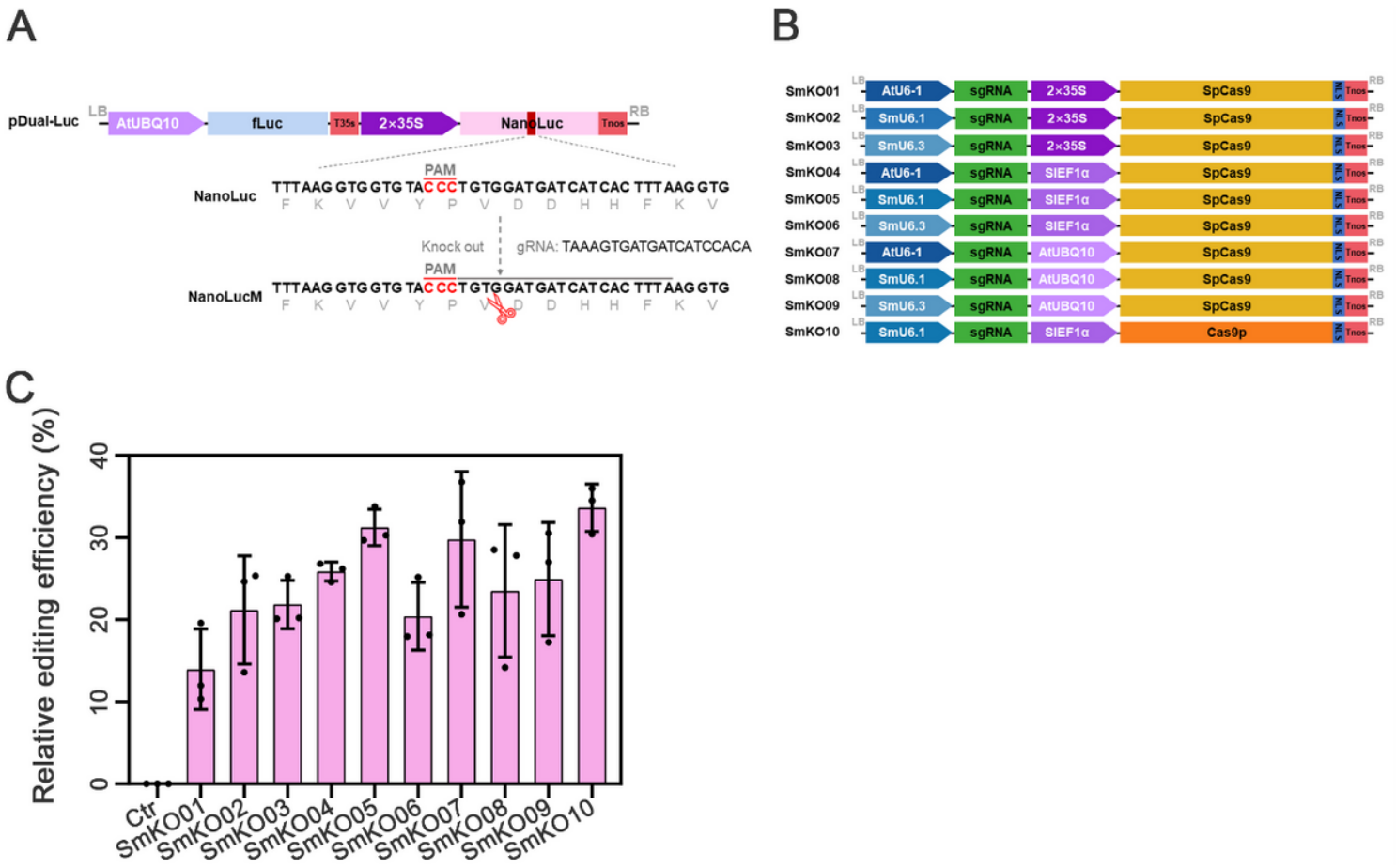


Figure 5

Screening of gene editing elements of *S. miltiorrhiza* by the protoplast transient. (A) Plant Dual-luciferase reporter (pDual-Luc) system for assessments of knock-out editors. Nano luciferase (NanoLuc) was inactivated by mutation (NanoLuM). *AtUBQ10*, *Arabidopsis thaliana* ubiquitin-10. fLuc, firefly luciferase. (B) Schematic illustration of knock-out editors with different edit element combinations used in this study (SmKO01-10). *SlEF1α*, tomato ELONGATION FACTOR-1α promoter. SpCas9, *Streptococcus pyogenes* Cas9. Cas9P, plant codon-optimized Cas9. NLS, nuclear localization signals. (C) Quantification of editing efficiency of *S. miltiorrhiza* by the protoplast. Three biological replications were performed for each treatment.

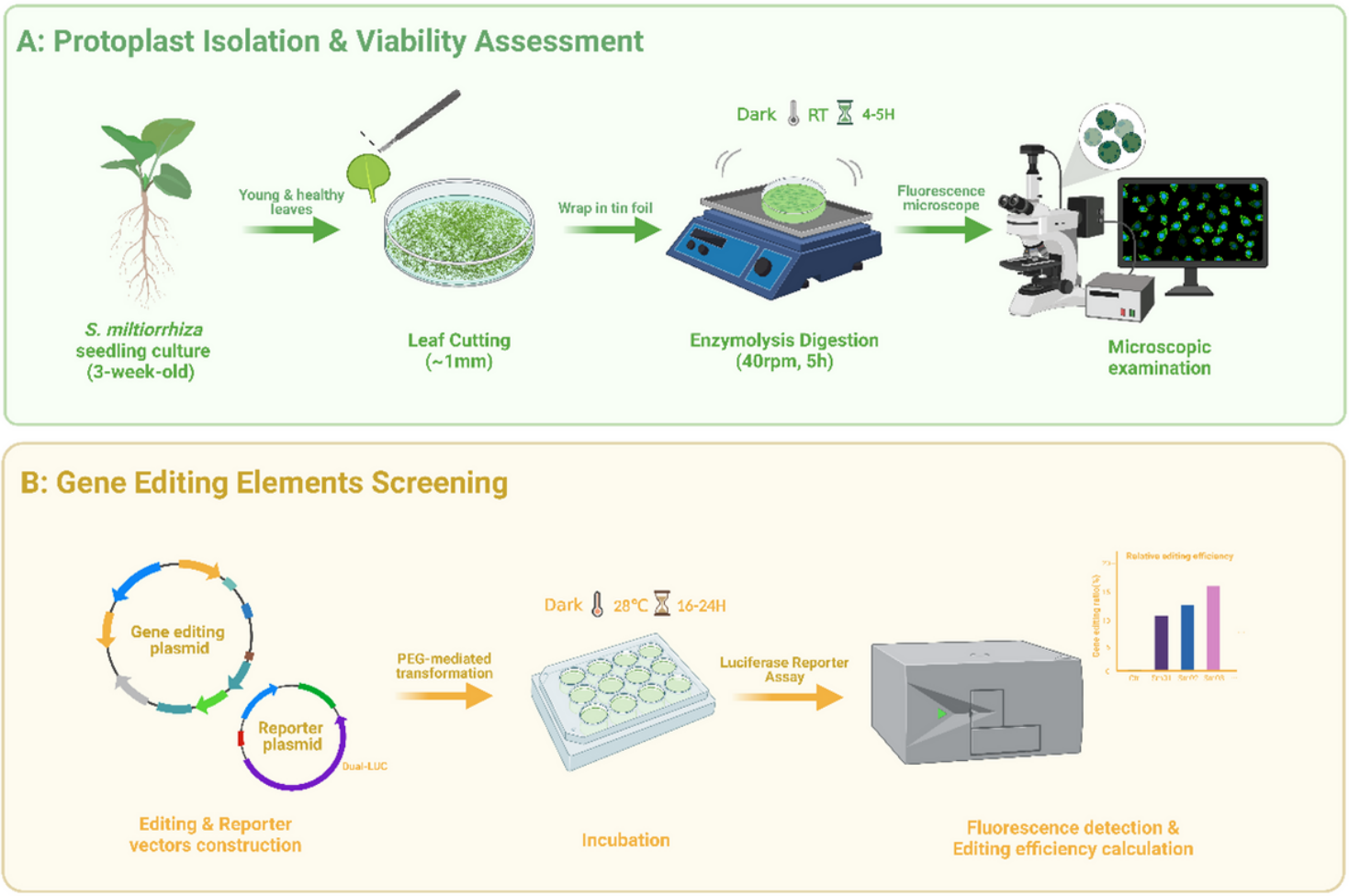


Figure 6

Protocol of *S. miltiorrhiza* gene editing elements screening by the protoplast transient. (A) Protoplast isolation and viability assessment. (B) Gene editing elements screening of *S. miltiorrhiza.*

Supplementary Files

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